Fibroblast Growth Factor Signaling Is Essential for Self-renewal of Dental Epithelial Stem Cells

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Background: Understanding of the self-renewal and differentiation of dental epithelial stem cells (DESCs) is important for tooth regeneration therapies.

Results: Depletion of FGF signaling suppressed self-renewal and led to differentiation of DESCs.

Conclusion: FGF signaling is essential for maintenance of DESCs.

Significance: The finding sheds new light on the mechanism by which the homeostasis, expansion, and differentiation of DESCs are regulated.

A constant supply of epithelial cells from dental epithelial stem cell (DESC) niches in the cervical loop (CL) enables mouse incisors to grow continuously throughout life. Elucidation of the cellular and molecular mechanisms underlying this unlimited growth potential is of broad interest for tooth regenerative therapies. Fibroblast growth factor (FGF) signaling is essential for the development of mouse incisors and for maintenance of the CL during prenatal development. However, how FGF signaling in DESCs controls the self-renewal and differentiation of the cells is not well understood. Herein, we report that FGF signaling is essential for self-renewal and the prevention of cell differentiation of DESCs in the CL as well as in DESC spheres. Inhibiting the FGF signaling pathway decreased proliferation and increased apoptosis of the cells in DESC spheres. Suppressing FGF or its downstream signal transduction pathways diminished Lgr5-expressing cells in the CL and promoted cell differentiation both in DESC spheres and the CL. Furthermore, disruption of the FGF pathway abrogated Wnt signaling to promote Lgr5 expression in DESCs both in vitro and in vivo. This study sheds new light on understanding the mechanism by which the homeostasis, expansion, and differentiation of DESCs are regulated.

Understanding the self-renewal and differentiation of stem cells (SCs) and the contribution of their microenvironment, designated as the “niche,” is a key issue for tissue engineering and regeneration therapies. Although the niche varies in nature and location in different tissue types (1, 2), it provides a protective environment to nurture SCs; it prevents SC reserves from undergoing differentiation and apoptosis, as well as preventing SCs from excessive propagation, to maintain tissue homeostasis (2). The cervical loop (CL) of mouse incisors is an SC niche where a population of self-renewing dental epithelial SCs (DESCs) resides that is responsive for the continuous growth and regenerative potential of mouse incisors throughout life (3–7). The CL is formed at the apical end of the developing tooth and is composed of the inner and outer enamel epithelia, stellate reticulum, and stratum intermedium (8). We recently reported that the CL contains both label-retaining and Lgr5-expressing cells, which represent slow-cycling and active DESCs, respectively (9). These Lgr5-expressing active DESCs highly express CD49f, form DESC spheres containing both quiescent and active SCs, and have the potential to differentiate into multiple lineages of tooth epithelial cells. Furthermore, the DESCs can be enriched by CD49fHigh-based cell sorting and maintained and expanded in vitro by the Matrigel-based sphere culture system (9). However, how cell signaling between DESCs and adjacent dental stromal cells controls DESC self-renewal and expansion and the generation of ameloblasts or other lineages of tooth epithelial cells is not well understood.

The fibroblast growth factor (FGF) and FGF receptor (FGFR) families have been shown to constitute reciprocal regulatory communication loops between the epithelial and mesenchymal compartments, playing important roles in...
FGF in Dental Epithelial Stem Cells

Animals—All animals were housed in the Program for Animal Resources of the Institute of Biosciences and Technology at Texas A&M University and were handled in accordance with the principles and procedures of that institution’s Guide for the Care and Use of Laboratory Animals; all experimental procedures were approved by the Institutional Animal Care and Use Committee. The mice carrying Fgfr1fl, Fgf2fl, Frs2af, Nkx3.1Cre, K5rtTA-H2BGFP, and Lgr5Ac alleles were bred and genotyped as described (4, 29–33). Activation of tetracycline-regulated K5rtTA-H2BGFP expression was achieved by administration of regular chow containing 0.0625% doxycycline (Teklad, Harlan Laboratories).

Cell and Organ Culture—For tooth or CL organ culture, incisors were dissected from maxilla. The apical end of the incisors were further separated from the rest of other tissues and placed in a 24-well cell culture plate containing 1 ml of high glucose DMEM with L-glutamate (Invitrogen) supplemented with 10% FBS, 100 IU of penicillin, and 100 μg/ml streptomycin. The tissues were cultured at 37 °C in a CO2 incubator for the time specified for each experiment. The Wnt3a conditioned medium or the control L-cell conditioned medium was collected according to the ATCC protocol. The medium conditioned either by Wnt3a-overexpressing or control L-cells was added to the culture medium at a ratio of 1:10; LiCl (100 ng/ml), NaCl (100 ng), and FGF1 (20 ng/ml) or FGF2 (20 ng/ml) was added as a supplement where indicated. An ERK inhibitor (SL327, EMD Millipore), PI3K inhibitor (LY294002, EMD Millipore), or FGR inhibitor (341608, EMD Millipore) was added to the medium to a final concentration of 50 μM unless otherwise specified. For DESC sphere cultures, the CLs were dissected from postnatal day 7 (P7) pups and digested with dispase and collagenase to obtain single cell suspensions as described (9). The cells were resuspended in 50 μl of oral epithelial progenitor medium (CnT-24, CELLNTEC Advanced Cell Systems, Bern, Switzerland) and mixed with Matrigel (BD Biosciences) at a 1:1 ratio. About 0.1 ml of the cell/Matrigel mixture (containing 50,000 cells) was seeded around the rims of wells in a 12-well tissue culture plate and cultured in a CO2 incubator at 37 °C for 10–14 days. The aforemention FGR, ERK, and PI3K inhibitors were added to the medium to a final concentration of 10 μM or as otherwise specified. Adenovirus-GFP and adenovirus-GFP-Cre were purchased from the Vector Development Laboratory of the Baylor College of Medicine. The infections were carried out by incubating 3.2 × 1011 pfu/ml virus/1 × 106 cells at 37 °C for 16 h. The cells were then washed twice with PBS and cultured in oral epithelial progenitor medium (CnT-24) at 37 °C. At least three independent experiments with at least three samples in each group were performed. Representative
whole-mount and section staining of the same experiments were shown.

Histology Analyses—DESC spheres were fixed in 4% paraformaldehyde (PFA) solution for 30 min at 4 °C. The dissected incisors, or the apical end of the incisors, which contains the CLs, were fixed in 4% PFA at 4 °C overnight. Fixed tissues were dehydrated serially with ethanol, embedded in paraffin, and completely sectioned according to standard procedures. Immunohistochemical analyses were performed on paraffin sections or frozen sections mounted on Superfrost Plus slides (Fisher Scientific). Antigen retrieval was performed by boiling samples in citrate buffer (10 mM) for 20 min or as suggested by the manufacturers. All sections were incubated at 4 °C overnight with primary antibodies diluted in PBS. The sources and concentrations of the primary antibodies were: rabbit anti-amelogenin (1:1000) for tissue section staining and 1:2000 for sphere and cell staining; a generous gift from Dr. Jan C. C. Hu, University of Michigan School Dentistry), mouse anti-CK14 (1:500, from Santa Cruz Biotechnology). Specifically bound antibodies were detected with FITC-conjugated secondary antibodies (Invitrogen) and visualized on a Zeiss LSM 510 confocal microscope. For LacZ staining, the dissected incisors or CLs were first fixed lightly with 0.2% glutaraldehyde for 45 min at room temperature. After washing with PBS for 10 min, the tissues were post-fixed with 4% PFA for 1 h, dehydrated, and paraffin-embedded for subsequent analyses. The dissected CLs were completely sectioned, and the most intensive LacZ-stained sections are shown.

Quantitative RT-PCR Analyses—Total RNA was extracted from freshly dissected or in vitro cultured tissues with the RiboPure kit (Ambion, Austin, TX). Reverse transcription was carried out with SuperScript III enzymes (Invitrogen) and random primers according to the manufacturer’s protocols. Real-time PCR was carried out with the SYBR Green RT-PCR kit (Qiagen, Valencia, CA) or TaqMan gene expression assays (Invitrogen) with pairs of primers specific for each transcript and following the manufacturer’s protocol. Relative mRNA abundance was calculated using the comparative threshold cycle method and normalized with β-actin. Data derived from at least three independent experiments are expressed as fold difference between experimental and control samples. The primer sequences are listed in Table 1.

Western Blotting Analysis—Dissected CLs or cultured cells were homogenized in lysis buffer (1% Triton X-100/PBS) with 1 mM PMSF and a 1:100 dilution of proteinase inhibitor and phosphatase inhibitor mixture I and II (Sigma). The protein extracts were harvested by centrifugation. Western blot analyses were done as described previously (34). Rabbit anti-ERK (1:3,000), rabbit anti-pERK (1:3,000), rabbit anti-amelogenin (1:2,000), and mouse anti-β-actin (1:3,000) antibodies were purchased from Santa Cruz Biotechnology. Rabbit anti-AKT (1:1000) and anti-pAKT (1:1000) were from Cell Signaling (Beverly, MA). The intensity of bands was quantitated by using NIH ImageJ software.

TUNEL Assays—For TUNEL assays, spheres were fixed with 4% PFA for 30 min, paraffin-embedded, and sectioned. Apoptotic cells in the sections were detected with the DeadEnd Fluorometric TUNEL system from Promega (Madison, WI).

Flow Cytometry and Cell Sorting Analysis—Disassociated DESCs were labeled with allophycocyanin (APC)-conjugated anti-CD49f (integrin α6) (BioLegend; clone GoH3, 20 μl/100 ul) or anti-CD44 (eBioscience; clone IM7, 1 mg/ml). Antibody labeling was conducted by a 20-min incubation at 4 °C with cell sorting buffer (1× PBS and 2% FBS) containing antibodies at the manufacturer’s suggested dilution in a volume of 100 μl/1 million cells. The cells were washed in 1 ml of cold cell sorting buffer, resuspended in 0.5 ml of cell sorting buffer, and analyzed. Disassociated K5-H2BGFP CLs were resuspended in cell sorting buffer for analysis. Analyses were conducted on a BD FACSaria I (Special Order Research Product program) or BD Accuri C6 flow cytometer, and a minimum of 10,000 cells was acquired for each experimental condition. Proper isotype controls were used according to the manufacturer’s suggestions.

Statistical Analyses—Western blots were scanned for quantitation with the ImageJ software. All quantitative data were expressed as the means ± S.D. from three independent experiments. A t test was used to determine whether the differences were statistically significant, and p values less than 0.05 were considered significant.

RESULTS

FGF Signaling in DESCs Is Required for Sphere Formation and Prevents Differentiation of the Cells—FGF signaling plays a role in SC self-renewal and differentiation (35–38). We first employed RT-PCR analyses to characterize the expression of Fgfr genes in the CLs and in DESC spheres (Fig. 1A). The results revealed that both the IIIb and IIIc isoforms of Fgfr1 and Frs2α were expressed in the CLs and in DESC spheres. The IIIb isoform of Fgfr2 was expressed in both CLs and DESC spheres;
however, the IIc isoform of Fgfr2 was detectable only in the CLs. To investigate the role of FGF signaling in DESCs, the cells were cultured in Matrigel for sphere formation analyses as described (9) with or without supplementation with FGF2, at a final concentration of 5 ng/ml. The sphere forming efficiency of DESCs grown in the presence of FGF2 was about 3 times that of those grown in the absence of FGF2 (Fig. 1B). Because low levels of endogenous FGFRs from cells or Matrigel might contribute to sphere forming activity, an FGFR inhibitor (341608) was added to the medium to block FGF signaling in order to reveal the role of endogenous FGFs from cells or Matrigel might contribute to sphere forming activity of DESCs. Together, the results indicate that FGF signaling is required for sphere forming activity of DESCs (Fig. 1F). Infecting the cells with GFP-bearing adenovirus did not inhibit the sphere forming activity of DESCs, indicating that virus infection per se did not affect the self-renewal activity of DESCs (Fig. 1F). The results suggest that Fgfr1 and Fgfr2 redundantly regulate the sphere forming activity of DESCs.

To further investigate the role of FGF signaling in regulating DESC sphere formation and differentiation, we then cultured DESC spheres in the presence or absence of FGFR inhibitors at a final concentration of 1 μM. At this intermediate concentration, the DESCs were viable and still formed spheres at a low efficiency. Phase contrast microscopy and H&E staining of sphere sections revealed that the DESC spheres formed a prominent, concentric, noncellular structure in the presence of FGFR inhibitors (Fig. 2, A and B). Real-time RT-PCR analyses demonstrated that expression of the differentiation markers, amelogenin and CK14, was increased in the FGFR inhibitor-treated groups (Fig. 2C). Furthermore, immunostaining revealed that suppression of FGF activity significantly increased amelogenin and CK14 expression in the spheres (Fig. 2D). These results suggest that suppressing FGF signaling inhibits sphere formation and promotes differentiation of DESCs. Together, the results indicate that FGF signaling is required to maintain self-renewal and prevent the differentiation of DESCs.

Both MAPK and AKT Pathways Are Required for Sphere Formation in DESCs—MAPK and PI3K/AKT pathways are the two major downstream transducers in the FGF signaling cascade. To determine whether the MAPK or PI3K pathways are required for the sphere forming ability of DESCs, DESC sphere cultures were treated with either an ERK inhibitor (SL327) or a PI3K inhibitor (LY294002). Similar to FGFR inhibitors, both the ERK and the PI3K inhibitor suppressed the sphere forming activity of DESCs in a dose-dependent manner (Fig. 3A). West-
ern blot analyses revealed that suppressing either the ERK or PI3K pathway significantly increased amelogenin expression in DESC spheres (Fig. 3B). The expression level of amelogenin was reduced in the presence of a high dose of either the ERK or PI3K inhibitor, likely due to increased cell death occurring under such conditions. The data were consistent with results showing that treating the DESC spheres with FGFR inhibitors increased DESC differentiation (Fig. 1), indicating that both pathways were required for FGFR to prevent differentiation of DESCs. In addition, the number of phosphorylated histone H3-positive cells was significantly reduced by treating the cells with FGFR, ERK, or PI3K inhibitor, indicating that the treatments reduced cell proliferation in DESC spheres (Fig. 3C). TUNEL assays further revealed that apoptosis in DESC spheres was increased by suppressing the PI3K and FGFR kinase activities (Fig. 3D). Interestingly, inhibiting ERK, but not PI3K or FGFR kinase, significantly increased the CD44+ cell population, which likely represented progenitors for ameloblasts (Fig. 3E), although no obvious difference was detected in the CD49f (integrin α6)+ population using the same treatment (Fig. 3F). Together, the results suggest that both the ERK and PI3K pathways promote DESC proliferation and prevent DESC differentiation, whereas the PI3K pathway also prevents cells from undergoing apoptosis.

Blocking FGF Signaling Promotes Cell Differentiation in the CL—We reported previously that ablation of Fgfr2 in the tooth epithelium leads to diminishment of the CL (13). To further study the roles of FGFR and its two major downstream pathways in the CL, the CL was dissected from incisors at the P7 stage for ex vivo tissue culture analyses. Treatment with the PI3K or FGFR inhibitors significantly disrupted the morphology of the CL (Fig. 4A). Unlike the DMSO-treated control group, where the epithelial cells were polarized and well aligned to form a CL, the epithelial cells in the PI3K or FGFR inhibitor-treated groups were poorly organized and the boundary between epithelial and stromal cells was poorly defined. Western blot analyses revealed that inhibiting ERK, but not PI3K, significantly increased expression of the epithelial marker CK14 (Fig. 4B). Treatment with the FGFR inhibitor significantly enhanced expression of amelogenin, the characteristic ameloblast matrix protein (Fig. 4B). Notably, suppressing the FGFR kinase only lightly impacted the activation of ERK and
PI3K, likely due to the existence of multiple upstream activators of ERK and PI3K in the CL. Because the effects of these inhibitors were different in CL organ cultures and DESC sphere cultures, a more detailed characterization is needed for resolving this discrepancy. Possible factors include the existence of both epithelial and mesenchymal components in the CL organ cultures, different media used in CL organ and DESC sphere cultures, and the short CL organ culture time (3 days) compared with the DESC sphere culture (10–14 days).

Blocking FGF Signaling Increases Slow-cycling Cells but Diminishes Lgr5-expressing Cells in the CL—To further investigate whether inhibiting FGF signaling affected slow-cycling or Lgr5-expressing cells in the CL, mice bearing the Lgr5LacZ reporter allele were treated with doxycycline to activate H2BGFP expression for 7 days. The teeth were then dissected and cultured in the presence of doxycycline to continue blocking H2BGFP expression. As the abundance of H2BGFP was reduced after each cell division, the quiescent cells would remain GFPbright, the fast-cycling cells would be GFP-negative, and the slow-cycling cells would be somewhat GFP-positive. Treating the tooth cultures with inhibitors of ERK, PI3K, or FGFR kinase did not affect the presence of H2BGFP-retaining cells in the CL region (Fig. 5A), suggesting that suppressing these pathways did not reduce slow-cycling DESCs. However, flow cytometry analyses revealed that the ratio of GFPbright (representing slow-cycling cells) to total GFP+ cells was increased, suggesting that inhibiting FGFR signaling prevented slow-cycling cells from entering into the cell cycle in the CL (Fig. 5B). Treatment with FGFR2 somewhat reduced the ratio, although statistical significance was lacking because of the large variation in the nature of the assay methods. On the other hand, treating the cultured teeth bearing the Lgr5lacZ reporter allele with ERK, PI3K, or FGFR inhibitors diminished Lgr5lacZ-expressing cells in the CL, suggesting that these signaling pathways were important for the maintenance of Lgr5-expressing cells in the CL (Fig. 5C).

FGF Signaling Is Required for Wnt Signaling to Up-regulate Lgr5 Expression in the CL—Lgr5 is a target gene of the Wnt signaling pathway in multiple organs, including the small intestine, hair follicles, and cochlea (3, 39, 40). Consistently, expression of the Lgr5lacZ reporter in the CL was up-regulated by treatment with Wnt3a-enriched conditioned medium or LiCl, a commonly used Wnt signaling activator (Fig. 6A), but not by the medium conditioned by nontransfected L-cells or NaCl. As FGFR inhibitors diminished Lgr5lacZ expression in the CL, we then further investigated whether inhibition of FGFR would abrogate the activity of Wnt signaling to enhance Lgr5 expression. The results clearly demonstrated that activation of Lgr5lacZ expression by either Wnt3a or LiCl was blocked by FGFR inhibitors. However, residual Lgr5lacZ expression was still noticeable in the LiCl- and FGFR inhibitor-treated CL (Fig. 6A). In line with the Lgr5lacZ reporter assays, treating the ex vivo cultured CLs with the Wnt3a-enriched conditioned medium or LiCl increased endogenous Lgr5lacZ expression at the mRNA level (Fig. 6B). Moreover, both FGFR1 and FGFR2 promoted expression of the Lgr5lacZ reporter in the CL (Fig. 6C). Together, the results suggest that FGF pathway is required for the Wnt pathway to activate Lgr5 expression in the CL of ex vivo cultured tooth. However, whether Wnt signaling regulated Lgr5 expression in the CL cells either directly or indirectly mediated by the stromal cells adjacent to the CL remained unknown. To address the issue, ex vivo cultures of incisors carrying the Wnt signaling reporter allele Axin2lacZ were used to track cells that had Wnt signaling activity. Similar to previous reports (7, 18), Axin2lacZ expression was not detected in the dental epithelium (Fig. 6D). However, the stromal cells in both control and Wnt3a-treated samples were highly LacZ-positive. The Wnt3a-treated samples appeared to have more intensive staining than that of the controls (Fig. 6D). Quantitative RT-PCR analyses further demonstrated that Wnt3a treatment significantly increased Axin2 expression (Fig. 6E). The results suggest that the regulation of Lgr5 expression by Wnt3a in the CL is likely mediated by stromal factors.

Ablation of Fgr2 in the tooth epithelium with Nkx3.1Cre diminishes the CL (13). Consistently, Lgr5lacZ expression in the Fgr2 conditional knock-out CL was dramatically reduced (Fig. 7A). Real-time RT-PCR analyses demonstrated that endogenous Lgr5 expression in the Fgr2 mutant CL areas was reduced to 30% of that in the wild type control (Fig. 7B), which was consistent with the decreased Lgr5 expression in spheres treated with FGFR inhibitor (Fig. 7C). Interestingly, expression of several inhibitors of the Wnt receptor Frizzled, including sFRP1, sFRP4, and Wif1, was increased in Fgr2 mutant CLs (Fig. 7D). However, expression of Wnt3a was reduced in Fgr2 mutant CLs. Of further note was the expression of Dkk1, an Lrp5/6 inhibitor, which was reduced in Fgr2 mutant CLs. Interestingly, the changes in expression of these genes were less than 2-fold, implying that Wnt signaling in the CL was delicately regulated. Together, the results indicated that the Wnt signaling pathway is suppressed in Fgr2 mutant CLs. Cells isolated from the CL regions of Fgr2 mutant had a significantly decreased sphere forming activity under the same conditions.
over time (Fig. 7E), indicating a decrease in DESCs in the degenerated CL of Fgfr2 mutant incisors.

**DISCUSSION**

Progress in studying the isolation and propagation of DESCs has been slow due to lack of reliable surface markers suitable for identifying and enriching DESCs, as well as the limited understanding of the molecular mechanisms governing DESC self-renewal and differentiation. Herein, we report, using newly developed methods of sphere culture, identification, and enrichment of DESCs (9), that FGF signaling is critical for maintaining the sphere forming capacity of DESCs and the homeostasis of Lgr5-expressing DESCs. Suppressing FGF signaling by inhibiting either FGFR kinase activity or its two major downstream pathways, ERK and PI3K, induced differentiation, decreased proliferation, and increased apoptosis in DESC spheres. Interestingly, inhibiting FGF signaling did not exhaust slow-cycling DESCs but diminished Lgr5 expression in the CL. In addition, inhibiting FGFR or its downstream ERK and PI3K/AKT pathways also diminished Lgr5 expression in the CL. It abrogated the activities of Wnt signaling to promote Lgr5 expression in the CL both *in vivo* and *in vitro*. As sphere forming capacity is normally used to evaluate the self-renewal activity of SCs, label retention for slow-cycling SCs, and Lgr5 expression for active cycling SCs (25–28), our results reveal that FGF signaling is required for maintaining active DESCs in the CL and preventing DESC differentiation.

Mouse incisors are organs that grow continuously through life and thus, require a constant supply of dental epithelial cells derived from DESCs residing in the CL. FGF signaling constitutes a reciprocal regulatory communication loop between the epithelial and mesenchymal compartments regulating tooth formation and regeneration (10–14). The FGF10-FGFR2IIIb signaling axis has been proposed as essential for the CL (5, 11). We reported previously that ablation of Fgfr2 in the tooth epithelium causes loss of the CL in maxillary incisors, likely due to loss of active DESCs in the CL (13). Our data here further demonstrate that FGF signaling is required for maintaining Lgr5-expressing but not label-retaining DESCs. This finding is consistent with the data that the sphere forming units were reversibly reduced by treatment with FGFR inhibitors and supports the report that expression of a dominant negative FGFR2 construct in postnatal tooth epithelial cells reversibly suppresses mouse incisor growth (14).

Suppressing FGFR kinase activity only slightly reduced the activation of ERK or PI3K (Fig. 4B), suggesting that both ERK and PI3K/AKT have multiple upstream regulatory pathways...
in the CL. Interestingly, suppressing the ERK pathway increased CK14 and amelogenin expression, whereas PI3K inhibition blocked the expression. However, suppressing FGFR kinase only slightly increased CK14 expression, whereas it dramatically increased amelogenin expression (Fig. 4B). These results suggest that activation of these two pathways favors commitment of different lineages and that FGFR signaling inhibits ameloblast differentiation either via combinatorial effects of the two pathways or other pathways independent of ERK and PI3K. Further rigorous characterization is needed to clarify this issue.

Wnt are secreted ligands that elicit receptor-mediated signals regulating the development, regeneration, and maintenance of progenitor cell pools, both via the canonical β-catenin-dependent and non-canonical β-catenin-independent pathways (41–47). Although it plays essential roles in early tooth development (48–50), the role of Wnt/β-catenin signaling in DESC homeostasis and differentiation is controversial. In situ hybridization experiments show that DESCs do not have the active Wnt/β-catenin signaling pathway (7), which is confirmed by using the three classic Wnt/β-catenin signaling reporters TOPGAL, BATGAL, and Axin2 in mice (18). However, expression of constitutive active β-catenin signaling reporters TOPGAL, BATGAL, and Axin2 in the CL. Interestingly, suppressing the ERK pathway increased CK14 and amelogenin expression, whereas PI3K inhibition blocked the expression. However, suppressing FGFR kinase only slightly increased CK14 expression, whereas it dramatically increased amelogenin expression (Fig. 4B). These results suggest that activation of these two pathways favors commitment of different lineages and that FGFR signaling inhibits ameloblast differentiation either via combinatorial effects of the two pathways or other pathways independent of ERK and PI3K. Further rigorous characterization is needed to clarify this issue.

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