p38α MAPK Is Required for Tooth Morphogenesis and Enamel Secretion*

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Matthew B. Greenblatt1,2, Jung-Min Kim1, Hwanee Oh1, Kwang Hwan Park3, Min-Kyung Choo1, Yasuyo Sano1, Coralee E. Tye1, Ziedonis Skobe1, Roger J. Davis1,5, Jin Mo Park1, Marianna Bei1,4, Laurie H. Glimcher1, and Jae-Hyuck Shim4

From the 1Department of Pathology, Brigham and Women’s Hospital, Boston, Massachusetts 02115, the 2Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York, New York 10065, the 3Department of Microbiology, Brain Korea 21 PLUS Project for Medical Sciences and Department of Orthopaedic Surgery, Yonsei University College of Medicine, Seoul, Republic of Korea, the 4Cutaneous Biology Research Center, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts 02129, the 5**Department of Biochemistry, University of Vermont College of Medicine, Burlington, Vermont, 05405, the 4Forasft Institute, Cambridge, Massachusetts 02142, the 4Howard Hughes Medical Institute and Program in Molecular Medicine, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01605, the 4Department of Surgery, Massachusetts General Hospital, Shriners Burns Hospital, Harvard Medical School, Boston, Massachusetts 02115, and the 4**Department of Medicine, Weill Cornell Medical College, New York, New York 10065.

Background: Little is known regarding the contribution of MAPKs to the development of ectodermal appendages.

Results: Mice with a deletion of p38α in ectoderm display defective secretion of dental enamel and the absence of dental cusps.

Conclusion: p38α mediates critical steps in tooth morphogenesis and enamel secretion.

Significance: This is the first in vivo study demonstrating that the p38 MAPK pathway is critical for tooth morphogenesis and enamel secretion.

An improved understanding of the molecular pathways that drive tooth morphogenesis and enamel secretion is needed to generate teeth from organ cultures for therapeutic implantation or to determine the pathogenesis of primary disorders of dentition (Abdollah, S., Macias-Silva, M., Tsukazaki, T., Hayashi, H., Attisano, L., and Wrana, J. L. (1997) J. Biol. Chem. 272, 27678–27685). Here we present a novel ectodermal dysplasia phenotype associated with conditional deletion of p38α MAPK in ectodermal appendages using K14-cre mice (p38αK14−/− mice). These mice display impaired patterning of dental cusps and a profound defect in the production and biomechanical strength of dental enamel because of defects in ameloblast differentiation and activity. In the absence of p38α, expression of amelogenin and β1 integrin in ameloblasts and p21 in the enamel knot was significantly reduced. Mice lacking the MAP2K MKK6, but not mice lacking MAP2K MKK3, also show the enamel defects, implying that MKK6 functions as an upstream kinase of p38α in ectodermal appendages. Lastly, stimulation with BMP2/7 in both explant culture and an ameloblast cell line confirm that p38α functions downstream of BMPs in this context. Thus, BMP-induced activation of the p38α MAPK pathway is critical for the morphogenesis of tooth cusps and the secretion of dental enamel.

Defining the molecular pathways that govern the development of teeth or the secretion of dental matrix proteins is fundamental to understand the basis of genetic disorders of dentition and ectodermal dysplasia syndromes (1–3). Additionally, this understanding forms the basis of strategies to generate teeth either in vitro for transplantation or in situ for regenerative applications. To date, a major focus of investigation in this area has been to define how the multiple secreted ligands derived from either the epithelium itself or from the underlying mesenchyme act at the molecular level (2).

In particular, multiple lines of evidence demonstrate that bone morphogenetic proteins (BMPs)4 play a critical role in this process. BMPs are secreted with spatial and temporal coordination relative to the formation of the enamel knot, a signaling center in the invaginated oral epithelium critical for the morphogenesis of the dental cusps. In this context, BMP4 is expressed by the underlying mesenchyme, and BMP2 and BMP7 are expressed predominantly in the oral epithelium (4). Consistent with a role of BMPs in promoting formation of dental cusps, mice deficient for the secreted inhibitor of BMP signaling, ectodin, display expanded dental cusps (4, 5). Likewise, suppression of BMP signaling by transgenic expression of follistatin, a secreted inhibitor of BMP/TGFβ superfamily signaling, driven by the K14 promoter blocks the differentiation and enamel matrix synthetic capacity of ameloblasts, the cell type responsible for enamel secretion and the absence or near absence of dental cusp formation (6). In particular, BMP stimulation appears to up-regulate expression of the cell cycle inhibitor p21 in the oral epithelium near the enamel knot, inhibition of which is needed for normal dental cusp formation (7).

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1 These authors contributed equally to this work.
2 To whom correspondence may be addressed: Dept. of Pathology, Brigham and Women’s Hospital, 75 Francis St., Boston, MA 02115. Tel.: 617-732-7510; Fax: 617-277-9015; E-mail: mag3003@med.cornell.edu.
3 To whom correspondence may be addressed: Dept. of Pathology and Laboratory Medicine, Weill Cornell Medical College, 1300 York Ave. E907, New York, NY 10065. Tel.: 212-746-2078; Fax: 212-746-9215; E-mail: jas2060@med.cornell.edu.
4 The abbreviations used are: BMP, bone morphogenetic proteins; En, embryonic day; ALC, ameloblast-like cell; DSPP, dentin sialophosphoprotein; μCT, micro-computed tomography.
because BMP4-soaked beads induce p21 expression. p21 is believed to contribute to the formation of dental cusps by slowing cellular proliferation in the ectodermal layer relative to that of the mesenchymal layer. p21 expression is itself inversely correlated with the expression of ectodin (7). Although p21-deficient mice have not been reported to have any defects in tooth cusp patterning, this may reflect redundancy with similar cell cycle inhibitors (8). Additionally, BMPs continue to play a role in the teeth of adult mice, because suppression of BMP signaling with transgenic expression of follistatin by the K14 promoter results in suppression of the differentiation and enamel matrix synthetic capacity of ameloblasts (6).

Generally BMPs signal through two main pathways: the SMAD transcription factors and MAPK pathways. In the context of osteoblast and chondrocyte differentiation, both the SMAD and the p38 MAPK pathways contribute to BMP function (9–11), whereas a prior study concluded that the p38 pathway and SMAD4 are functionally redundant in the oral epithelium, because inactivation of the p38 pathway produced no effect unless the SMAD pathway was also targeted with genetic ablation of SMAD4 (12). However, this conclusion was drawn on the basis of treatment of mandibular organ cultures with the p38 inhibitor SB203580, and the effect of genetic ablation of p38 signaling had not been studied. Moreover, in addition to a lack of definitive data regarding the role of MAPKs in mediating the effects of BMPs, nearly every other secreted ligand implicated in morphogenesis of ectodermal structures, including FGFs and WNTs, has the capacity to activate MAPKs (13). Thus, the relative contribution of each of these pathways to MAPK activation in this context remains to be fully elucidated.

Here, we have sought to determine the contribution of the p38 MAPK pathway to dental development and enamel production. In particular, we find that epithelial-specific deletion of p38α in p38α<sup>K14</sup> mice results in defects in the morphogenesis of dental cusps and enamel secretion, because of defective signal transduction by BMPs in the absence of p38α.

**EXPERIMENTAL PROCEDURES**

**Mice**—Both the p38α floxed allele and p38α<sup>K14</sup> mice were previously described (14, 15). Mice bearing Mkk3 and Mkk6 null alleles were also previously described (16, 17). Mkk6<sup>+/−</sup>, Mkk3<sup>−/−</sup>, and Tak1<sup>K14</sup> mice were all maintained on the C57BL/6 background and housed in accordance with the policies of the institutional animal care and use committee. For all mice analyzed, age- and gender-matched WT C57BL/6 mice housed in the same facility or littermate control mice were used.

**In Situ Hybridization and Immunohistochemistry**—In situ hybridization was performed as previously described (9). Briefly, digoxigenin-labeled antisense probes were generated to the indicated targets, hybridized with paraffin sections, and visualized using an anti-digoxigenin HRP conjugate system. Immunohistochemistry was also performed as previously described (11).

**Vickers Microhardness Testing**—Maxillary and mandibular incisors were resin embedded, polished, and tested for enamel microhardness on a LECO M 400 HI testing machine (LECO, St. Joseph, MI), using a load of 25 g for 5 s with a Vickers tip. Thirty indentations per enamel sample were scored.

**Scanning Electron Microscopy**—Scanning electron microscopy was performed as previously described (18). Briefly, incisors were dried in air and acetone, fastened to stubs, sputter-coated, and visualized on a JEOL 6400 scanning electron microscope.

**μCT Analysis of Dental Enamel**—Skulls of the indicated mice were stripped of skin, immersed in 70% EtOH, and scanned in a μCT-35 (Scanco Medical Co.) at 70 kV, 114 μA, and 0.012 isotropic voxels. For quantitative analysis, individual mandibular incisors were contoured and analyzed using the Scanco software platform.

**Tissue Culture and in Situ Hybridization**—Oral epithelium from E14.5 embryos was dissected and cultured as previously described (4, 6). Tissue explants were treated with BSA (1 ng/ml; Sigma) or BMP2/7 (100 ng/ml; R&D Systems) in the presence or absence of SB203580, a p38α and β MAPK inhibitor (10 μM; EMD Millipore) for 16 h at 37 °C. After culture, explants were treated with cold MeOH for 2 min, fixed in 4% paraformaldehyde overnight at 4 °C and processed for whole mount in situ hybridization as previously described (6). The p21 probe was kindly provided by Dr. Irma Thesleff (7).

**FIGURE 1. Expression and activation of p38 signaling components in ameloblasts.** A, immunohistochemical staining for phospho-MKK3/6, MKK3, and MKK6 on mandibular incisors from 4-day-old WT mice. The expressions in ameloblasts are indicated with red arrows. B, immunohistochemical (IHC) staining for phospho-p38 on secretory ameloblasts from the mandibular incisors of 6-week-old p38α<sup>K14</sup> and WT mice.
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A

WT

p38αK14

B

WT

p38αK14

C

WT

p38αK14

Mandibular incisors

Molar, cross section

D

6 week old

3 week old

E

Dentin

Soft tissue

Vessels

mg Hydroxyapatite/cm³

F

WT

p38αK14
**Cell Culture and Antibodies**—The ameloblast-like cell (ALC) line was kindly provided by Dr. Toshihiro Sugiyama (19) and maintained in DMEM containing 10% dialyzed FBS, 2 mM L-glutamine, 1% HEPES, 1% nonessential amino acids, and 1% penicillin/streptomycin. The following antibodies were used: anti-GAPDH (Affinity Bioreagents); anti-phospho-MK3/6, MKK3, phospho-p38, p38α, phospho-Smad1/5, Smad4, and RhoA (Cell Signaling Technology); and anti-β-integrin and proliferating cell nuclear antigen (Santa Cruz Biotechnology).

**p21 Luciferase Reporter Assay**—For reporter gene assay, the ALC line was plated at 2 × 10^3 cells/well in 12-well plates. After 24 h post-transfection with the p21-luc reporter gene (a generous gift from Dr. Arthur Skoultchi) and Renilla along with vector or M KK6-CA mutant using Effectene transfection reagent (Qiagen), cells were treated with vehicle or BMP2/7 in the absence or presence of SB203580. Alternatively, cell were infected with lentivirus expressing control, murine Smad4 or RhoA shRNA and then transfected with the p21-luc reporter gene and Renilla. The luciferase activity of cell lysates was measured using a luciferase reporter assay system (Promega). Reporter activity was normalized to the protein content of the cell lysates.

**Statistics**—All statistical analysis was performed in the GraphPad prism software package. All values graphed are means ± standard deviation.

## RESULTS

**Expression and Activation of p38 Signaling Components in Ameloblasts**—To examine activation of the p38 MAPK pathway in ameloblasts in vivo, immunohistochemistry was performed for the MAP2Ks upstream of p38, MKK3, and MMK6 and their phosphorylation levels in mandibular incisors (Fig. 1A). MKK3 and MMK6 are highly expressed and phosphorylated in both ameloblasts and odontoblasts. Likewise, phosphorylation of p38 MAPKs is observed in both pressecretory and secretory ameloblasts and was nearly ablated in p38α/C14 mice. (Fig. 1B) Thus, the p38 MAPK pathway is present and activated in ameloblasts in vivo.

**Mice Lacking p38α in Ectoderm Display Defects in Dental Enamel Production and Impaired Dental Cusp Morphogenesis**—To determine the contribution of the p38 MAPK pathway to tooth development, mice with a skin-specific deletion of the p38 gene and p38/ were examined using μCT (Fig. 2A and quantitative (Fig. 2E) analyses does not result from shifting the enamel peak so that it falls below an arbitrary threshold, but the enamel peak is greatly reduced in size, without a shift in the mode toward lower densities. Consistent with this μCT analysis, histologic analysis of p38α/C14 incisors shows occasional disruption of the ameloblast layer, implying that adhesion between ameloblasts and enamel is defective in the absence of p38α (Fig. 2F).

Next, the Vickers hardness test was performed to assess the biomechanical properties of the remaining enamel. As shown in Fig. 3A, the hardness of the enamel covering both the maxillary and mandibular incisors was significantly reduced in p38α/C14 mice. Likewise, scanning electron microscopy confirmed the reduction in enamel thickness and demonstrated that, although the remaining enamel displays abnormal biomechanical properties, it shows a relatively normal ultrastructure (Fig. 3B). Additionally, we examined expression of ameloblast marker genes in mandibular incisors of p38α/C14 mice using immunohistochemistry and in situ hybridization analyses. As expected, β-integrin, amelogenin, and dentin sialophosphoprotein (DSPP) are expressed in WT ameloblasts. However, their expression levels were substantially reduced in p38α/C14 ameloblasts (Fig. 3, C and D) (20).

Previously, both p38α and p38β were demonstrated to contribute to skeletal mineralization and osteoblast differentiation (11). Surprisingly, both tooth morphogenesis and quantitative measures of enamel production are normal in p38β/ mice, suggesting that the contribution of p38α to tooth biology is highly selective (Fig. 3, E and F). Therefore, p38α activity is necessary for both the morphogenesis of dental cusps and effective enamel secretion.

**MAP2K MKK6 but Not MAP2K MKK3 Contributes to the Formation of Dental Enamel**—To determine the roles of the MAP2Ks upstream of p38, MKK3, and MKK6 in tooth formation, MKK3/−/−, M KK6/−/−, and MKK3/−/− M KK6/−/− mice were
FIGURE 3. Defects in dental enamel production and ameloblast activity in p38αK14 mice. A, nanoindentation studies of incisors from 6-week-old p38αK14 and WT mice showing decreased enamel hardness, both by quantitative measure (left panels) and by visualization of the probe indentation site (right panels). ** indicates p < 0.01 by a two-tailed unpaired Student's t test; * indicates p < 0.05. VHN, Vickers hardness number. B, scanning electron microscopy analysis of mandibular incisors from 6-week-old p38αK14 and WT mice showing decreased thickness of the enamel layer (red arrows). Bar, 100 μm. C, immunohistochemical (IHC) staining for β4-integrin on mandibular incisors from 4-day-old WT and p38αK14 mice. D, in situ hybridization (ISH) for amelogenin and DSPP on mandibular incisors from 4-day-old WT and p38αK14 mice. The region of DSPP expression in ameloblasts is indicated with a red arrow. E, histogram plotting density on the x-axis versus the volume in voxels corresponding to that density in mandibular incisors from 4-week-old p38β−/− and WT mice. F, quantitation of enamel thickness and enamel volume relative to total tooth volume in the mandibular incisors of 4-week-old p38β−/− and WT mice.
examined. Because Mkk3<sup>−/−</sup>Mkk6<sup>−/−</sup> mice are embryonic lethal, tooth phenotypes were analyzed in Mkk3<sup>−/−</sup>Mkk6<sup>+/−</sup> mice. In μCT analysis windowed to display only tooth enamel, Mkk6<sup>−/−</sup> mice showed relatively sparse and uneven enamel deposition most evident along the maxillary and mandibular incisors (Fig. 4A). This was confirmed by histogram analysis.
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A

![Image](image_url)

FIGURE 5. p38α acts in the enamel knot to influence dental cusp formation. A, coronal sections were cut from p38αK14 and WT embryos at the indicated ages. B–D, coronal sections were cut from E14.5 p38αK14 and WT embryos, and in situ hybridization (ISH) or immunohistochemistry (IHC) was performed as indicated. Arrows indicate cusp formation areas.

showing a reduced peak in the enamel density range (Fig. 4B). Accordingly, contouring of individual mandibular incisors demonstrated a decrease in both total enamel volume and enamel thickness (Fig. 4C). Similar to the phenotype of p38αK14 mice, scanning electron microscopy of mandibular incisors showed a decrease in the thickness of the enamel layer and an apparently normal ultrastructural enamel lattice pattern in Mkk6−/− mice (Fig. 4D). In contrast to these findings, both Mkk3−/− and Mkk3−/−Mkk6+/- mice showed no defects in dental enamel, suggesting that contribution of M KK6 to tooth development is highly selective (Fig. 4, E and F). Thus, M KK6-deficient mice display phenotypic defects consistent with a contribution to p38α activation in ameloblasts; however, the mild phenotype argues that other mechanisms of p38α activation are likely also involved.

p38α Regulates Cell Proliferation in the Enamel Knot—Pattern of dental cusps occurs in a specialized region of developing teeth termed the enamel knot. Because teeth form at the interface between invaginated oral epithelium and the underlying mesenchyme, local variations in cell proliferation create “ripples” in this interface, and these ripples result in dental cusps (21). First, to examine embryonic tooth development in p38αK14 mice, tooth development was examined in E13.5, E15.5, and E18.5 mouse embryos (Fig. 5A). Consistent with the major role of p38α in tooth morphogenesis being restricted to participating in cusp formation at the enamel knot, defects in morphology were not detected in the bud stage of E13.5 embryos prior to formation of the enamel knot, and time points thereafter displayed impairment in the folding of the mesenchymal/epithelial interface characteristic of cusp formation. To focus on the role of p38α in the formation of dental cusps, activation of the p38 MAPK pathway was characterized in the primary enamel knot of E14.5 WT and p38αK14 embryos using immunohistochemistry for phospho-MKK3/6. As shown in Fig. 5B, activation of the p38 pathway occurs throughout the oral epithelium in the tooth bud. Because altered proliferation is an important mechanism for dental cusp morphogenesis, we next assessed cell proliferation in the primary enamel knot using immunohistochemistry for proliferating cell nuclear antigen (Fig. 5C). WT mice show an absence of cell proliferation corresponding to the enamel knot (21), whereas a corresponding region was not present in p38αK14 mice (indicated with arrows). Induction of the cyclin-dependent kinase inhibitor p21 has been reported to contribute to the halt in proliferation at the enamel knot (7). Consistent with immunohistochemistry for proliferating cell nuclear antigen, induction of p21 is impaired in p38αK14 mice (indicated with an arrow), providing a mechanism for the dysregulated proliferation in enamel knots lacking p38α (Fig. 5D).

p38α Mediates BMP Signaling during Tooth Development—The defects in dental cusp formation and enamel secretion present in p38αK14 mice are very similar to those in mice with transgenic expression of the BMP inhibitor follistatin in dental epithelium (4, 6). Together with a previous report showing that p21 expression is directly regulated by BMPs in the enamel knot (7), this suggests that p38 may function primarily downstream of BMPs in the context of tooth development and enamel secretion. To directly examine BMP-induced activation of the p38 MAPK pathway in ameloblasts, the ameloblast-like cell line ALC (19) was stimulated with BMP2/7 at various time points, and the phosphorylation levels of p38 and MKK3/6 were examined by immunoblotting analysis (Fig. 6A). Phosphorylation levels of SMAD1/5, BMP receptor-regulated SMAD proteins, peaked at 15 min of stimulation and gradually decreased, whereas phosphorylation levels of MKK3/6 and p38 reached highest levels after 60 min of stimulation and were sustained thereafter. As expected, BMP2/7-induced phosphorylation of p38, but not MKK3/6, was blocked by the p38 inhibitor SB203580 (Fig. 6B).

To examine the importance of the p38 MAPK pathway for BMP-induced gene expression in ameloblasts, the ALC line was incubated with BMP2/7 for 3 h in the presence or absence of SB203580, and gene induction was measured by RT-PCR analysis. BMP2/7 stimulation modestly induced expression of the enamel matrix components amelogenin and ameloblastin, and this was blocked by addition of SB203580 (Fig. 6C). This indicates that p38 activation is necessary for BMP-induced expression of amelogenin and ameloblastin. Additionally, BMP2/7-induced expression of p21 was also blocked by SB203580, which is consistent with the defective p21 expression in the enamel knot of p38αK14 mice (Fig. 5D). Likewise, p21 transcriptional activity was modestly increased by BMP2/7 stimulation in the ALC line, and this was inhibited by SB203580, indicating
FIGURE 6. The p38 pathway transduces BMP signals in ameloblasts. A, ALC line was stimulated with BMP2/7 (100 ng/ml) for the indicated duration, and lysates were immunoblotted with the indicated antibodies. B, ALC line was pretreated with SB203580 (1 or 10 μM) for 1 h and then treated with BMP2/7 (100 ng/ml) for 30 min. Lysates were immunoblotted (IB) as indicated. C, after pretreatment with SB203580 (10 ng/ml) for 1 h, ALC line was stimulated with BMP2/7 (100 ng/ml) for 3 h, and the relative levels of the indicated transcripts were determined by RT-PCR. Pretreatment with SB203580 resulted in a significant decrease for expression of amelogenin, ameloblastin, and p21 (**p < 0.01, indicated by a two-tailed Student’s t test). D, ALC line was transfected with a luciferase reporter construct driven by the p21 promoter (p21-luc). After pretreatment with SB203580 for 30 min, cells were incubated with BMP2/7 for 48 h, and luciferase activity was measured. p21 luciferase activity was normalized to Renilla. Vehicle versus BMP, **p < 0.01, indicated by a two-tailed Student’s t test; p < 0.05, indicated with *, all by a two-tailed Student’s t test. E and F, total RNAs were isolated from the ALC line expressing control vector or a constitutive active (CA) mutant of MKK6, and the effect on gene expression determined by RT-PCR (E). Alternatively, ALC line was transfected with p21-luc reporter gene and Renilla in the absence or presence of MKK6-CA and p21-luc activity was analyzed at 24 h after transfection (F). p21 luciferase activity was normalized to Renilla. ** indicates p < 0.01, and * indicates p < 0.05 by a two-tailed Student’s t test. G, explants of embryonic oral epithelium isolated from E14.5 embryos were treated with vehicle, BMP2/7 alone, or BMP2/7 and SB203580 for 16 h, and whole mount in situ hybridization for p21 was performed. Shown are multiple representative images for each condition.
that the p38 MAPK pathway is required for BMP-induced expression and activation of p21 in ameloblasts (Fig. 6, C and D). To directly examine the contribution of p38 activation to induce these genes, a constitutively active MKK6 mutant (MKK6-Ca) that enforced activation of the p38 pathway was stably expressed in the ALC line via lentivirus-mediated delivery. As expected, exogenous expression of MKK6-Ca was sufficient to induce expression of amelogenin and ameloblastin (Fig. 6E). A similar effect of constitutively active MKK6 to induce transcriptional activation of p21 was also observed (Fig. 6F). Thus, p38 activation is essential for gene induction by BMPs in ameloblasts.

To demonstrate the relevance of this observation in a physiological system, we conducted ex vivo culture of explants of embryonic dental epithelium isolated from E14.5 WT embryos. The explants were stimulated with BMP2/7 in the presence or absence of SB203580, and whole mount in situ staining for p21 was performed to assess BMP-induced expression of p21 (Fig. 6G). BMP2/7 stimulation induced p21 expression in the oral epithelium, and this was blocked by addition of SB203580. Thus, both in vivo and in vitro evidence demonstrates that p38 acts in oral epithelium to induce p21 expression.

**SMAD4-dependent Pathways Are dispensable for Induction of Ameloblastin, Amelogenin, and p21 in Vitro**—Because stimulation with BMPs activates both MAPK and SMAD pathways, we examined whether the SMAD pathway is required for the ability of BMP2/7 to induce expression of these targets. To this end, SMAD4, which is classically required for SMAD signaling, was deleted in the ALC line via lentivirus-mediated delivery of murine Smad4 shRNA, although recent work has proven the existence of SMAD4-independent SMAD signaling pathways (Fig. 7A) (10). SMAD4 deficiency had no effect on the ability of BMP2/7 to induce transcriptional activation of p21 or expression of amelogenin or ameloblastin (Fig. 7, B and C). Thus, BMP-induced SMAD activation is likely to be dispensable for gene induction and p21 activation in ameloblasts.

**RhoA Mediates Activation of the p38 Pathway Downstream of BMP Signaling**—Next, we sought to identify upstream signal transducers of the MKK6/p38α signaling axis in BMP signaling. Given that MAP3K TAK1 functions as a critical upstream activator of the MKK3/6-p38α/β MAPK signaling axis in long bones (11), we examined whether TAK1 plays a similar role upstream of p38 in ameloblasts in vivo by using Tak1K14 mice. Consistent with a previous study (22), these mice develop a lethal dermatitis within 2 weeks of age. Despite limiting analysis of their teeth to the first 2 weeks of life, Tak1K14 mice show no apparent defects in cusp morphogenesis (Fig. 8A). Thus, although TAK1 is able to activate the p38 MAPK pathway in long bones, it is dispensable for the ectodermal contribution to tooth development. A number of mice lacking other MAP3Ks, including Mekk2 (MAP3K2), Tpl2 (MAP3K8), and Taok3 (MAP3K18), were also obtained, and their tooth phenotypes were examined. Similar to Tak1K14 mice, no defects were found to display detectable by μCT in adult mice (data not shown), whereas Mlk3 (MAP3K11)-deficient mice display severe defects in dentin formation as we previously reported (23). Taken together, these findings demonstrate that the MAP3Ks mediating p38 activation in ameloblasts are distinct from those in other tissues, although further investigation will be needed to identify the exact genes responsible.

Therefore, we examined whether a small G protein, which has been known to activate various MAP3Ks, might be required for activation of the p38 pathway in ameloblasts. In particular, we tested involvement of RhoA in ameloblast gene induction and p21 activity, given that it has been described to contribute to dentin enamel formation (24, 25). Intriguingly, knockdown of RhoA results in a significant reduction in BMP-induced...
phosphorylation of both MKK3/6-p38 and SMAD1/5 in the ALC line (Fig. 8, B and C). Likewise, BMP2/7-induced expression of ameloblastin, amelogenin, and p21 was reduced in RhoA-deficient ALC line (Fig. 8D). BMP2/7-induced transcriptional activation of p21 was also ablated by RhoA knockdown in the ALC line (Fig. 8E).

DISCUSSION

Here we demonstrate that p38α MAPK is a crucial mediator of the effects of BMPs in tooth development and enamel secretion. In these contexts, p38α is required for expression of p21 in embryonic enamel knot and for expression of amelogenin and β3-integrin in ameloblasts. Biochemical analysis using ALC ameloblast-like cell line showed that the RhoA-MKK6-p38α signaling axis is crucial for induction of p21, amelogenin, and ameloblastin genes and p21 transcriptional activation by BMP2/7, and in this in vitro system, the p38 pathway plays a dominant role over SMAD4-dependent pathway in expression of these targets and p21 activation. However, this conclusion is subject to the limitations of the existence of SMAD4-independent SMAD signaling pathways and the in vitro knockdown system used.

The role of p38α in the formation of ectodermal appendages is not limited to teeth, because p38αK14 mice display defects in the development of hair, including loss of coat hair and a complete absence of awl hairs, one of the morphologic classes of pellage hairs in mice up to 3 weeks of age. The meibomian glands, footpad glands, and nails in p38αK14 mice were morphologically intact. Thus, p38α selectively mediates the development of a subset of ectodermal appendages (data not shown).

Consistent with the in vivo finding of defective tooth cusp morphogenesis in the absence of p38α, in vitro models demonstrate that the p38 MAPK pathway mediates expression of p21, amelogenin, and ameloblastin in response to BMP2/7. This finding is also consistent with reports demonstrating that p21 expression is regulated by the p38 MAPK pathway in other tissues (26). Interestingly, in the context of gamma radiation-induced arrest in the cell cycle, the effect of p38 on p21 mRNA levels is post-transcriptional, mediated via phosphorylation and accumulation of the mRNA stability factor HuR (27). This raises the possibility that a similar mechanism may apply to p38 regulation of p21 expression in the enamel knot.

In addition to mice lacking p38α in oral epithelium, the dentition of mice lacking p38β was also examined, and no defects were detected. Thus, whereas both p38α and p38β contribute to osteoblast differentiation and long bone mineralization, only p38α functions in ameloblasts. Consistent with the lack of a dental phenotype in p38β−/− mice, immunohistochemical staining with an antibody recognizing pan isoform phosphop38 is nearly absent in p38αK14 mice, arguing that p38α is the predominant p38 isoform active in ameloblasts.
The finding that expression of $\beta_4$-integrin, amelogenin and DSPP in ameloblasts are all defective in the absence of p38$\alpha$ indicates that the functional defects in adult p38$\alpha$K14 ameloblasts are likely to be multifactorial, involving defective expression of both enamel matrix genes and adhesion molecules. These defects, along with the finding that the p38 MAPK pathway is activated in both neonatal and adult ameloblasts, suggest that the requirement for p38 activation in ameloblasts is continual and that the reduction in enamel secretion in p38$\alpha$K14 mice is unlikely to be secondary to developmental effects. Notably, $\beta_4$-integrin deficiency in either humans or mice results in impaired epidermal basement membrane adherence, absence of hemidesmosomes, and the clinical syndrome of epidermolysis bullosa (28, 29). Deficient expression of molecules such as integrin-$\alpha 4$ involved in basement membrane adhesion may explain the observation that the ameloblast cell layer is disrupted and displays intussusception relative to the enamel surface.

Additionally, the contribution of MKK3 and MKK6 MAP2Ks upstream of p38$\alpha$ to tooth biology was examined. Because both MKK3 and MKK6 can mediate the two activation loop phosphorylation events required for p38$\alpha$ activation, they are believed to be partially redundant at the biochemical level (30). However, in the setting of skeletal mineralization, both MKK3 and MKK6 contributed to long bone mineralization, whereas only MKK3 contributed significantly to calvarial mineralization. This implies that two MAP2Ks can have partially overlapping biochemical roles but distinct functions in vivo (11). In our analysis of dentition, Mkk6$^{-/-}$ but not Mkk3$^{-/-}$ mice displayed a mild defect in enamel production, and both Mkk3$^{-/-}$ and Mkk6$^{-/-}$ mice had normal dental cusp formation and coat hair development. Because only MKK6 but not MKK3 has a role in dental enamel production, and the phenotype of Mkk6$^{-/-}$ is substantially less severe than that of p38$\alpha$K14 mice, one may propose that another MAP2K plays a partially redundant role in enamel production. One obvious candidate for this is Mkk4, which has been observed to contribute to residual p38 activation by UV light in Mkk3$^{-/-}$/Mkk6$^{-/-}$ embryonic fibroblasts (31). If Mkk4 acts in ameloblasts to regulate enamel production, this would be the first observation of a physiologic role for Mkk4 in p38 activation.

Although ectodysplasins are also associated with morphogenesis of enamel knot and development of certain types of pellage hair, several lines of evidence argue that p38$\alpha$ does mediate their effects in vivo (32). Defects in EDA signaling result in impaired morphogenesis of lacrimial and meibomian glands, but they are intact in p38$\alpha$K14 mice (33). Similarly, the transcriptional hallmark of impaired EDA signaling in the enamel knot is dysregulated expression of Shh (sonic hedgehog), not p21 as we observe in p38$\alpha$K14 mice (34). This apparent lack of a role for p38$\alpha$ in ectodysplasin signaling may correlate with in vitro observations that, contrary to many other TNF family receptors, overexpression of the ectodysplasin receptor EDAR was not associated with induction of JNK or p38 MAPK activity in vitro (35).

Lastly, whereas TAK1 is necessary for the activation of p38 by BMPs in osteoblast and chondrocyte differentiation (9, 11), TAK1K14 mice do not recapitulate the phenotype of p38$\alpha$K14 mice. A similar lack of effect was observed in mice lacking the MAP3Ks Tpl2, Mekk2, or Taok3. Mice lacking the MAP3K Mlk3 have a profound defect in dentin production and corresponding spontaneous tooth fracture, which complicates assessment of ameloblast function (23). However, Mlk3-deficient mice clearly do not display the defects in tooth cusp formation seen in p38$\alpha$K14 mice. This argues that substantial differences exist between the means of proximal activation of the p38 MAPK pathway in the oral epithelium versus other tissues. This discordance between the BMP signaling pathways in ameloblasts and those observed in other contexts suggests that an alternative pathway to the well studied SMAD or TAK1-dependent MAPK pathways is involved. Supporting this conclusion, RhoA was found to contribute to both SMAD and p38 activation in ameloblasts. This raises the possibility that the RhoA/ROCK pathway is mediating activation of the p38 pathway in this context (36). Although mice expressing a dominant negative RhoA transgene have defects in dental enamel, it is unclear whether they recapitulate the same defects in morphogenesis as seen in p38$\alpha$K14 mice (24, 25). Further in vivo studies with RhoA conditional mice and mice deficient in ROCK1 and 2 are necessary to further substantiate that RhoA acts upstream of the p38 pathway in the oral epithelium.

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REFERENCES

1. Abdollah, S., Macias-Silva, M., Tsukazaki, T., Hayashi, H., Attisano, L., and Wrana, J. L. (1997) TbetaRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. J. Biol. Chem. 272, 27678–27685
2. Jernvall, J., and Thesleff, I. (2012) Tooth shape formation and tooth renewal: evolving with the same signals. Development 139, 3487–3497
3. Mikkola, M. L. (2008) TNF superfamily in skin appendage development. Cytokine Growth Factor Rev. 19, 219–230
4. Wang, X. P., Suomalainen, M., Jorgesson, C. J., Matzuk, M. M., Wanker, M., Werner, S., and Thesleff, I. (2004) Modulation of activin/bone morphogenetic protein signaling by follistatin is required for the morphogenesis of mouse molar teeth. Dev. Dyn. 231, 98–108
5. Laurikka, J., Kassai, Y., Pakkasjarvi, L., Thesleff, I., and Itoh, N. (2003) Identification of a secreted BMP antagonist, ectodin, integrating BMP, FGF, and SHH signals from the tooth enamel knot. Dev. Biol. 264, 91–105
6. Wang, X. P., Suomalainen, M., Jorgesson, C. J., Matzuk, M. M., Werner, S., and Thesleff, I. (2004) Follistatin regulates enamel patterning in mouse incisors by asymmetrically inhibiting BMP signaling and ameloblast differentiation. Dev. Cell 7, 719–730
7. Jernvall, J., Aberg, T., Kettunen, P., Keranen, S., and Thesleff, I. (1998) The life history of an embryonic signaling center: BMP-4 induces p21 and is associated with apoptosis in the mouse tooth enamel knot. Development 125, 161–169
8. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J., and Leder, P. (1995) Mice lacking p21Cip1/Waf1 undergo normal development, but are defective in G1 checkpoint control. Cell 82, 675–684
9. Shim, J. H., Greenblatt, M. B., Xie, M., Schneider, M. D., Zou, W., Zhai, B., Gygi, S., and Gimlich, L. H. (2009) TAK1 is an essential regulator of BMP signalling in cartilage. EMBO J. 28, 2028–2041
10. Retting, K. N., Song, B., Yoon, B. S., and Lyons, K. M. (2009) BMP canonical Smad signaling through Smad1 and Smad5 is required for endochondral bone formation. Development 136, 1093–1104
11. Greenblatt, M. B., Shim, J. H., Zou, W., Sitara, D., Schweitzer, M., Hu, D., Lotinun, S., Sano, Y., Baron, R., Park, J. M., Arthur, S., Xie, M., Schneider,
M. D., Zhai, B., Gygi, S., Davis, R., and Glimcher, L. H. (2010) The p38 MAPK pathway is essential for skeletogenesis and bone homeostasis in mice. J. Clin. Invest. 120, 2457–2473

12. Xu, X., Han, J., Ito, Y., Brinas, P. Jr., Deng, C., and Chai, Y. (2008) Ectodermal Smad4 and p38 MAPK are functionally redundant in mediating TGF-beta/BMP signaling during tooth and palate development. Dev. Cell 15, 322–329

13. Shim, J. H., Greenblatt, M. B., Zou, W., Huang, Z., Wein, M. N., Brady, N., Hu, D., Charron, J., Brodkin, H. R., Petsko, G. A., Zaller, D., Zhai, B., Gygi, S., Glimcher, L. H., and Jones, D. C. (2013) Schnurri-3 regulates ERK downstream of WNT signaling in osteoblasts. J. Clin. Invest. 123, 4010–4022

14. Kim, C., Sano, Y., Tutoda, M., Carlson, B. A., Arpa, L., Celada, A., Lawrence, T., Otsu, K., Brissette, J. L., Arthur, J. S., and Park, J. M. (2008) The kinase p38alpha serves cell type-specific inflammatory functions in skin injury and coordinates pro- and anti-inflammatory gene expression. Nat. Immunol. 9, 1019–1027

15. Nishida, K., Yamaguchi, O., Hirotsu, S., Hikosai, S., Higuchi, N., Watanabe, T., Takeda, T., Osaka, S., Morita, T., Kondo, G., Uno, Y., Kashiwase, K., Tanaka, A., Nakai, A., Matsumura, Y., Miyazaki, J., Sudo, T., Hongo, K., Kusakari, Y., Kurihara, S., Chien, K. R., Takeda, J., Horii, M., and Otsu, K. (2004) p38alpha mitogen-activated protein kinase plays a critical role in cardiomyocyte survival but not in cardiac hypertrophic growth in response to pressure overload. Mol. Cell. Biol. 24, 10611–10620

16. Wysk, M., Yang, D. D., Lu, H. T., Flavell, R. A., and Davis, R. J. (1999) Requirement of mitogen-activated protein kinase kinase 3 (MKK3) for tumor necrosis factor-induced cytokine expression. Proc. Natl. Acad. Sci. U.S.A. 96, 3763–3768

17. Tanaka, N., Kamanaka, M., Enslin, H., Dong, C., Wysk, M., Davis, R. J., and Flavell, R. A. (2002) Differential involvement of p38 mitogen-activated protein kinase kinases MKK3 and MKK6 in T-cell apoptosis. EMBO Reports 3, 785–791

18. Bartlett, J. D., Dobeck, J. M., Tye, C. E., Perez-Moreno, M., Stokes, N., Reynolds, A. B., Fuchs, E., and Skobe, Z. (2010) Targeted p120-catenin ablation disrupts dental enamel development. Dev. Cell 18, 785–791

19. Nakata, A., Kameda, T., Nagai, H., Ikegami, K., Duan, Y., Terada, K., and Otsu, K. (2004) p38alpha mitogen-activated protein kinase plays a critical role in cardiomyocyte survival but not in cardiac hypertrophic growth in response to pressure overload. Mol. Cell. Biol. 24, 10611–10620

20. Wysk, M., Yang, D. D., Lu, H. T., Flavell, R. A., and Davis, R. J. (1999) Requirement of mitogen-activated protein kinase kinase 3 (MKK3) for tumor necrosis factor-induced cytokine expression. Proc. Natl. Acad. Sci. U.S.A. 96, 3763–3768

21. Tanaka, N., Kamanaka, M., Enslin, H., Dong, C., Wysk, M., Davis, R. J., and Flavell, R. A. (2002) Differential involvement of p38 mitogen-activated protein kinase kinases MKK3 and MKK6 in T-cell apoptosis. EMBO Reports 3, 785–791

22. Bartlett, J. D., Dobeck, J. M., Tye, C. E., Perez-Moreno, M., Stokes, N., Reynolds, A. B., Fuchs, E., and Skobe, Z. (2010) Targeted p120-catenin ablation disrupts dental enamel development. Dev. Cell 18, 785–791

23. Zou, W., Greenblatt, M. B., Shim, J. H., Kant, S., Zhai, B., Lotinun, S., Brady, N., Hu, D. Z., Gygi, S. P., Baron, R., Davis, R. J., Jones, D., and Glimcher, L. H. (2011) MKK3 regulates bone development downstream of the faciogenital dysplasia protein FGFD1 in mice. J. Clin. Invest. 121, 4383–4392

24. Xue, H., Li, Y., Everett, E. T., Ryan, K., Peng, L., Porecha, R., Yan, Y., Lucchese, A. M., Kuehl, M. A., Pugach, M. K., Bouchard, J., and Gibson, C. W. (2013) Ameloblasts require active RhoA to generate normal dental enamel. Eur. J. Oral Sci. 121, 293–302

25. Li, Y., Pugach, M. K., Kuehl, M. A., Peng, L., Bouchard, J., Hwang, S. Y., and Gibson, C. W. (2011) Dental enamel structure is altered by expression of dominant negative RhoA in ameloblasts. Cells Tissues Organs 194, 227–231

26. Lavelle, D., DeSimone, J., Hankewych, M., Kousnetzova, T., and Chen, Y. H. (2003) Decitabine induces cell cycle arrest at the G1 phase via p21(WAF1) and the G2/M phase via the p38 MAP kinase pathway. Leuk. Res. 27, 999–1007

27. Lafarga, V., Cuadrado, A., Lopex de Silanes, I., Bengoechea, R., Fernandez-Capetillo, O., and Nebreda, A. R. (2009) p38 Mitogen-activated protein kinase- and HuR-dependent stabilization of p21(Cip1)mRNA mediates the G1/S checkpoint. Mol. Cell. Biol. 29, 4341–4351

28. van der Neut, R., Krimpenfort, P., Calafat, J., Niessen, C. M., and Sonenberg, A. (1996) Epithelial detachment due to absence of hemidesmosomes in integrin beta 4 null mice. Nat. Genet. 13, 366–369

29. Vidal, F., Aberdam, D., Miquel, C., Christiano, A. M., Pulkkinen, L., Uitto, J., Ortonne, J. P., and Meneguzzi, G. (1995) Integrin beta 4 mutations associated with junctional epidermolysis bullosa with pyloric atresia. Nat. Genet. 10, 229–234

30. Zarubin, T., and Han, J. (2005) Activation and signaling of the p38 MAP kinase pathway. Cell Res. 15, 11–18

31. Branco, D., Tanaka, N., Jaeschke, A., Ventura, J. J., Kelkar, N., Tanaka, Y., Kyuuma, M., Takeshita, T., Flavell, R. A., and Davis, R. J. (2003) Mechanism of p38 MAP kinase activation in vivo. Genes Dev. 17, 1969–1978

32. Kere, J., Srivastava, A. K., Montonen, O., Zonana, J., Thomas, N., Ferguson, B. M., Morgan, D., Clarke, A., Baybayan, P., Chen, E. Y., Ezer, S., Saarialho-Kere, U., de la Chapelle, A., and Schlesinger, D. (1996) X-linked anhidrotic (hypohidrotic) ectodermal dysplasia is caused by mutation in a novel transmembrane protein. Nat. Genet. 13, 409–416

33. Gründberg, H. (1971) The glandular aspects of the tabby syndrome in the mouse. J. Embryol. Exp. Morphol. 25, 1–19

34. Tucker, A. S., Headon, D. J., Schneider, P., Ferguson, B. M., Overbeek, P., Tschopp, J., and Sharpe, P. T. (2000) Edar/Eda interactions regulate enamel knot formation in tooth morphogenesis. Development 127, 4691–4700

35. Koppinen, P., Pispa, J., Laurikka, J., Thesleff, I., and Mikkola, M. L. (2001) Signaling and subcellular localization of the TNF receptor Edar. Exp. Cell Res. 269, 180–192

36. Wang, Y. K., Yu, X., Cohen, D. M., Wozniak, M. A., Yang, M. T., Gao, L., Eyckmans, J., and Chen, C. S. (2012) Bone morphogenetic protein-2-induced signaling and osteogenesis is regulated by cell shape, RhoA/ROCK, and cytoskeletal tension. Stem Cells Dev. 21, 1176–1186