Neurotrophic Factor Neurotrophin-4 Regulates Ameloblastin Expression via Full-length TrkB

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Neurotrophic factors play an important role in the development and maintenance of not only neural but also nonneural tissues. Several neurotrophic factors are expressed in dental tissues, but their role in tooth development is not clear. Here, we report that neurotrophic factor neurotrophin (NT)-4 promotes differentiation of dental epithelial cells and enhances the expression of enamel matrix genes. Dental epithelial cells from 3-day-old mice expressed NT-4 and three variants of TrkB receptors for neurotrophins (full-length TrkB-FL and truncated TrkB-T1 and T2). Dental epithelial cell line HAT-7 expressed these genes, similar to those in dental epithelial cells. We found that NT-4 reduced HAT-7 cell proliferation and induced the expression of enamel matrix genes, such as ameloblastin (Ambn), a protein that represents the most abundant non-Aamel enamel matrix protein. We previously created Ambn-null mice, which develop severe enamel hypoplasia in which ameloblasts detached from the matrix, lost cell polarity, resumed proliferation, and formed multiple cell layers. These results suggest that Ambn is essential for ameloblast differentiation and enamel formation.

Mammalian development is a complex and highly orchestrated process that involves intricate cross-talk between growth factors and other regulatory molecules. The interaction between the epithelium and mesenchyme induces specific molecular and cellular changes that lead to organogenesis. These interactions are particularly crucial during the initiation of the development of ectodermal organs, such as teeth, skin, hair, and mammary and prostate glands (1). The oral epithelium provides the initial signaling for neuronal crest-derived ectomesenchyme development, and then both tissues interact during tooth formation. Various transcription factors, growth factors, and extracellular matrices are expressed by enamel matrix-producing ameloblasts during tooth development (2–4). The principal components of the enamel matrix that are synthesized by secretory ameloblasts can be classified into two major categories, amelogenin (Amel) and non-Amel, which includes ameloblastin (Ambn) and enamelin (Enam) (5). Ambn, also known as amelin or sheathlin, is a tooth-specific glycoprotein that represents the most abundant non-Amel enamel matrix protein. We previously created Ambn-null mice, which develop severe enamel hypoplasia in which ameloblasts detached from the matrix, lost cell polarity, resumed proliferation, and formed multiple cell layers. These results suggest that Ambn is essential for ameloblast differentiation and enamel formation.

Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 and -4 (NT-3 and NT-4, respectively) are structurally and functionally related and belong to the neurotrophin family, which promotes the development and survival of the vertebrate nervous system (7). Neurotrophins interact with two classes of cell surface receptors. The first class is Trk tyrosine kinase receptors that bind neurotrophins with a high affinity (8). TrkA mediates the biological...
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response of NGF, TrkC is activated by NT-3, and BDNF and NT-4/5 are the preferred ligands for TrkB (7). TrkB and TrkC have truncated transcripts at the C terminus (9–12). The second class is the common low affinity neurotrophin receptor, p75, which does not have a tyrosine kinase domain (13, 14). Further, neurotrophins have other regulatory roles during embryogenesis. NGF is a mitogenic factor for human epithelial cells (15), and NT-3 stimulates the proliferation of migratory neural crest cells (16). The expression of p75 may be required for kidney morphogenesis (17) and also promote apoptosis (18, 19). In the skin, the expression of BDNF and NT-4 is strikingly dependent on the hair cycle and peaks during spontaneous, apoptosis-driven hair follicle regression, known as catagen. NT-4 was also reported to accelerate catagen development in murine skin organ cultures. These results suggest that NT-4 is useful as an agent of hair growth control.

During tooth development, neurotrophic factors and their receptors are expressed in the tooth germ (20). However, their role in tooth development has not been elucidated. At the initiation stages of tooth germ development, NGF is expressed in the dental mesenchyme and weakly in the dental epithelium. At the bud stage, the majority of dental epithelial cells have lost their NGF expression, although NGF is still expressed in the inner dental epithelium and condensed mesenchyme. During later embryonic and early postnatal tooth development, NGF can be observed in the dental follicles. At the bell stage, NGF appears in epithelial cells of the stratum intermedium, whereas after birth it is restricted to cells located in the cervical part of the enamel organ. In the postnatal period, NGF is also detected in the dental papilla mesenchyme. BDNF is expressed in the region of developing rat teeth as well as in the mesenchyme under the developing skin of the mandibular process (20). In postnatal animals, BDNF is mainly detected in the dental papilla, and its expression pattern is correlated with the onset of dental innervation (21). NT-3 is expressed throughout the mesenchyme of the mandibular process at the initiation stage, whereas it appears in the epithelial cervical loops in the cap stage. During later stages, NT-3 expression is gradually restricted to the more cervical parts of the inner enamel epithelium and is no longer detected in postnatal tooth germs (20). The expression of NT-4 is restricted to epithelial cells. During subsequent development, expression persists in all dental epithelium components, including ameloblasts and the outer enamel epithelium as well as in the dental lamina (20, 21). Among neurotrophic factors, NT-4 is the only one detected in differentiated ameloblasts. These findings suggest that NT-4 may be important for dental epithelium differentiation and maintenance of ameloblast functions. However, the role of NT-4 in tooth development is unknown.

In the present study, we investigated the roles of NT-4 and TrkB in tooth development in vitro using dental epithelium cultures and in vivo using NT-4 knock-out mice. NT-4 and TrkB receptors were expressed in the dental epithelium of 3-day-old mice and in the HAT-7 dental epithelial cell line. We found that NT-4 inhibited proliferation and induced differentiation of HAT-7 cells. NT-4 treatment of HAT-7 cells increased mRNA expression for enamel matrix proteins Ambn, Enam, and dentin sialophosphoprotein. Further, NT-4-mediated induction of Ambn expression was regulated by the full-length TrkB-FL receptor and ERK1/2 pathway. In NT-4 knock-out mice, Ambn expression was dramatically reduced, and the enamel layer was thin. Our findings suggest that NT-4 plays a role in proliferation and differentiation of the dental epithelium and is required for the expression of enamel matrix genes.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HAT-7 cells, an epithelial cell line, and mDP cells, a dental mesenchymal cell line, were maintained in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin at 37 °C in a humidified atmosphere containing 5% CO2 (22). To transfect with the expression vectors for TrkB, HAT-7 cells were plated in a 60-mm plastic tissue culture plate (Falcon) at a density of 1 × 105 cells/3 ml/plate. To facilitate the detection of protein expression, V5 and His tags were fused to the C terminus of the two rat TrkB isoforms, TrkB-FL and TrkB-T1. TrkB-FL and TrkB-T1 cDNA were prepared from adult rat brain mRNA by RT-PCR and confirmed by DNA sequencing. The forward primer for TrkB-FL and TrkB-T1 was 5’-CTCTGACTGACTGGACCTGG-3’, and the reverse primer was 5’-GCCATAGGATGTCCAGGTTAGCGGC-3’ for TrkB-FL or 5’-CCATCCAGGTTAGCGGC-3’ for TrkB-T1. PCR was performed at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The PCR products were cloned into pEFe6/5-His-TOPO® (Invitrogen) according to the manufacturer’s protocol. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Stable transfectant cells for TrkB-FL and TrkB-T1 were selected in the presence of 5 μg/ml Blasticidin (Invitrogen).

Cell Proliferation and Bromodeoxyuridine (BrdUrd) Incorporation—Cells were plated at 1 × 105 cells/ml/well in 12-well plates for 24 h. Cell numbers were determined using a trypan blue dye exclusion method. For the BrdUrd incorporation assay, cells were incubated at the same cell density described above for 24 h prior to the addition of various growth factors. After treatments with various growth factors, BrdUrd (Sigma) was added to the plates (10 μM) for 30 min, and then the cells were fixed with cold methanol for 10 min, rehydrated in phosphate-buffered saline (PBS), and incubated for 30 min in 1.5 M HCl. After washing three times in PBS, the plates were rinsed with cold phosphate-buffered saline (PBS), and incubated for 30 min in 1.5 M HCl. After washing three times in PBS, the plates were incubated with a 1:50 dilution of fluorescein isothiocyanate-conjugated anti-BrdUrd antibody (Roche Applied Science) for 30 min at room temperature. Finally, the cells were washed in PBS three times and incubated with 10 μg/ml propidium iodide (Sigma) in PBS for 30 min at room temperature. BrdUrd-positive cells were examined under a microscope (Biozero-8000; Keyence, Japan).

Western Blotting—Cells were plated in 12-well plates at 1 × 105 cells/well for 1 day prior to NT-4 treatment. The cells were then treated with 100 ng/ml NT-4 for 0–60 min at 37 °C. Thereafter, they were washed twice with ice-cold 1 mM sodium orthovanadate (Sigma) in PBS, lysed with Nonidet P-40 buffer supplemented with a proteinase inhibitor mixture (Sigma) and phenylmethylsulfonyl fluoride at 4 °C for 10 min, and centrifuged, and then the supernatants were transferred to a fresh
tube. The cell lysates were separated by 12% SDS-PAGE and analyzed by Western blotting. The blotted membrane was incubated with antibodies, and the signals were detected with an ECL kit (Amersham Biosciences). ERK and second antibodies were purchased from Cell Signaling.

**RNA Isolation and RT-PCR—**Developing molars were dissected from mice on postnatal day 1 (P1), P3, and P7. Epithelial and mesenchymal tissues were separated from tooth germ from P3 mice under a microscope. Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s protocol. First strand cDNA was synthesized at 42 °C for 90 min using oligo(dT)$_{14}$ primer with SuperScript III (Invitrogen). PCR amplification was performed using the primers listed in supplemental Table 1. The PCR products were separated on a 1.5% agarose gel. The relative expression level was deduced from a standard curve constructed using the positive control sample and normalized against the expression level of HPRT in each sample.

**Protein Kinase-inhibitory Assay—**Serum-deprived HAT-7 cells were plated in 6-well plates and treated with 0.5 µM K-252a (Trk tyrosine kinase inhibitor; Calbiochem) and 100 nM TAT-Pep5 (p75NTR signaling inhibitor; Calbiochem) prior to treatment with NT-4 for 30 min. After 48 h, total RNA was extracted, and RT-PCR was performed.

**Preparation of Tissue Sections and HE Staining—**Mouse heads from P1, P3, and P7 wild-type and NT-4 null mice were fixed with 4% paraformaldehyde in PBS overnight at 4 °C. The tissues were decalcified with 250 mM EDTA/PBS for 3 days, dehydrated in xylene through a graded ethanol series, and then embedded in paraffin. Sections were sliced at 8 µm using a microtome (RM2125RT; Leica). For detailed morphological analysis of the molars, the sections were stained with Harris hematoxylin (Sigma) and Eosine Y (Sigma). The widths of enamel matrix and dentin were measured under a microscope (Biosozer-8000).

**RESULTS**

**Expression of NT-4 and TrkB Receptors in the Tooth Germs and Dental Cell Lines—**We first examined the expression of NT-4 and TrkB receptors in tooth germs and dental cell lines by RT-PCR. In tooth germs of P3 mice, NT-4 was highly expressed in the dental epithelium and weakly expressed in the dental mesenchyme (Fig. 1A). The full-length TrkB-FL and truncated TrkB-T1 and T2 were expressed in the dental epithelium. On the other hand, TrkB-T1, but not TrkB-FL or TrkB-T2, was expressed in the mesenchyme. Further, p75 expression levels were low in both the epithelium and mesenchyme (Fig. 1A). The expression patterns of NT-4, TrkB, and p75 in dental epithelial cell line HAT-7 and dental mesenchymal cell line mDP were similar to those in the tooth germ tissues, except for a low expression level of TrkB-FL in mDP cells and a high expression level of p75 in HAT-7 cells (Fig. 1B). These results suggest that NT-4, TrkB-FL, TrkB-T1, and TrkB-T2 are expressed in the dental epithelium and may regulate differentiation of the dental epithelium.

**Inhibition of Proliferation of HAT-7 Cells by NT-4—**We next examined the effect of NT-4 on HAT-7 cell proliferation (Fig. 2). HAT-7 cells were treated with NT-4, and cell proliferation was analyzed by BrdUrd incorporation for 1 h. The number of BrdUrd-positive cells was decreased by 30% after stimulation with NT-4 (Fig. 2A). We also found that the number of HAT-7 cells was decreased by about 25% when the cells were cultured with NT-4 for 24 h. BrdUrd incorporation after 1 h was analyzed using a fluorescence microscope (A and B). Cell numbers of HAT-7 cells cultured with or without NT-4 were counted using a trypan blue exclusion method after 1, 3, 5, and 7 days of culture (C). These experiments were repeated at least five times with similar results. Statistical analysis was performed using analysis of variance (*, p < 0.01).

**NT-4 Regulates Ameloblastin Expression—**To analyze the effects of neurotrophic factors on dental epithelium differentiation, NGF, BDNF, or NT-4 was added to HAT-7 cell cultures. After 48 h, total RNA was analyzed for the expression of ameloblast differentiation markers by RT-PCR. Ambn, Enam, dentin sialophosphoprotein (Dsp), osteopontin (Ost), and osteonecin (Osl) were induced by NGF, BDNF, and NT-4 (Fig. 3, A and B).
Amel was also induced by NT-4 (data not shown). This effect was similar on all of the various amelogenin isoforms. The expression level of Ambn induced by NT-4 was higher than those by NGF or BDNF (Fig. 3B). The induction of Ambn expression by BDNF or NT-4 was dose-dependent (Fig. 3C), with the higher level by NT-4. The expression of gap junctional proteins (Gja1) and transforming growth factor–β1 was the same between the control and neurotrophic factor–treated cells (Fig. 3D). These results indicate that NT-4 induces enamel matrix genes and promotes ameloblast differentiation.

**NT-4 and BDNF Induce Expression of Their Receptor but Not p75**—Since the expression level of TrkB receptors is important for NT-4 signaling, we examined their expression in HAT-7 cells with or without NT-4 by RT-PCR (supplemental Fig. 1). We found that TrkB-FL, TrkB-T1, and TrkB-T2 were highly induced by NT-4 and BDNF. NGF also induced the expression of TrkB-FL and TrkB-T1, but not TrkB-T2 (supplemental Fig. 1, A and B).

**Overexpression of TrkB-FL Enhances NT-4-mediated Ambn Induction**—NT-4 induced expression of Ambn, TrkB-FL, and truncated TrkB. However, it is not clear which receptor is active for NT-4-mediated Ambn induction. To assess this question, we stably transected HAT-7 cells with the TrkB-FL or TrkB-T1 expression construct, cultured them with NT-4, and analyzed Ambn expression by RT-PCR. Ambn expression was induced by NT-4 in untransfected cells as shown in Figs. 3, A and B, and after 30 min (Fig. 5A). Further, the level of phosphorylation of ERK2 was higher than that of ERK1 (Fig. 5B). Next, we examined the ERK1/2 phosphorylation level in TrkB-FL-transfected HAT-7 cells in the presence of NT-4 (Fig. 6, A and B). TrkB-FL-transfected cells showed strong activation of ERK1/2 at 5 min after the NT-4 treatment. However, the activation of ERK1/2 was not observed in TrkB-T1-transfected cells. These results indicate that full-length TrkB-FL is a major TrkB receptor for NT-4 signaling, and truncated TrkB-T1 acts as a dominant negative factor for dental epithelial cells.

**TrkB Inhibitor K252a Inhibits Ambn Expression**—NT-4 binds to TrkB and the low affinity receptor p75 and transduces downstream cellular signaling (8). To identify the signaling pathway involved in Ambn expression, we treated HAT-7 cells with the TrkB inhibitor K252a or p75 inhibitor TAT-pep5 prior to stimulation with NT-4 (Fig. 7, A and B). NT-4-mediated induction of Ambn was significantly inhibited by K252a and TAT-pep5. Moreover, the induction of TrkB receptors by NT-4 was also inhibited by K252a and TAT-pep5. The inhibitory effect by K252a was higher than that by TAT-pep5. The MEK inhibitor PD98059 inhibited phosphorylation of neurotrophic factor–induced ERK1/2, and PD98059 treatment also inhibited the NT-4-mediated Ambn induction in HAT-7 cells (data not shown). These results suggest that ligand-induced activation of TrkB and p75 is important for the expression of Ambn, TrkB-FL, and TrkB-T1.

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**FIGURE 3. Expression of tooth marker genes in HAT-7 cells with neurotrophic factors.** HAT-7 cells were cultured with 100 ng/ml NGF, BDNF, or NT-4 for 48 h. Total miRNA was analyzed for the expression of various genes by semiquantitative RT-PCR. Ambn, Enam, dentin sialophosphoprotein (Dspp), osteoepitin (Opn), osteonectin (Osn), connexin 43 (Cx43), and transforming growth factor–β1 (Tgf-β1) (A). Hprt was used as an internal control. The level of gene expression in the absence of growth factors was set at 1 for comparison (B and D). HAT-7 cells were stimulated with various amounts of NT-4 and BDNF for 48 h. Hprt expression showed no significant difference between each culture. The level of Ambn expression in cells without factors was set at 1 for comparison (C).
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NT-4 Null Mice Develop a Thin Enamel Layer and Have Reduced Ambn Expression—We demonstrated that NT-4 promoted epithelium cell differentiation in culture. To examine in vivo function of NT-4 in tooth development, we analyzed molars of NT-4 null mice (Fig. 8). NT-4 expression was completely absent in tooth germs of P1, P3, and P7 mice (supplemental Fig. 3). The expression of TrkB in NT-4 null tooth germs was similar to that of heterozygotes and wild-type mice (data not shown). We found that P3 molars had a thinner enamel matrix layer than control, whereas there was no significant difference in the predentin and dentin (Fig. 8, A and B). The size, shape, and polarization of ameloblasts in the mutant molars were normal as compared with those of the heterozygotes. Furthermore, Ambn expression in NT-4 null tooth germs was reduced as compared with that in heterozygotes (Fig. 8, C and D). These results suggest that NT-4 regulates Ambn expression and enamel layer formation.

DISCUSSION

Our results show that NT-4 regulates dental epithelial cell differentiation and enamel matrix gene expression via TrkB-FL but not via truncated TrkB forms. NT-4 inhibited cell proliferation and also induced enamel matrix genes, such as Ambn, in dental epithelial cells. NT-4-deficient teeth resulted in a thin enamel layer during the initial stage of amelogenesis. Our findings are the first to show that a neurotrophic factor plays an important role in tooth development.

The functional roles of NT-4 and its receptors have been reported mainly in neuronal tissues. Complete ablation of p75 in mice causes defects in both the nervous and vascular systems (23). Those animals displayed sensory and sympathetic defects, thus demonstrating that the p75 receptor is required for proper neuronal development. TrkB mutants display severe phenotypes that result in the death of most mutant mice in the first postnatal week because of their inability to feed (24), whereas NT-4 knock-out mice are viable and fertile but have a 50% loss of neurons in the nodose-petrosal and geniculate ganglia (25, 26). BDNF knock-out mice are characterized by selective sensory disorders and have a reduced number of neurons in sensory ganglia; they do not survive longer than 3–4 weeks after birth (27, 28). Although NT-4 and BDNF use TrkB as a receptor, phenotypes of TrkB, NT-4, and BDNF knock-out mice differ each other. Thus, it is suggested that NT-4 has a different expression pattern and function from that of BDNF. In fact, NT-4, but not BDNF, is expressed in the inner dental epithelium. During tooth germ development, NGF is expressed in the dental mesenchyme but not in the dental epithelium (20). In contrast, BDNF is found in the dental mesenchyme in the human tooth germ but not in that of mice. In the present study, both NGF and BDNF induced expression of the ameloblast markers, Ambn and Enam, and NT-4 receptors, TrkB-FL and TrkB-T1. NGF and BDNF may be important for mesenchymal and epithelial interactions. NT-4 was expressed mostly in dental epithelia in tooth germs and has been detected in both dental epithelial and mesenchymal cell lines, suggesting that it functions in an autocrine manner in dental epithelium. We found that p75 was expressed in an undifferentiated dental epithelial cell line but was not detectable in the tooth germ. It was
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FIGURE 6. Increase in phosphorylation of ERK1/2 in TrkB-FL transfectant cells by NT-4. The time course of phosphorylation of ERK1/2 in TrkB-FL- and T1-transfected HAT-7 cells after treatment with NT-4 was analyzed by Western blotting (IB) using the anti-phospho-MAPK antibody (A). The Western blots with anti-MAPK showed equivalent amounts of total ERK proteins in each lane. The relative intensity of p-ERK1 in the control cells at 0 min was set at 1 for comparison (B).

FIGURE 7. Inhibition of NT-4-mediated induction of Ambn and TrkB by K252a and TAT-pep5. HAT-7 cells were treated with NT-4 in the presence of K252a or TAT-pep5. The expressions of Ambn, TrkB-FL, TrkB-T1, and TrkB-T2 were analyzed by semiquantitative RT-PCR with specific primer sets (A). The level of gene expression in the control cells without NT-4 was set at 1 for comparison (B).

FIGURE 8. Decrease in the enamel matrix width and expression of Ambn in NT-4 null mice. Hematoxylin and eosin staining of P3 mouse molars were performed (A). The widths of the enamel matrix, dentin, and predentin were measured (B). Developing molars from heterozygote and mutant mice were dissected from P1, P3, and P7 mice, and total mRNA was prepared. The expression of Ambn was analyzed by semiquantitative RT-PCR with an Ambn primer set (C). The level of Ambn expression in P1 heterozygote mice was set at 1 for comparison (D).

reported that in incisors, p75 is expressed in the inner dental epithelium but is completely absent in differentiated ameloblasts (6). Further, the possibility of epithelial-mesenchymal communication within the intact tooth germ, whereas there is complete absence of those effects in the individual cells cultures, may be at the root of the differences of p75 expression between tooth germ and dental epithelial cell cultures. Moreover, p75 expression was not changed after stimulation with neurotrophins, whereas the p75 inhibitor TAT-pep5 was less effective on the expression of Ambn than Trk inhibitor K252a. These results suggest that p75 may not be important for the expression of Ambn in ameloblasts.

Truncated TrkB receptors have dominant inhibitory effects on BDNF and presynaptic signaling for BDNF-induced synaptic potentiation in cultured hippocampal neurons (29, 30). Truncated TrkB-T1 mediates neurotrophin-evoked calcium signaling in glia cells (31) and plays a direct signaling role in mediating inositol-1,4,5-trisphosphate-dependent calcium release. In developing teeth, TrkB-T1, but not TrkB-FL or TrkB-T2, is detected by in situ hybridization (32). In the present study, all types of TrkB were detected in P3 tooth germ epithelium and a dental epithelial cell line. This discrepancy of TrkB expression may have occurred because of different detection efficiencies of the methods used. Although both TrkB-FL and truncated TrkB were induced by NT-4, overexpression of TrkB-FL enhanced the expression of Ambn, but TrkB-T1 had a dominant negative effect on NT-4-induced Ambn expression. In NT-4-null mice, the expression of TrkB-T1 and -T2 was not changed from normal levels. These results suggest that truncated TrkB does not have an inhibitory effect on Ambn expression induced by NT-4.

Ambn plays an important role in maintaining the differentiation state of ameloblasts, serves as a cell...
adhesion molecule, and inhibits the proliferation of the dental epithelium (6). A deficiency of Ambn causes severe enamel hypoplasia, accelerates proliferation of the dental epithelium, and decreases the expression of amelogenin. The Ambn promoter functions in a cell type-specific manner and contains cis-acting elements that function to enhance and suppress transcription (33). The transcription factor Runx2, known as an essential factor for transcription of mineralized tissue genes, is also required for Ambn transcription (33). Site-directed mutagenesis of the Runx2-binding site in the Ambn promoter decreases Ambn promoter activity in the dental epithelium (33). These results indicate that Runx2 and Sp3 are necessary for a high level of the expression of Ambn. We showed that NT-4 did not have an effect on the expression of Runx2 in the dental epithelium (supplemental Fig. 2). Further, K252a treatment also did not cause any differences in Runx2 or Sp3 expressions. Thus, neurotrophic factor signaling is not required to regulate the expression of Runx2 and Sp3. The ERK-MAPK pathway provides a major link between the cell surface and nucleus to control proliferation and differentiation. The inhibition of MAPK signaling blocks osteoblast-specific gene expression in mature osteoblasts, whereas a constitutive active form of the MAPK intermediate, MEK1, is stimulatory (36). Runx2 is required for cells to respond to MAPK in vitro (37). FGF2 induces osteocalcin expression through MAPK activation in osteoblast cell line and bone marrow stromal cells (40). We demonstrated that in the dental epithelium, ERK phosphorylation was induced by NT-4 and necessary for the phosphorylation of Ambn expression. In fact, the MEK inhibitor PD98059 inhibited ERK phosphorylation and Ambn expression in dental epithelium (data not shown). These results suggest that NT-4-TrkB-ERK signaling is important for Ambn expression and ameloblast differentiation.

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