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Wnt-RhoA signaling is involved in dental enamel development

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Abstract

Transgenic mice that express dominant negative RhoA (RhoA^{DN}) in ameloblasts have hypoplastic enamel, with defects in molar cusps. β -catenin and Wnt5a were up-regulated in enamel organs of RhoA^{DN} transgenic mice, which indicated that both canonical and non-canonical Wnt pathways were implicated in the process of enamel defect formation. It was hypothesized that RhoA^{DN} expressed in ameloblasts interfered with normal enamel development through the pathways that were induced by fluoride. The Wnt and RhoA pathways were further investigated in an ameloblast-lineage cell line (ALC) with treatment by fluoride. The activities of RhoA and ROCK II decreased significantly, similar to activities in RhoA^{DN} transgenic mice. Both the canonical and non-canonical Wnt pathways were activated by treatment of NaF, which were verified by Western blot and TOPflash (β -catenin-TCF/LEF reporter gene) assay. β -catenin localization to both cytoplasm and nucleus was up-regulated in fluoride treated ALC while Gsk-3 β , the negative regulator of the Wnt pathway, showed a decreased expression pattern. The current results indicated that both Wnt and RhoA pathways are implicated in fluoride induced signaling transductions in ALC as well as the development of enamel defects in RhoA^{DN} transgenic mice.

Keywords

RhoA^{DN} transgenic mice; Wnt; RhoA; dental enamel

Teeth develop from a series of successive and reciprocal interactions between dental epithelium and mesenchyme, which are mediated by signaling molecules and pathways (1–3). Dental enamel is the product of a single layer of inner epithelial cells which undergoes remarkable lengthening in appearance and secretion of enamel extracellular matrix to form the enamel layer. Of the increasing number of signaling pathways which are thought to be involved in the process of tooth formation and especially dental enamel development, the Wnt signaling pathway has caught much attention. Nineteen Wnt family members as well as a large number of effectors have important roles during the development of the enamel organ (1–3). The canonical Wnt pathway is activated when β -catenin enters the nucleus and interacts with TCF/LEF transcription factors to promote downstream gene expression (4). β -catenin was strongly expressed in the enamel knot and inner dental epithelium during cap and bell stages, with an intimate association with enamel morphogenesis (5). The noncanonical Wnt pathway is subcategorized into the Wnt-Ca²⁺ pathway and the planar-cell-polarity (PCP) pathway (6–7). Wnt5a, a representative noncanonical Wnt ligand, could trigger the development of tumorigenic characteristics of enamel epithelium cells when it was overexpressed (8).

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RhoA is one of the critical molecules out of more than 20 identified Rho GTPases and plays a role in regulating the cytoskeleton as well as other cell activities (9–10). RhoA was localized in secretory ameloblasts and the treatment of ameloblasts with fluoride led to rapid intracellular actin cytoskeleton changes which may be mediated by the Rho/ROCK pathway (11). RhoA and effector ROCKS are important for expression of amelogenin and ameloblast differentiation (12). A transgenic mouse model which expressed a dominant negative RhoA (RhoA^{DN}) in secretory ameloblasts was generated, in which the activity of endogenous RhoA was partially blocked by the N19 dominant negative mutation (13–14). The mottled and pitted molar enamel revealed a critical role of the RhoA pathway in enamel development.

Both canonical and noncanonical Wnt signaling pathways are involved in activation of the RhoA pathway. In the canonical Wnt pathway, Wnt3a could stimulate osteoblastic differentiation, increase Chinese hamster ovary cell motility and induce neurite retraction through both β -catenin and RhoA/ROCK dependent pathways and is partially mediated by Dishevelled (Dvl) proteins (15–17). In the noncanonical or planar cell polarity (PCP) signaling, the Wnt signaling pathway mediated cytoskeleton changes through activation of RhoA (18). This showed that RhoA/ROCK acted as the downstream factors of two noncanonical Wnt ligands, wnt11 and wnt5, regulated convergence and extension movements of embryonic tissues in zebrafish embryos (19) as well as dorsal cell elongation during zebrafish gastrulation (20).

In this study, based on the enamel defects in RhoA^{DN} transgenic molars, the canonical and noncanonical Wnt signaling transductions were investigated within enamel organs of RhoA^{DN} transgenic mice. Furthermore, since receiving too much fluoride during tooth development will result in fluorosis which shows some similarities in phenotype, the transductions of RhoA and Wnt signaling pathways in an ameloblast-lineage cell line (ALC) treated with fluoride were also analyzed to illustrate the role of these two signaling pathways during dental enamel development. The ALC cells were derived from tooth germs of newborn C57BL/6J mouse mandibular molars (21).

MATERIALS AND METHODS

Reagents

Antibodies: anti-RhoA, anti-GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), anti- β -catenin, anti-p- β -catenin(Ser33/37/Thr41), anti-p- β -catenin(Ser45), anti-p- β -catenin(Ser552), anti-p- β -catenin(Ser675), anti-Gsk-3 β , anti-Dvl2, anti-Dvl3 (Cell Signaling Technology Inc., Danvers, MA, USA), anti-ROCK II(Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GFP, anti-Lamin B1, anti-Wnt5a and anti-Wnt3a antibody (Abcam, Cambridge, MA, USA), anti-phospho-MYPT1 (Thr850) (Millipore, Bedford, MA, USA), anti- β -actin (Sigma, St. Louis, MO, USA), anti-rabbit HRP-linked secondary antibody (Vectastain ABC kit PK-4001, Vector Laboratories, Burlingame, CA, USA) and DAB substrate (Vector). Protein G beads (Santa Cruz), rMYPT1 (Millipore), NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, Rockford, IL, USA), Bradford Protein Concentration Assay kit (Pierce), protease inhibitor cocktail (Roche Diagnostics, City?, Germany), phosphatase inhibitor cocktail (Sigma), TOPflash luciferase reporter plasmid (Upstate Biotechnology, Lake Placid, NY, USA), pRL-SV40 (Promega, Madison, WI, USA), Lipofectamine Plus Reagent (Invitrogen, Carlsbad, CA, USA), dual luciferase assay kit (Promega), Rho Activation Assay Kit (Millipore).

Transgenic mice

TgEGFP-RhoA^{DN}-13 mice were generated through the University of Pennsylvania Transgenic Core Facility, yielding strains with germ-line transfer (14). All work was performed in accordance with the regulations of the University of Pennsylvania Institutional Animal Care and Use Committee.

Immunohistochemistry

Mandibles dissected from postnatal (PN) 4, 6 and 8 day WT (wild-type) and TgEGFP-RhoA^{DN}-13 mice were fixed in 4% paraformaldehyde overnight at 4°C and demineralized in 5% EDTA for 2, 4 or 6 d respectively. Consecutive 6 µm paraffin-embedded tooth germ sections were prepared; slides were blocked in 1% bovine serum albumin at room temperature for 30 min and incubated overnight at 4°C with rabbit polyclonal antibodies against β -catenin (1:100). Control sections were incubated without primary antibody. After rinsing with PBS, the sections were incubated with anti-rabbit HRP-linked secondary antibody for 30 min at 37°C and DAB substrate.

Cell culture

ALC was cultured in minimum essential medium Eagle Spinner Modification (Sigma), supplemented with 10% fetal bovine serum (Sigma), 1% Penicillin-Streptomycin (Invitrogen), 1% L-glutamine (Invitrogen), 10 ng/ml Recombinant hEGF (Sigma) and 0.2 mM CaCl₂ (Sigma) (21).

Western blot

The enamel organs from 20 mandibular first molar teeth from PN 0, 2, 4, 6 or 8 WT and TgEGFP-RhoA^{DN}-13 pups were isolated and incubated for 30 min on ice in protein lysis buffer (10 mM Tris pH 7.6, 100 mM NaCl, 2 mM EDTA, 0.5% CHAPS) containing protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. Tooth samples were homogenized and centrifuged at 14,000×g for 10 min. ALC which was treated with or without 1.5 mM NaF, were lysed with the same buffer. Protein concentrations were measured with Bradford Protein Concentration Assay according to the manufacturer's instructions. NE-PER Nuclear and Cytoplasmic Extraction Kit was used according to manufacturer's recommendations if extraction of separate cytoplasmic and nuclear protein fractions was necessary. Thirty micrograms of protein for each sample were loaded onto pre-cast 4–20% gels (Thermo Fisher Scientific, Rockford, IL, USA) followed by electrophoresis and transfer to polyvinylidene fluoride (PVDF) membranes using 300 mA for 2 h. Membranes were blocked with 5% nonfat milk for two h at room temperature and incubated with primary antibodies at 4°C overnight. After rinsing, the membranes were incubated with HRP-conjugated secondary antibodies (1:5000 dilution) at room temperature for 2 h. Immunoreactive proteins were visualized by incubating membranes with supersignal west femto thermo Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA, USA). Target proteins were detected using antibody for RhoA, GAPDH, β -catenin, p- β -catenin (Ser33/37/Thr41), p- β -catenin (Ser45), p- β -catenin (Ser552), p- β -catenin (Ser675), Gsk-3 β , Dvl2, Dvl3, ROCK II, GFP, Lamin B1, Wnt5a, Wnt3a, phospho-MYPT1 (Thr850), β -actin (1:1000 dilution).

Immunoprecipitation analysis for ROCK II

Protein lysate (25 µg) of enamel organ tissue or ALC was added to 100 µl pre-diluted protein G beads suspension. The mixed slurry was rotated on a shaker for 10 min and centrifuged. Supernatant was collected and incubated with 0.5 µl anti-ROCK II for 30 min while rotating on a shaker at 4°C. Fresh 100 µl pre-diluted protein G beads solution was added and shaken for 15 min. 15 µl protein lysis buffer, 1 µl of 100 uM ATP and 0.5 µl

rMYPT1 were added, and the tubes were shaken for 30 min at 37°C. Six µl of 4× SDS sample buffer (0.25 M Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 0.02% bromophenol blue, 0.2 M DTT) was added to the tube, boiled for 10 min, and run on SDS-PAGE. ROCK II activity was indicated by p-MYPT-1 band density normalized by that of total ROCK II.

Semi-quantitative RT-PCR

RNA was extracted from ALC treated with or without 1.5 mM NaF for 24 h. Two micrograms total RNA was reverse transcribed and PCR was performed with a Veriti Thermal Cycler (Applied Biosystems) with Applied Biosystems PCR kit. The sequences of PCR primers were: β -catenin sense primer 5'-ACCTTTCAGATGCAGCGACT-3', anti-sense primer 5'-GTCGTGGAATAGCACCTGT-3'; GAPDH sense primer 5'-TCACCACCATGGAGAAGGC-3', anti-sense primer 5'-GCTAAGCAGTTGGTGGTGCA-3'. The thermocycling profile was: 8 min at 95°C for denature, 30 s at 95°C, 30 s at 58°C, 45 s at 72°C for 30 cycles, with 10 min extension at 72°C. PCR products were electrophoresed on 1.5% agarose gels. β -catenin band intensities were normalized against GAPDH as a control.

TOPflash dual-luciferase reporter assays

ALC was plated in 24-well plates. One µg TOPflash (or FOPflash, which harbors mutant TCF-binding sites) luciferase reporter plasmid and 20 ng pRL-SV40 (RENILLA luciferase driven by the SV40 promoter) were transfected with the Lipofectamine Plus Reagent according to the manufacturer's protocol. After 24 h, cells were treated with or without 1.5 mM NaF for 12 h. Luciferase activity was evaluated using the dual luciferase assay kit. Transcriptional activity was measured as relative light units (RLU), a ratio of firefly luciferase activity to Renilla activity.

RhoA Activity Assay

RhoA activity was analyzed with a Rho Activation Assay Kit following the manufacturer's instructions. The Rho binding domain (RBD) can specifically bind to GTP-bound RhoA and immunoprecipitate active RhoA. ALC which were treated with or without 1.5 mM NaF for 1, 6, 12 and 24 h were lysed and protein concentration assay was performed. 200 µg of protein sample in each group was incubated with Rho Assay Reagent slurry for 45 min at 4°C with agitation. After incubation, the beads were washed 3 times with lysis buffer. The amount of immunoprecipitated GTP-RhoA was analyzed by immunoblot. The RhoA activity was expressed as the intensity of GTP-RhoA band normalized by total RhoA.

Statistics

Data are reported as means $\pm$ SD. Statistical significance was assessed using Student's t tests for two groups or one-way analysis of variance (ANOVA) and Tukey's post test for more than two groups. Significance was defined as $P < 0.05$.

RESULTS

Expression of β -catenin in the ameloblast layer of TgEGFP-RhoA^{DN}-13 molars is higher than in WT mouse molars

The ameloblast layer in PN4 TgEGFP-RhoA^{DN}-13 mice molars exhibited intensely positive staining, while it was lighter in PN4 WT mice molars (Fig. 1B,E). At PN6, β -catenin staining decreased in the ameloblast layer of both strains (Fig. 1C,F). PN8 WT molar sections showed negligible staining (Fig. 1D), similar to the negative control (Fig. 1A). The PN8 TgEGFP-RhoA^{DN}-13 mice group had weak staining which was higher than PN8 WT

(Fig. 1G). The results indicated that β -catenin is up-regulated in ameloblasts in RhoA^{DN} transgenic mice.

The RhoA signaling pathway is blocked in enamel organs of TgEGFP-RhoA^{DN}-13 molars

Western blots were probed with anti-GFP and anti-RhoA to check the expression of GFP-RhoA fusion protein in WT and TgEGFP-RhoA^{DN}-13 mice. The fusion protein could be detected in dental epithelium from PN2 TgEGFP-RhoA^{DN}-13 mice and decreased sharply 6 d later. Two Western blot membranes showed similar size bands at 55–60 kDa. RhoA and ROCK II expression were up-regulated from PN0 and for the following 6 d in TgEGFP-RhoA^{DN}-13 mice, however ROCK activity was downregulated when RhoA signaling was blocked in TgEGFP-RhoA^{DN}-13 molars. β -actin expression reached a peak on PN6 and decreased sharply 2 d later in TgEGFP-RhoA^{DN}-13 molars while a steady increase in amount was seen in WT mice (Fig. 2A,B).

Both canonical and non-canonical Wnt signaling pathways are activated in TgEGFP-RhoA^{DN}-13 molars

Both cytosolic and nuclear β -catenin expression levels were evaluated by Western blot. The results showed that levels were significantly higher after birth of TgEGFP-RhoA^{DN}-13 mice compared to WT mice from PN0-PN6. Blots were probed for GAPDH and Lamin B1 as the loading controls for cytosolic and nuclear proteins, respectively. Wnt3a and Wnt5a were selected as the representative factors for canonical and noncanonical Wnt pathways. The up-regulation of Wnt3a in dental epithelial tissue of PN0-PN6 TgEGFP-RhoA^{DN}-13 transgenic mice verified that Wnt3a is one of the canonical Wnt family members to play a role in transgenic mice. It is not clear whether the rest of the canonical Wnt factors are involved in the process. Increased Wnt5a suggested that the noncanonical Wnt pathway is also involved. Gsk-3 β , an important factor of the β -catenin degradation complex, increased at PN2 and decreased at PN8 in TgEGFP-RhoA^{DN}-13 molars, which indicated that the canonical Wnt pathway is not regulated by Gsk-3 β alone (Fig. 3A,B).

NaF activated both canonical and non-canonical Wnt signaling pathways in ALC *in vitro*

Western immunoblots revealed that cytosolic and nuclear β -catenin increased significantly when ALC was treated with 1.5 mM NaF. The TOPflash dual-luciferase reporter assay result verified up-regulated β -catenin transcriptional activity (Fig. 4A,B) and indicated NaF could activate the canonical Wnt signaling pathway in ALC *in vitro*. The results showed that p- β -catenin (Ser33/37/Thr41) decreased while p- β -catenin (Ser552) increased significantly in NaF treated ALC compared to the control group. It implied that more than one signal pathway regulated β -catenin phosphorylation in NaF treated ALC (Fig. 4C,D). The expression levels of β -catenin degradation complex effectors and Wnt5a were also investigated in ALC with or without NaF treatment. Gsk-3 β and Axin 1 decreased significantly after 1 d of stimulation with 1.5 mM NaF. Dvl3 was up-regulated significantly with 2 d of NaF treatment (Fig. 5A,B). Wnt3a and Wnt5a, the representatives of canonical and non-canonical Wnt family members, could be up-regulated by NaF (Fig. 5A,B). Moreover, NaF did not change β -catenin gene expression in ALC (Fig. 5C,D).

NaF impacted the RhoA pathway by regulating RhoA and ROCK activity

Active GTP-bound RhoA increased considerably after 4 h of treatment with 1.5 mM NaF and began to decrease at 8 h through 24 h. The activity of downstream effector ROCK underwent a similar pattern, which revealed the increase within 4 h of NaF incubation and decrease in the following hours (Fig. 6A,B). Furthermore, 1.5 mM NaF could up-regulate total RhoA expression but not ROCK after 2 d of treatment, when RhoA and ROCK activities were down-regulated at that time point. This indicated that NaF could regulate

RhoA and ROCK activity very quickly and that there is no obvious correlation between protein activity and expression level in this case (Fig. 6C,D).

DISCUSSION

Amelx mRNA transcripts were first detected at E15 and increased through newborn postnatal stages (22). Since the expression of TgEGFP-RhoA^{DN} in transgenic mice was regulated by the *Amelx* promoter, RhoA signaling activity decreased when the *Amelx* transcripts increased in amount. Therefore, it was reasonable that canonical Wnt pathway activity was only up-regulated in this period of time and decreased dramatically in PN8 in TgEGFP-RhoA^{DN}-13 molars. This suggested that the elevated canonical Wnt pathway in molar ameloblasts in late bell stage can also interfere with the differentiation of ameloblasts and the development of enamel, which has been shown in incisors (23). However, Gsk-3 β , an important factor of the β -catenin degradation complex, increased when the canonical Wnt signaling pathway was active, which indicated that Wnt signaling activity is not Gsk-3 β dependent in TgEGFP-RhoA^{DN}-13 mouse molars. The Wnt signaling pathway plays a critical role during tooth morphogenesis. The moderate immunostaining in WT ameloblast cytoplasm and the expression of Wnt3a showed that Wnt signaling is active in the process of enamel formation. When Wnt3a and β -catenin are up-regulated in ameloblasts of TgEGFP-RhoA^{DN}-13 mouse molars, mottled and pitted molar enamel with obvious defects at cusps and occlusion surface could be observed at 2 and 8 wk. The enamel thickness of transgenic molars was reduced significantly compared to that in WT mice (14). It was reported that up-regulated canonical Wnt pathway activity in the epithelial cells of the postnatal incisor caused reduced enamel as well as abnormal or absent ameloblasts (23). Active Wnt/ β -catenin signaling in dental epithelium from bud stage resulted in supernumerary tooth generation and gross defects in morphogenesis (24–25). Our results are consistent with these findings in the above publications.

Sharpe and his group observed *Wnt-3*, *Wnt-4*, *Wnt-6*, *Wnt10b* and Wnt receptor *MFz6* in the enamel knots or dental epithelium (26). However, they also reported that canonical Wnt pathway activity is absent in ameloblasts of incisors and molars from bud to bell stages, which indicated that Wnt does not regulate ameloblast differentiation but maintains proliferation and other biological characteristics (27). From the immunostaining and Western blot results, cytoplasmic β -catenin was weakly expressed while nuclear β -catenin was consistently present in PN0-PN8 WT mice, which supports the idea that Wnt signaling could be almost inactive during ameloblast differentiation, but the Wnt ligands and β -catenin are indispensable for biological functions. Canonical Wnt signaling was regulated in a delicate and accurate way in epithelium and showed temporal and spatial variation in different stages of epithelial tissues including enamel knot and ameloblasts.

When TgEGFP-RhoA^{DN} transgenic mice showed enamel defects somewhat similar to dental fluorosis, we wondered whether TgEGFP-RhoA^{DN} and fluoride triggered similar cell signaling pathways and whether canonical Wnt signaling was implicated. IHC and Western blot results showed that Wnt signaling was increased significantly in TgEGFP-RhoA^{DN}. Furthermore, ALC culture analysis verified that both the canonical and noncanonical Wnt pathways could be triggered with NaF treatment. This indicated that dental fluorosis may partially occur through crosstalk between the Wnt and RhoA pathways. RhoA/ROCK could be the downstream effectors of the Wnt pathway and could feedback to induce overexpression of β -catenin or increase Wnt signaling pathway activity when it had been blocked.

β -catenin expression can be regulated through two different ways: from gene to RNA-transcription and translation, or by phosphorylation to prevent proteasomal degradation.

Axin and casein kinase I (CKI) induce β -catenin phosphorylation at Ser45, which is a priming site to initiate phosphorylation at Ser33, Ser37 and Thr41 regulated by co-expression of Axin and Gsk-3 β . Phosphorylation of β -catenin by GSK3 leads to the degradation of β -catenin. Overexpression of Dvl could suppress Ser45 phosphorylation, thereby precluding β -catenin degradation (28). Moreover, two other β -catenin phosphorylation sites Ser552 and Ser675, which are phosphorylated by Akt and Protein kinase A (PKA) respectively, can cause β -catenin accumulation and increase its transcriptional activity, thereby activating the canonical Wnt pathway (29–31). In this experiment, NaF could upregulate Wnt3a and Wnt5a in ALC and resulted in reducing β -catenin phosphorylation at Ser33, Ser37 and Thr41 while slightly increasing β -catenin phosphorylation at Ser552. These results indicated that fluoride could regulate β -catenin expression through phosphorylation at Ser33, Ser37, Thr41 and Ser552 to reduce β -catenin degradation while increasing accumulation in the cytoplasm and nuclei simultaneously. Besides Wnt3a and Wnt5a, there is little evidence that other Wnt family members are involved in this process. Furthermore, because expression of β -catenin is not significantly different between ALC treated with or without NaF for 24 h, the increased accumulation of β -catenin in the cytoplasm should only be due to phosphorylation.

ROCK activity decreased in TgEGFP-RhoA^{DN}-13 molars, which indicated that RhoA^{DN} may negatively regulate the RhoA pathway by decreasing ROCK activity rather than at the protein expression level. F-actin is a downstream target of the RhoA/ROCK signaling pathway, and was elevated in NaF treated teeth but reduced when teeth were pretreated by the ROCK inhibitor Y-27632 (8). In the current experiment, the RhoA/ROCK signaling pathway was blocked in ameloblasts of TgEGFP-RhoA^{DN}-13 transgenic mice where the β -actin expression level was elevated 2 days earlier than WT and retained high expression for 4 d. It was assumed that any severe alterations in F-actin will negatively affect the cytoskeleton of ameloblasts, potentially altering enamel matrix secretion and cell signal transduction. RhoA/ROCK activity showed a dramatic increase within 4 h in ALC treated with NaF and was followed by a quick decrease, which indicated that fluoride could potentially affect the actin cytoskeleton and cell biological functions partially by regulating the activities of RhoA and ROCK.

In conclusion, fluoride induced dental fluorosis may share cell signaling mechanisms with TgEGFP-RhoA^{DN} transgenic mice. RhoA and ROCK play an important role in regulating the actin cytoskeleton and cell signaling within ameloblasts. Both canonical and non-canonical Wnt signaling pathways are implicated in enamel defects resulting from excess fluoride or down regulation of the RhoA pathway.

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Figure 1.

Immunohistochemical analysis of β -catenin expression in the ameloblast layer of WT and TgEGFP-RhoA^{DN}-13 molars. (A) negative control lacking primary antibody (PN6 TgEGFP-RhoA^{DN}-13 molar). Staining for β -catenin was not observed in TgEGFP-RhoA^{DN}-13 control sections (arrows). (B)(C)(D) A gradually decreased β -catenin amount was observed in WT molar sections from positive staining in PN4 (B), a weak positive reaction in PN6 (C) to almost negative expression in PN8(D) (arrows). (E)(F)(G) Compared to the PN4-8 WT molars, darker β -catenin staining was observed in the ameloblast layer in PN4-8 TgEGFP-RhoA^{DN}-13 molars (arrows).

A

B

Figure 2.

The RhoA signaling pathway was significantly impacted in enamel organs of TgEGFP-RhoA^{DN}-13 molars. (A) Western immunoblots of dental epithelial tissue in WT and TgEGFP-RhoA^{DN}-13 molars of PN 0, 2, 4, 6 and 8 d. Blots were probed for GFP, GFP-RhoA fusion protein, RhoA, ROCK II, p-MYPT-1, β-actin and GAPDH. (B) Normalized quantitative data from Western blots. The expression of TgEGFP-RhoA^{DN} fusion protein was evaluated with GFP and RhoA antibody, which indicated that the fusion protein can be detected from PN2 and peaked in the following 4 d, but decreased dramatically in PN8 TgEGFP-RhoA^{DN}-13 molars. TgEGFP-RhoA^{DN} fusion protein was not detected in WT molar. Compared to WT, RhoA and ROCK increased significantly from PN0 and lasted to

PN6 and PN8 in TgEGFP-RhoA^{DN}-13 molars respectively. However, the IP assay showed that ROCK activity (p-MYPT-1) was downregulated by RhoA^{DN}, which indicated that the RhoA pathway was reduced in TgEGFP-RhoA^{DN}-13 molars. β -actin expression reached a peak on PN6 and decreased sharply 2 d later in TgEGFP-RhoA^{DN}-13 molars while a steady increase in expression was seen in WT mice. These experiments were repeated three times.

* $P < 0.05$ (W=WT, E= TgEGFP-RhoA^{DN}-13, fusion α Rho=a fusion protein in which RhoA^{DN} was fused to the EGFP reporter, end RhoA=endogenous RhoA, W2=PN2 WT, E2= PN2 TgEGFP-RhoA^{DN}-13)

A

B

Figure 3.

Both canonical and non-canonical Wnt signaling pathways are activated in TgEGFP-RhoA^{DN}-13 molars. (A) Western immunoblots of dental epithelium in PN0-PN8 WT and TgEGFP-RhoA^{DN}-13 molars. Proteins from the cytoplasm and nuclei were isolated separately and blots were probed for β-catenin (cytosolic and nuclear β-catenin), Lamin B1, Gsk-3β, Wnt3a, Wnt5a and GAPDH. (B) Normalized quantitative results from Western blot. Cytosolic, nuclear β-catenin, Wnt3a and Wnt5a amounts were significantly greater in TgEGFP-RhoA^{DN}-13 molars compared to WT. Gsk-3β increased at PN2 and decreased at PN8 in TgEGFP-RhoA^{DN}-13 molars, which indicated that the canonical Wnt pathway is not regulated by Gsk-3β alone. These experiments were repeated three times. * P<0.05 (W=WT, E= TgEGFP-RhoA^{DN}-13, W2=PN2 WT, E2= PN2 TgEGFP-RhoA^{DN}-13)

Figure 4.

NaF activated the canonical Wnt signaling pathway in ALC in vitro. (A) Western immunoblots of cytosolic and nuclear β -catenin from ALC treated with or without 1.5 mM NaF. Blots were probed for β -catenin (cytosolic and nuclear β -catenin), Lamin B1 and GAPDH. (B) Normalized quantitation of Western blot data. The significantly increased cytosolic and nuclear β -catenin indicated that NaF could activate the canonical Wnt signaling pathway in ALC in vitro. The TOPflash dual-luciferase reporter assay verified that β -catenin transcriptional activity increased sharply in NaF treated ALC compared to the control group. * $P < 0.05$ (C) Western immunoblots of cytosolic protein from ALC treated with or without 1.5 mM NaF. Blots were probed for p- β -catenin (Ser33/37/Thr41), p- β -

catenin (Ser45), p- β -catenin (Ser552), p- β -catenin (Ser675) and GAPDH. (D) Quantitation of data in (C). NaF treatment led to β -catenin phosphorylation at Ser552 and reduced phosphorylation of β -catenin at Ser33, Ser37 or Thr41. The phosphorylation of β -catenin at Ser45 and Ser675 was not changed significantly with the treatment of NaF. These experiments were repeated three times. * $P < 0.05$ (C=Control group, F=1.5 mM NaF treatment group, C6=6h in control group, F6=6h in NaF treatment group)

Figure 5.

NaF activated Wnt signaling by regulating Wnt family members, β -catenin degradation complex factors and Dvl family members. (A) Western immunoblots of cytosolic protein from ALC treated with or without 1.5 mM NaF for 1–2 d. Blots were probed for Gsk-3 β , Axin 1, Dvl2, Dvl3, Wnt3a, Wnt5a and GAPDH. (B) Quantitation of data in (A). Gsk-3 β and Axin 1 decreased significantly with stimulation by 1.5 mM NaF while Dvl3 was up-regulated significantly. Wnt3a and Wnt5a, the representatives of canonical and non-canonical Wnt family members were up-regulated by NaF. Dvl2 was not significantly changed with NaF. (C=Control group, F=1.5 mM NaF treatment group, C1=1d in control group, F1=1d in NaF treatment group) * $P < 0.05$ (C) Expression of β -catenin mRNA in

control and experimental groups. (D) There is no significant difference between ALC treated with or without NaF in terms of β -catenin. These experiments were repeated three times. (C=Control group, F=1.5 mM NaF treatment group, C24=24h in control group, F24=24h in NaF treatment group)

Figure 6.

NaF could activate the RhoA pathway in ALC within 4 h. (A) Active RhoA pull down assay and immunoprecipitation were performed to analyze the activity of RhoA and ROCK. Immunoblots for GTP-RhoA, p-MYPT-1, total RhoA and ROCK are shown. (B) Activity measurements. The amounts of immunoprecipitated GTP-RhoA and active ROCK increased significantly within 4 h, followed by a decrease. (C=Control group, F=1.5 mM NaF treatment group, C24=24h in control group, F24=24h in NaF treatment group) (C) Western immunoblots of ALC treated with or without 1.5 mM NaF for 1 and 2 d. Blots were probed for RhoA and ROCK. (D) Quantitation of data in (C). After 2 d of treatment with NaF, total RhoA was up-regulated while no difference was observed for total ROCK. These

experiments were repeated three times. * $P < 0.05$ (C=Control group, F=1.5 mM NaF treatment group, C1=1 d in control group, F1=1 d in NaF treatment group).