Wnt-RhoA Signaling Pathways in Fluoride-Treated Ameloblast-Lineage Cells

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Key Words

Ameloblast-like cells · RhoA pathway · Signal transduction · Wnt pathway

Abstract

This study examined the effect of sodium fluoride (NaF) on the Wnt and RhoA signaling pathways in murine ameloblast-lineage cells (ALCs) to better understand the developmental mechanisms of dental fluorosis. Wnt and Rho pathway activities were investigated when ALCs were treated with 1.5 mM NaF, dickkopf-related protein-1 (Dkk-1), secreted frizzled related-protein-2 (sFRP-2), β -catenin siRNA dominant negative RhoA (RhoA^{DN}) plasmid and Y-27632. Wnt pathway activity was investigated via RT-PCR, Western blot and Topflash luciferase assay. The activity of the RhoA pathway was analyzed via Rho pull-down assay and immunoprecipitation. The differentiation of ALCs was analyzed by alkaline phosphatase assay. Western blot and Topflash luciferase assay results verified that both the Wnt and Rho pathways were up-regulated by 1.5 mM NaF. Wnt was discovered to be located upstream from the Rho pathway, as confirmed by treatment with Wnt pathway cell receptor inhibitors Dkk-1 and sFRP-2, leading to a decrease in RhoA and ROCK activity. Inhibition

of the Rho pathway with RhoA^{DN} plasmid and Y-27632 caused upregulation of Wnt pathway activity which could be further increased by 1.5 mM NaF. The increased Wnt pathway activity was found to negatively regulate ALC differentiation. These data suggest that fluoride could induce the cross-talk between Wnt and RhoA signaling pathways, and these responses are predicted to contribute to the development of enamel fluorosis.

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Abbreviations used in this paper

ALCs	ameloblast-lineage cells
ALP	alkaline phosphatase
Dkk-1	dickkopf-related protein-1
LEF/TCF	lymphoid enhancer DNA-binding factor 1/ T cell-specific transcription factor
LRP5/6	lipoprotein receptor-related proteins 5 and 6
MEM	minimum essential medium
NaF	sodium fluoride
RhoADN	dominant negative RhoA
RLU	relative light units
sFRP-2	secreted frizzled-related protein-2

Introduction

Fluoride has been the most effective treatment for the prevention of caries since the 1940s [Wong et al., 2011]. However, when consumed in excess during tooth development, fluoride will lead to a developmental disturbance of dental enamel in terms of enamel rod size, number, shape and quality of interaction, known as dental fluorosis [Eanes and Hailer, 1998]. Mild dental fluorosis is characterized by opaque white striations that run horizontally across the tooth [Alvarez et al., 2009]. In severe cases, the enamel pitting and porous regions increase in size, and discoloration of the enamel surface is seen [Bronckers et al., 2009]. When a transgenic mouse model expressing a dominant negative RhoA (RhoA^{DN}) in secretory ameloblasts was generated, some similarities to enamel fluorotic defects in the cutting edge of incisors as well as cusps in the molar were observed [Li et al., 2011a]. Moreover, three independent transgenic mouse strains showed that fluoride-induced enamel alterations have similar changes in cell signaling mechanisms. We hypothesized that Wnt and RhoA pathways are implicated in fluoride-induced signal transduction in ameloblast-lineage cells (ALCs) as well as the enamel defects in RhoA^{DN} transgenic mice [Peng et al., 2011].

Rho-mediated signaling potentially has an influential role in amelogenesis [Hatakeyama et al., 2009; Biz et al., 2010]. RhoA is one of the critical Rho GTPases that play a role in regulating the cytoskeleton as well as other cell activities [van Aelst and Symons, 2002; Burridge and Wennerberg, 2004]. RhoA is recognized as a molecular switch that normally cycles from the active GTP-bound form to the inactive GDP-bound form, and regulates downstream effector ROCK (Rho-associated protein kinase) leading to structural changes in the cytoskeleton of ameloblasts [Li et al., 2005].

The canonical Wnt pathway is β -catenin dependent and is activated when β -catenin enters the nucleus and interacts with lymphoid enhancer DNA-binding factor 1/T cell-specific transcription factor (LEF/TCF) transcription factors to promote downstream gene expression [Clevers, 2006]. Activation of canonical Wnt signaling is initiated by Wnt ligand binding to two receptors: frizzled and lipoprotein receptor-related proteins 5 and 6 (LRP5/6) [Nelson and Nusse, 2004]. Upon interaction, Dishevelled inhibits the β -catenin degradation complex, which includes adenomatous polyposis coli, Axin proteins and glycogen synthase kinase-3 β . β -Catenin accumulates in the cytoplasm and nucleus, and nuclear β -catenin interacts with LEF/TCF to promote downstream gene expression [Clevers, 2006; MacDonald et al., 2009]. Various ap-

proaches have indicated that the canonical Wnt pathway does not induce or even inhibit dental epithelial cell differentiation, but could maintain dental epithelial cells with normal biological character [Millar et al., 2003; Jarvinen et al., 2006; Chen et al., 2009; Lohi et al., 2010; Suomalainen and Thesleff, 2010].

Several studies have revealed cross-talk between Wnt and RhoA signaling pathways. Wnt3a, a canonical Wnt pathway family member, could stimulate osteoblastic differentiation, increase Chinese hamster ovary cell motility and induce neurite retraction through both β -catenin and RhoA/ROCK-dependent pathways [Kishida et al., 2004; Endo et al., 2005; Rossol-Allison et al., 2009]. RhoA/ROCK could also act as the downstream factors of two noncanonical Wnt ligands, Wnt11 and Wnt5, to regulate convergence and extension movements of embryonic tissues in zebrafish embryos [Zhu et al., 2006] as well as dorsal cell elongation during zebrafish gastrulation [Marlow et al., 2002]. In contrast, RhoA signaling could impact on Wnt pathways. β -Catenin and Wnt5a were upregulated in enamel organs of RhoA^{DN} transgenic mice, which expressed the RhoA^{DN} transgene in ameloblasts [Peng et al., 2011]. This indicated that both canonical and noncanonical Wnt pathways were implicated in the formation of enamel defects in these mice.

The aim of this study was to better understand the interaction between the Wnt and Rho pathways when cells are treated with sodium fluoride (NaF). This investigation will provide a better understanding of the mechanisms involved in fluoride-induced cell signal transduction.

Materials and Methods

Reagents

The following antibodies were sourced for this investigation: anti-RhoA, anti-GAPDH, anti- β -catenin, anti-p- β -catenin (Ser33/37/Thr41) from Cell Signaling Technology Inc. (Danvers, Mass., USA), anti-lamin B1 from Abcam Inc. (Cambridge, Mass., USA), anti-ROCK II from Santa Cruz Biotechnology Inc. (Santa Cruz, Calif., USA) and anti-phospho-MYPT1 (Thr850) from Millipore (Billerica, Mass., USA). The NE-PER Nuclear and Cytoplasmic Extraction Kit and Bradford Protein Concentration Assay Kit (Pierce, Rockford, Ill., USA), Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and Phosphatase Inhibitor Cocktail (Sigma-Aldrich, St. Louis, Mo., USA) were also used. The inhibitors used were recombinant mouse secreted frizzled-related protein-2 (sFRP-2), recombinant mouse dickkopf-related protein-1 (Dkk-1; R&D Systems, Minneapolis, Minn., USA). InSolution Y-27632 came from Calbiochem (La Jolla, Calif., USA). β -Catenin siRNA, control siRNA-A and siRNA transfection reagent were from Santa Cruz Biotechnology Inc. Topflash luciferase reporter plasmid (Upstate Biotechnology, Lake Placid, N.Y., USA),

pRL-SV40 (Promega, Madison, Wis., USA), Lipofectamine Plus reagent (Invitrogen, Carlsbad, Calif., USA), Dual Luciferase Assay Kit (Promega, Madison, Wis., USA) and Rho Activation Assay Kit (Millipore) were all also obtained. StemTAG Alkaline Phosphatase Activity Assay Kit (Colormetric) came from Cell Biolabs Inc. (San Diego, Calif., USA).

Cell Culture

ALCs from a wild-type mouse [Takahashi et al., 2007] were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine, 0.2 mM calcium chloride and 10 ng/ml recombinant human epidermal growth factor (Sigma-Aldrich), and cells were passaged as needed. The medium was changed every other day, and fresh L-glutamine (1% of current medium volume) was added weekly.

Treatment with Wnt Receptor Inhibitors

When ALCs reached 70–80% confluence, 0.1 µg/ml Dkk1, 0.15 µg/ml SFRP2 or both were added to their respective groups. NaF was added to 1.5 mM 6 h later. The RhoA and ROCK activities were then checked 4 h later, while cytoplasmic β -catenin and nuclear β -catenin levels were checked 8 h later. This experiment was repeated three times.

Treatment with RhoA^{DN} Plasmid and ROCK Inhibitor

The RhoA^{DN} (dominant negative RhoA) plasmid includes amelogenin regulatory sequences and partially blocks endogenous RhoA in ameloblasts by the N19 dominant negative mutation [Qiu et al., 1995; Li et al., 2011b]. Four micrograms of RhoA^{DN} plasmid was transfected to the RhoA pathway blocking groups using 10 µl of Lipofectamine 2000 in serum-free Opti-MEM (Invitrogen). Medium was replaced by MEM with 10% FBS 6 h later and 1.5 mM NaF was added 24 h after the medium replacement. Y-27632 was provided to ROCK-inhibited groups 6 h before 1.5 mM NaF was added. This experiment was repeated three times.

Treatment of β -Catenin siRNA

A total of 6.5 µl of control siRNA or β -catenin siRNA were transfected into cells with 1 ml of siRNA transfection reagent in siRNA transfection medium. After 6 h of treatment, 1 ml of 4× normal growth medium was added to the cell plates. Cell samples in every treatment group were collected at different times depending on the target proteins being investigated. This experiment was repeated three times.

Semiquantitative RT-PCR

RNA was extracted from ALCs treated with or without 1.5 mM NaF for 24 h. Two micrograms of total RNA was reverse transcribed and PCR was performed with a Veriti™ Thermal Cycler (Applied Biosystems, Foster City, Calif., USA) with Applied Biosystems PCR kit. The sequences of PCR primers were: β -catenin sense primer 5'-ACCTTTCAGATGCAGCGACT-3', antisense primer 5'-GTCGTGGAATAGCACCTGT-3'; GAPDH sense primer 5'-TCACCACCATGGAGAAGGC-3', antisense primer 5'-GCTAAGCAGTTGGTGGTGCA-3'. The thermocycling profile was 8 min at 95°C for denaturing, 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 separated by electrophoresis on 1.5% agarose gels. β -Catenin band intensities were normalized against GAPDH as a control. This experiment was repeated three times.

Western Blot

ALCs in each group were incubated on ice with 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. Protein concentrations were measured with Bradford Protein Concentration Assay according to the manufacturer's instructions. The NE-PER Nuclear and Cytoplasmic Extraction Kit was used according to the manufacturer's recommendations if extraction of separate cytoplasmic and nuclear protein fractions was necessary. In this experiment, cytoplasmic and nuclear β -catenin were extracted when ALCs were treated with Wnt receptor inhibitors. Total β -catenin and phosphorylated β -catenin expressions were investigated when the RhoA pathway was blocked. For each sample, 30 µg of protein were loaded onto 4–20% gels (Thermo Fisher Scientific, Rockford, Ill., USA) followed by electrophoresis and transfer to polyvinylidene fluoride membranes using 300 mA for 2 h. Membranes were blocked with 5% nonfat milk for 2 h at room temperature and incubated with primary antibodies at 4°C overnight. After rinsing, membranes were incubated with HRP-conjugated secondary antibodies (1:5,000 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, Mass., USA). Target proteins were detected using antibody for RhoA, GAPDH, β -catenin, p- β -catenin (Ser33/37/Thr41), ROCK II and phospho-MYPT1 (Thr850; 1:1,000 dilution). This experiment was repeated three times.

Topflash Dual-Luciferase Reporter Assays

ALCs were plated in 24-well plates. According to the manufacturer's protocol, 1 µg of Topflash (or Fopflash, which harbors mutant TCF-binding sites) luciferase reporter plasmid and 20 ng of pRL-SV40 (RENILLA luciferase driven by the SV40 promoter) were transfected with the Lipofectamine Plus reagent. Topflash or Fopflash was transferred along with RhoA^{DN} plasmid, control siRNA or β -catenin siRNA. After 24 h, cells were treated with 1.5 mM NaF for 8 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. Each treatment was carried out in duplicate and the experiment was repeated three times.

RhoA Activity Assay

Fifteen microliters of Rho Assay Reagent slurry (Millipore) were added to each cell extract centrifuge tube. The reaction mixtures were incubated for 45 min at 4°C with gentle agitation followed by brief centrifugation (10 s, 14,000 g, 4°C). After incubation, the supernatant was removed and the remaining beads were washed three times with Mg²⁺ Lysis/Wash Buffer (Millipore). The amount of immunoprecipitated GTP-RhoA was analyzed by immunoblot. The RhoA activity was expressed as the intensity of the GTP-RhoA band normalized by total RhoA. This experiment was repeated three times.

Immunoprecipitation Analysis of ROCK II Activity

Twenty-five micrograms of protein ALC lysate was added to 100 µl of prediluted protein G beads solution. The mixed solution was rotated on a shaker at 4°C for 10 min and centrifuged for 20 s at 3,000 rpm. 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 prediluted protein G beads solution was added and tubes were

Fig. 1. Wnt pathway upregulation by NaF showed an increase of β-catenin protein expression and Topflash activity. Both Wnt pathway cell receptor inhibitors and β-catenin siRNA could inhibit the increase. **a** β-Catenin mRNA was downregulated by β-catenin siRNA. RT-PCR results showed that NaF did not alter β-catenin mRNA with or without control siRNA. However, β-catenin siRNA could decrease the β-catenin message significantly. **b** Western blot results showed that β-catenin was increased with NaF treatment and β-catenin siRNA could inhibit the increase. β-Catenin was normalized with GAPDH. **c** Topflash activity increased in NaF and the control siRNA + NaF group, but decreased in the β-catenin siRNA + NaF-treated groups. Fopflash activity

was not changed among all four groups. **d** Cytosolic and nuclear β-catenin protein levels with treatment of Wnt cell receptor inhibitors Dkk-1 and sFRP-2. Western blot showing the levels of cytosolic and nuclear β-catenin. Cytosolic β-catenin was normalized with GAPDH and nuclear β-catenin was normalized with lamin B1. Normalized band intensity of cytosolic and nuclear β-catenin showed that cytosolic β-catenin significantly decreased within all inhibitor groups and nuclear β-catenin decreased significantly in the FDS-treated group. C = Control; F = NaF; FD = NaF + Dkk1; FS = NaF + sFRP2; FDS = NaF + Dkk1 + sFRP2; siC+F = control siRNA + NaF; si+F = β-catenin siRNA + NaF. * $p < 0.05$.

shaken for 15 min, and immediately centrifuged for 20 s. Supernatant was discarded. To the immunoprecipitated ROCK beads, 20 μl of lysis buffer, 1.5 μl of 100 μM ATP and 0.5 μl of rMYPT1 was added. Sample tubes were incubated in a shaker (300 rpm) at 37°C for 30 min. Samples were boiled and run on SDS-PAGE. ROCK II activity was indicated by p-MYPT-1 band density normalized by that of total ROCK II. This experiment was repeated three times.

Alkaline Phosphatase Assay

Alkaline phosphatase assay (ALP) activity was quantified in ALCs harvested after 60 h in culture following the treatments described above, and the protein concentration was determined. Cell lysates were transferred to a 96-well plate and were processed for ALP activity using StemTAG Alkaline Phosphatase Activity Assay Kit according to the manufacturer's instruction. Absorbance of each well was read at 405 nm by using a Dynatech Laboratories MRX Microplate Reader. The activity was determined by absor-

bance at 405 and normalized against the amount of protein in the reaction. Each treatment was carried out in duplicate and the experiment was repeated three times.

Statistics

Data are reported as means ± SD. Statistical significance was assessed using Student's *t* tests for two groups or one-way analysis of variance and Tukey's post hoc test for more than two groups. Significance was defined as $p < 0.05$.

Results

The Wnt pathway core transcription factor β-catenin and Wnt ligand receptors were investigated to confirm Wnt signal transduction in NaF-treated ALCs. ALCs

Fig. 2. RhoA^{DN} and Y-27632 could inhibit the activity of the Rho pathway in ALC treated with NaF. RhoA^{DN} plasmid caused a significant decrease in RhoA activity ($p < 0.05$) and inhibition with Y-27632 led to a significant decrease in ROCK activity ($p < 0.05$). C = Control; F = NaF; R = RhoA^{DN}; FR = NaF + RhoA^{DN}; Y = Y-27632; FY = NaF + Y-27632. * $p < 0.05$.

treated with control siRNA and β -catenin siRNA showed that the β -catenin siRNA downregulated both β -catenin mRNA and protein, while NaF could upregulate the β -catenin protein amount and downstream transcriptional activity (fig. 1a–c). Cells treated with β -catenin siRNA showed a significant decrease in Topflash activity ($p < 0.05$) in comparison to the NaF plus control siRNA-treated groups (fig. 1c). The role of Wnt receptors in NaF-treated ALCs was also analyzed. ALCs treated with Wnt signal antagonists and NaF were analyzed using Western blot. Samples were collected 4 h following treatment with 1.5 mM NaF. In order to confirm the efficiency of the Wnt cell receptor inhibitors Dkk-1 and sFRP-2, the levels of cytoplasmic and nuclear β -catenin were analyzed. Treatment with NaF led to high expression of both cytoplasmic and nuclear β -catenin. This upregulation of β -catenin could have occurred through either the conventional receptors, LRP5/6 or frizzled. Treatment with both of the Wnt cell receptor antagonists inhibited the Wnt signal, as demonstrated by a significant decrease in accumulation of cytoplasmic β -catenin and nuclear β -catenin ($p < 0.05$; fig. 1d).

Rho pathway activity was also evaluated in NaF-treated ALCs. Rho pull-down and immunoprecipitation assays showed that NaF significantly upregulated activity of

the Rho pathway components RhoA and ROCK. RhoA^{DN} and Y-27632 could inhibit the increase ($p < 0.05$; fig. 2).

In order to investigate how the Rho pathway activity was affected by the Wnt pathway, ALCs treated with Wnt signal antagonists and NaF were analyzed using a Rho pull-down assay and immunoprecipitation for activity of ROCK and Western blot. Treatment with Wnt pathway cell receptor inhibitors Dkk-1 and/or sFRP-2 caused a decrease in RhoA and ROCK activity. The greatest decrease in activity was seen when both Dkk-1 and sFRP-2 were used together (fig. 3a). With verification that siRNA downregulates the Wnt pathway, a Western blot was run for siRNA samples and incubated with RhoA antibodies to measure the amount of activated RhoA compared to total RhoA. NaF upregulated RhoA activity ($p < 0.05$) in comparison to the control group. The level of RhoA activity significantly decreased with β -catenin siRNA treatment ($p < 0.05$; fig. 3b). In cells treated with β -catenin siRNA prior to treatment with NaF, the Wnt pathway, and presumably β -catenin expression, will be downregulated. A significant recovery was seen in ALP activity for NaF + β -catenin siRNA-treated cells. This result was statistically significant ($p < 0.05$) in comparison to all other treatment groups (fig. 3c).

Finally, the role of the Rho pathway in relation to the canonical Wnt pathway was investigated. The expression

Fig. 3. RhoA and ROCK activities were decreased when the Wnt pathway was blocked by either receptor inhibitors or β -catenin siRNA. **a** RhoA and ROCK activities are downregulated by treatment with Wnt pathway cell receptor inhibitors Dkk-1 and sFRP-2. Western blot showing activated RhoA and ROCK. Activated RhoA was normalized with total RhoA, and activated ROCK, measured by phosphorylated MYPT-1, was normalized with total ROCK, showing that both RhoA and ROCK activity were significantly increased by treatment with NaF ($p < 0.05$). A decrease in RhoA or ROCK activity is observed with treatment of receptor inhibitors Dkk-1, sFRP-2 or both ($* p < 0.05$). **b** Activity of RhoA and ROCK decreased with β -catenin siRNA treatment. Western

blot showing activated RhoA and ROCK. Activated RhoA was normalized with total RhoA, and activated ROCK, measured by phosphorylated MYPT-1, was normalized with total ROCK. RhoA and ROCK activity increased with treatment of NaF in comparison to the control group, and decreased with β -catenin siRNA treatment in comparison to the NaF-treated group ($* p < 0.05$). **c** Alkaline phosphatase activity increased with β -catenin siRNA treatment. Alkaline phosphatase activity decreased in the NaF-treated group and increased significantly with β -catenin siRNA treatment ($* p < 0.05$). C = Control; F = NaF; FD = NaF + Dkk1; FS = NaF + sFRP2; FDS = NaF + Dkk1 + sFRP2; siC+F = control siRNA + NaF; si+F = β -catenin siRNA + NaF.

of β -catenin and phosphorylated β -catenin proteins was investigated in ALCs treated by transfection with RhoA^{DN} or Y-27632. Blots were probed for β -catenin, p- β -catenin (Ser33/37/Thr41) and GAPDH. β -Catenin was increased by treatment with NaF, RhoA^{DN} and Y-27632, while p- β -catenin (Ser33/37/Thr41) was downregulated. The combination of NaF and RhoA^{DN} significantly increased

β -catenin expression ($p < 0.05$) in comparison to the NaF- or RhoA^{DN}-treated groups (fig. 4a, b). Transfection of the Rho pathway inhibitors RhoA^{DN} plasmid and treatment with Y-27632 showed an increase in Topflash luciferase activity compared to the control group. The addition of NaF to the RhoA^{DN}- or Y-27632-treated group showed significant increases ($p < 0.05$) in Topflash lucif-

Fig. 4. Fluoride could upregulate the Wnt pathway independently of the RhoA pathway activity. The decreased RhoA pathway activity could also activate the Wnt pathway. **a** Canonical Wnt pathway was activated by treatment with RhoA^{DN} and Y-27632. Western blot showing expression of β -catenin and phosphorylated β -catenin protein with the treatment of NaF, RhoA^{DN} and Y-27632. Blots were probed for β -catenin, p- β -catenin (Ser33/37/Thr41) and GAPDH. **b** Normalized band intensity of β -catenin and phosphorylated β -catenin protein. β -Catenin was increased by treatment with NaF, RhoA^{DN} and Y-27632, while p- β -catenin (Ser33/37/Thr41) was downregulated. The combination of NaF and RhoA^{DN} significantly upregulate β -catenin ($p < 0.05$). **c** Both RhoA^{DN}- and Y-27632-treated groups had significantly higher luciferase activity ($p < 0.05$) in comparison to the control group. The addition of NaF to the RhoA^{DN}-treated group had significantly higher luciferase activity ($p < 0.05$) in comparison to the NaF- or RhoA^{DN}-treated groups. **d** Treatment with NaF, RhoA^{DN} or Y-27632 could significantly decrease the ALP activity in ALCs ($p < 0.05$). The addition of NaF to the RhoA^{DN}- or Y-27632-treated groups had the lowest ALP ($p < 0.05$). C = Control; F = NaF; R = RhoA^{DN}; FR = NaF + RhoA^{DN}; Y = Y-27632; FY = NaF + Y-27632. * $p < 0.05$.

erase activity and, therefore, an upregulation of the Wnt pathway (fig. 4c). After 60 h, ALC treated with 1.5 mM NaF showed a decrease in ALP activity. A statistically significant decrease in ALP activity was observed in groups treated with RhoA^{DN} and Y-27632 ($p < 0.05$) in comparison to the control group. The addition of NaF to the RhoA^{DN}- or Y-27632-treated group showed significant decreases ($p < 0.05$) in comparison with the RhoA^{DN}- or Y-27632-treated groups (fig. 4d).

In summary, both Wnt and Rho pathways could be upregulated by NaF. The Wnt pathway was activated through its two receptors: frizzled and LRP5/6. NaF could upregulate RhoA pathway, which was blocked by RhoA^{DN} and Y-27632. The RhoA pathway could also be activated by the Wnt pathway. Both Wnt pathway cell receptor inhibitors (DKK-1 and sFRP-2) and β -catenin siRNA could block RhoA pathway activity. Decreased RhoA pathway activity could also activate the Wnt pathway (fig. 5).

Discussion

Previous *in vivo* studies done with RhoA^{DN} transgenic mice that express RhoA with a dominant negative N19 mutation to decrease RhoA activity in ameloblasts [Qiu et al., 1995] concluded that interference with RhoA activity leads to surface defects in enamel [Li et al., 2011a]. Because the defects seen in RhoA^{DN} transgenic mice were somewhat similar to those seen in cases of human enamel fluorosis, in the present study ALCs were treated with NaF to determine if the cultured cells respond through cell signaling pathways and how treatment affects the cross-talk between Wnt and Rho in ameloblast cells, leading to alterations in pathway component expression or activity.

The specific inhibitors used in this study were chosen because of their efficiency shown in previous experiments. Dkk-1 and sFRP-2 have been used in multiple

Fig. 5. Wnt and Rho pathways could be upregulated by NaF. Both Wnt pathway cell receptor inhibitors (Dkk-1 and sFRP-2) and β -catenin siRNA could block RhoA pathway activity. Decreased RhoA pathway activity could also activate the Wnt pathway. The whole cell signal pathway was divided into three main lines and two sublines. ①②④ The tree main lines – NaF could upregulate the Wnt pathway through its two receptors, frizzled and LRP5/6. ⑤⑥ NaF could upregulate the RhoA pathway, which could be blocked by RhoA^{DN} and Y-27632. ①③⑦ The RhoA pathway could also be activated by the Wnt pathway. ⑦⑧ Two sublines – activity of RhoA and ROCK decreased with β -catenin siRNA treatment. ⑥④ The decreased RhoA pathway activity could also activate the Wnt pathway. Dashed lines indicate that the cell signal mechanism is as yet unclear; solid lines indicate that the mechanism has been reported.

studies to downregulate Wnt signaling through the inhibition of LRP5/6 and frizzled [Oshima et al., 2005; Liu et al., 2011]. Two efficient options for inhibition of β -catenin have been described, namely β -catenin siRNA and quercetin. Based on a literature review, siRNA has been used in ameloblast treatment [Duan et al., 2011; Otsu et al., 2011] and was determined to work efficiently. Also, RhoA^{DN} plasmid, which decreases intracellular RhoA activity, and Y-27632, a conventional inhibitor of ROCK, has been proven to inhibit RhoA and ROCK in ameloblasts [Qiu et al., 1995; Ishizaki et al., 2000]. The assays used measure different aspects of the pathways to avoid false results.

Since activation of Wnt signaling uncouples the degradation complex allowing an accumulation of β -catenin in the cytoplasm [Liu and Millar, 2010], a decrease in cytoplasmic β -catenin after the addition of Wnt pathway cell receptor inhibitors was expected. Although the sFRP-2 group showed higher levels of cytoplasmic β -catenin than the groups treated with Dkk-1 or both inhibitors, an overall decrease in cytoplasmic β -catenin accumulation was seen when the Wnt pathway was downregulated. This verifies the effectiveness of the Wnt cell receptor inhibitors. The results for nuclear β -catenin did not seem to follow this trend. A decrease in all three inhibited groups (Dkk-1, sFRP-2 and both inhibitors) was expected; however, the only significant result was seen when both inhibitors were added together. This result could be caused by a translocation of accumulated β -catenin to the nucleus prior to the addition of inhibitors and collection of samples. It is apparent that nuclear β -catenin was not significantly influenced by either inhibitor when used alone; however, when used together the inhibitors proved to be efficient.

These experiments illustrated that 1.5 mM NaF increased RhoA activity within 4 h. The treatment of ameloblast cells with NaF led to a change in cellular activities through the inactivation of RhoGAP, which activates RhoA [Vincent et al., 1998; Li et al., 2005]. The addition of Wnt cell receptor antagonists Dkk-1 and sFRP-2 to cultured ALCs led to a decrease in RhoA activity, signifying that RhoA may be downstream of the Wnt pathway in these cells. The results of the RhoA activity assay help to explain how ROCK (measured by phosphorylated MYPT-1) is affected by Dkk-1 and sFRP-2. Again, an increase in activated ROCK was seen after treatment with NaF. The Wnt pathway inhibitors caused a decrease in activated ROCK, thus further confirming that RhoA and its effector ROCK are downstream from the Wnt pathway. When the Wnt pathway was blocked, the Rho pathway was downregulated.

β -Catenin siRNA efficiency was verified by downregulation of β -catenin target expression in the nucleus using a Topflash gene report assay. Treatment with β -catenin siRNA led to a decrease in RhoA activity, again validating that Wnt and β -catenin are located upstream from RhoA. Lastly, treatment with β -catenin siRNA caused a significant restoration of ALP activity. This indicates that when the Wnt pathway is blocked, ALP activity increases. Since alkaline phosphatase activity is associated with cell differentiation [O’Gorman et al., 2012], high alkaline phosphatase activity in this case implies that the Wnt pathway may negatively regulate ALC differentiation. This is an

expected result because Wnt signaling may act to restrict terminal differentiation in ameloblasts, and essentially arrest cells in a state of proliferation [Lohi et al., 2010].

NaF could activate the RhoA pathway in ALC within 4 h [Peng et al., 2011]. Therefore, treatment with RhoA^{DN} plasmid or Y-27632 in the presence of NaF causes a significant inhibition in RhoA or ROCK activity 4 h later. Blocking the Rho pathway may lead to an increase in Wnt pathway activity. Otsu et al. [2011] discovered that by blocking ROCK with Y-27632, β -catenin was able to translocate from the cell membrane to the nucleus. The Topflash gene report assay showed an increase in Wnt pathway activity in ALCs treated with RhoA^{DN} or Y-27632. Western blot incubated with β -catenin antibody further confirmed the upregulation of β -catenin when the Rho pathway components RhoA and ROCK were inhibited. NaF could further upregulate Wnt pathway activity compared to RhoA^{DN} treatment alone. However, treatment with Y-27632 and NaF did not cause a significant increase in Wnt activity, implying that NaF and ROCK may regulate Wnt signaling pathway activity through a pathway independent of Rho.

It is clear from previous experiments that treatment with NaF upregulates the Wnt pathway, as do inhibitors of the Rho pathway. Treatment with both of these up-regulating factors presumably caused an increase in Wnt signaling, which led to a decrease in alkaline phosphatase activity. It is also possible that NaF can influence the differentiation of ALCs, as was discussed by Riksen et al. [2011], who confirmed that high concentrations of fluoride cause a reduction in ALP activity. In addition, Milan et al. [2001] validated that 0.09 M (378 ppm) of fluoride inhibits ALP activity. Furthermore, previous studies have found a direct relationship between high ALP activity of ameloblasts and increased enamel mineralization [Gomez and Boyde, 1994]; thus, Wnt signaling may negatively regulate enamel mineralization.

It is most likely an apparent contradiction that fluoride results in both activation of Rho and Wnt signaling, and inhibits ALP production. Yet inhibition of Rho also inhibits ALP production. Thus, signals that oppositely affect Rho have the same effect on ALP activation. To the best of our knowledge, based on our data and that in the literature [Milan et al., 2001; Riksen et al., 2011], fluoride inhibits ALP activity. Even if fluoride results in both increased Rho activation and β -catenin stabilization, it does not mean that increased activation of Rho and Wnt signals lead to decreased ALP activity. Again, it is NaF, not RhoA, Wnt or β -catenin, that inhibits ALP activity. Figure 3c shows that β -catenin stabilization had a negative

effect on ALP activity, which is consistent with the effect of NaF. Figure 4d shows that RhoA activity had a similar trend to ALP activity. From this result, it indicated that increased RhoA may increase ALP activity. Fluoride could activate RhoA, Wnt and other unknown pathways. The common effect of fluoride is a decrease in ALP activity, and it is interesting to speculate the potential mechanisms. Firstly, as was mentioned above, ALP activity is decreased by common effects which include Wnt, Rho and other so far unknown signals. Next, it was verified that diminished β -catenin could upregulate ALP activity (fig. 1b, 3c). It is possible that activated Rho could up-regulate ALP activity by decreasing Wnt secretion or β -catenin nuclear translocation. To clarify this, extensive experimentation has been performed.

The present study focused on NaF-induced cross-talk between the Wnt and Rho pathways to better understand the developmental mechanisms of enamel and enamel fluorosis. It is possible that enamel fluorosis can occur through the cross-talk of these pathways since both the Wnt and Rho pathways have increased activity when ALCs are treated with NaF. Figure 5 attempts to summarize these pathways in ALCs treated with NaF. This relationship is particularly important given that exposure to fluoride can occur through water, food and other common sources. Although the amount of fluoride ingested can be theoretically measured, there is a lack of knowledge in respect to the exact amount of fluoride that reaches the surface of ameloblasts. RhoA has been previously shown to have a significant role in the development of enamel, and high levels of Wnt signaling may lead to decreased enamel mineralization. These signaling pathways are involved in the development of other oral diseases, including ectopic teeth and taste papillae [Liu and Millar, 2010]; therefore, it is important to thoroughly understand how they function.

From the analysis of cells in vitro, it was proven that the Wnt and RhoA pathways play an important role in ALCs when treated with fluoride. The cross-talk between the Wnt and RhoA pathway, as well as its effects on the cell skeleton, is a promising and proven way to disclose the mechanisms of dental fluorosis.

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