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Effect of Fluoride on Dentin Formation

by

Yu-Hsing Kao

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Oral and Craniofacial Sciences

in the

GRADUATE DIVISION

of the

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by

(Yu-Hsing Kao)

DEDICATION AND ACKNOWLEDGEMENTS

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The research presented in this thesis is entirely my own and Dr. Den Besten's lab.

ABSTRACT

It is known that fluoride helps to prevent tooth decay, however an excess of fluoride can cause enamel fluorosis. Although a great deal is known about enamel fluorosis, not much is known on the effects of excess fluoride on forming dentin, and its relationship to the tooth structure. To study the effects of fluoride on dentin, 4 week-old fluoride sensitive A/J mice were exposed to either 0 (control) or 50 ppm fluoride in drinking water for 3 weeks, after which they were sacrificed and serum fluoride levels were measured. The incisors were histologically analyzed for changes in dentin formation and relative expression of dentin matrix proteins and regulative proteins for its expression. These studies revealed that when exposed to excess fluoride, A/J mice exhibited a thicker pre-dentin layer. The relative amount of dentin sialoprotein was reduced in fluoride exposed incisors, possibly related to a direct effect of fluoride on Wnt signaling pathways. Taken together, these findings show that fluoride can alter dentin formation. These findings suggest that further human studies to further determine whether enamel fluorosis is predictive for altered dentin formation are needed. It is possible that if the quality of the dentin is affected by overexposure to fluoride, this may affect the incidence and rate of progression of dentin caries.

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1. INTRODUCTION

1.1 Fluoride and Enamel Fluorosis

1.1.1 Fluoride Protects Against Dental Caries

Dental caries occurs when bacteria in a biofilm produce lactic acid by saccharolytic fermentation. This acid can dissolve the hydroxyapatite crystals in enamel and produce an amorphous sodium monohydrogen phosphate from hydroxyapatite [1], which results in a loss of tooth surface. Fluoride has two mechanisms of protection from caries. First, it is incorporated into forming enamel crystals, but it also affects enzymes responsible for enamel formation. The presence of fluoride ion in the crystal slows the transformation to amorphous calcium monohydrogen phosphate. Therefore, fluoroapatite formed at the enamel surface in presence of fluoride reduces the rate of caries development. Increased fluoride concentration at the enamel surface also inhibits lactate production, possibly related to changes in the bacterial biofilm. In the second mechanism of protection, fluoride is incorporated into bacterial biofilms where it inhibits enolase. Enolase catalyzes the production of phosphoenolpyruvate, a precursor of lactate from 2-phosphoglycerate, during glycolysis. Oral bacterial uptake of mono- and disaccharides mostly utilizes the phosphoenolpyruvate transport system to transfer them

into the cytosol. Fluoride not only inhibits lactic acid production, but also the phosphoenolpyruvate transport system-mediated uptake of saccharide substrates [2].

1.1.2 Dental uses

Fluoride is widely used by dental providers to help prevent dental caries. Fluoride is available in different preparations ranging from low (0.25-1 mg per tablet; 1000-1500 mg fluorine per kg in toothpaste) to high concentrations (liquids containing 10,000 mg/L, gels containing 4000-6000 mg/kg, and varnishes may be used for local topical applications) [3].

1.1.3 Fluoride supplementation in public water supply

Studies on fluoride in the water supply have shown it is beneficial in caries prevention. By comparing caries experience with fluoride concentration in public water supplies and enamel fluorosis, it was shown that 1 ppm (1 mg/L fluoride) is beneficial in preventing caries and results in minimal enamel fluorosis [2]. These studies were largely the credit of Frederick Summer McKay, who graduated dentistry in 1901. When Dr. McKay moved to Colorado Springs, he noticed mottled enamel was a common problem with his patients. In 1908 he sent these tooth samples to G. V. Black who was Dean of Northwestern University Dental School of Chicago. McKay was able to

determine geographic boundaries where mottled enamel was found and he ultimately determined that the cause of the mottled enamel was fluoride in the water supply. Later studies by Dr. H. Trendley Dean, a dental officer of the U.S. Public Health Service, found that fluoride levels up to 1 ppm in the drinking water do not cause obvious dental fluorosis and also reported the correlation between fluoride content in the water and a reduced incidence of dental caries. In 1942, he showed that at fluoride concentrations of 0.9-1.2mg/L (ppm), 12-33% of the population exhibited signs of mild fluorosis. In 1945, community-wide studies were conducted in Grand Rapids, Michigan to evaluate the effect of fluoride in the drinking water on caries, by adding sodium fluoride to the water supply in fluoride-deficient areas. These studies showed that in areas where fluoride was 8-10 ppm, severe fluorosis was observed. In one area where the water supply had no fluoride, the population DMFT was 6-8. However, as the fluoride concentration increased to 1 ppm, the mean DMFT of the same population decreased approximately 50%. Furthermore, increasing fluoride above 1ppm slightly decreased the DMFT further, though also resulted in markedly increased prevalence of fluorosis [2]. In the United States, water fluoridation became readily available after 1955 and fluoridated toothpastes after 1975.

1.1.4 Dental fluorosis

Dental fluorosis occurs as a result of excess fluoride ingestion during tooth formation [1]. Fluorosed enamel presents varying phenotypes. Mildly fluorosed teeth have a white opaque appearance, the result of hypomineralization. More severe fluorosis is characterized by pitting and loss of enamel, with increasing porosity relative to the severity of fluorosis [4]. Pitting can lead to secondary staining causing a yellow to brown discoloration. During the early maturation stage in fluorosed enamel, the amount of amelogenin protein is increased. When fluoride is incorporated into the mineral of the tooth, more protein binds to the forming mineral. As a result, protein removal by proteinases is delayed which results in hypomineralization. Fluoride also seems to enhance mineral precipitation in forming teeth, thus resulting in hypermineralized bands of enamel [1].

The level of fluorosis is determined by examining the buccal surface of each tooth and classifying it as either: Normal (score 0) enamel has a translucent appearance with a smooth, glossy surface, pale creamy white color; Questionable (score 1) enamel may contain white flecks to occasional white spots; Very mild (score 2) small, opaque, paper-white areas scattered over less than 25% of the tooth surface or a premolar or molar showing no more than 2mm of white opacity at the cusp tips; Mild (score 3) white

opaque areas are discontinuous or a continuous white opaque affect less than 50% of the tooth surface; Moderate (score 4) more than 50%, but not all of the enamel surface is affected with white opacity and little or no brown spots are present; Severe (score 5) all the enamel displays continuous white opacity of more than half of the tooth surface and is stained brown with evidence of mild attrition [2].

1.1.5 Fluoride Toxicity

Fluoride may be essential for humans and animals, however there is currently no data indicating the minimum nutritional requirement in humans. Fluoride toxicity occurs when fluoride is ingested at high levels. A minimum oral dose of 1 mg fluoride per kg of body weight can produce signs of acute fluoride toxicity [5]. Studies have shown fluoride can cause mottled enamel at 1 ppm, toxic effects on bone at >10 ppm, and more general effects at > 50 ppm.

1.1.6 Fluoride Guidelines

WHO guideline; value of fluoride in drinking water of 1.5 mg/L (ppm), was set in 1984 and reaffirmed in 1993. Fluoride levels higher than 1.5 mg/L show an increased risk of dental fluorosis and much higher levels can cause skeletal fluorosis [6] [7]. Studies in China and India report an increased prevalence of skeletal fluorosis above 1.4

mg/L in drinking water. This value is higher than the recommended value for artificial fluoridation of water supplies, which is usually 0.5-1.0 mg/L [8]. The United States Department of Health and Human services recommends 0.7-1.2 ppm fluoride in drinking water. CDC Water Fluoridation Statistics state that of the 308,745,538 US population, 204.5 million (73.9%) Americans received fluoridated water. This is an increase of nearly 9 million since 2008. In 2010, 62.1% California residents received fluoridated water (2010 Water Fluoridation Statistics, <http://www.cdc.gov/fluoridation/statistics/2010stats.htm>) [9].

1.2 Dentin Fluorosis

Although much is known about enamel fluorosis, there is a paucity of literature on the effects of fluoride on dentin.

1.2.1 Dentin

Dentin is a mineralized connective tissue of the tooth and is produced by odontoblasts from the dental papilla mesenchymal cells. It is very similar to bone and they share many extracellular matrix proteins associated with mineralization such as DMP1, fibronectin, collagen type I, alkaline phosphatase, osteonectin, osteopontin, bone sialoprotein (BSP), bone-1, and osteocalcin. Once formed dentin does not undergo

remodeling, however it can respond to stimulation by forming reparative dentin to protect the pulp. Differentiation of odontoblasts is regulated by epithelial-mesenchymal interactions which provide instructions for both morphogenesis and cell differentiation. Odontoblasts are columnar polarized cells with eccentric nuclei and long cellular processes (Fig 1). This single layer of polarized cells lies adjacent to the basement membrane of the inner dental epithelium. Odontoblast differentiation is controlled by the inner dental epithelium. Terminal odontoblast differentiation is initiated during the bell stage at the sites of the future cusps [10].

During dentin formation, odontoblasts secrete unmineralized type I collagen-rich matrices called predentin, a precursor of dentin. This unmineralized organic phase lies between the mineralization front and their forming cells and is transformed to the

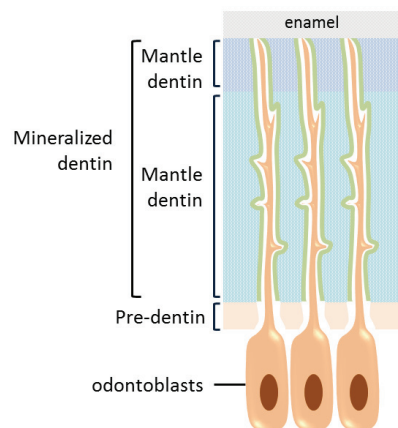


Figure 1: Dentin structure

mineralized phase after hydroxyapatite (HA) crystals are deposited [11].
Biom mineralization involves interactions between type I collagen and multiple non-collagenous proteins (NCPs). NCPs are believed to promote control of mineralization of collagen fibers and crystal growth within predentin (Fig 1). Dentin sialophosphoprotein (DSPP) is a member of one category of NCPs called SIBLING (Small Integrin-Binding Ligand, N-linked Glycoprotein) family [12].

1.2.2 DSPP in Biom mineralization

Dentin phosphoprotein (DPP) and dentin sialoprotein (DSP) are two proteins found significantly in the extracellular matrix (ECM) of dentin [11]. DPP and DSP are the cleavage products of dentin sialophosphoprotein (DSPP). Human and mouse genetic studies show DSPP gene knockout results in mineralization defects in dentin and bone [11], [13]. DSPP is believed to contribute to the early events of amelogenesis and to the formation of the dentinoenamel junction and the adjacent “aprismatic” enamel [14]. DSPP mRNA is primarily expressed in teeth by terminally differentiated [15] odontoblasts and transiently by presecretory preameloblasts, and at low levels in osteoblasts [10]. It is believed to play a crucial role in converting predentin to mineralized dentin. DSPP is a large 940-amino acid polypeptide that is proteolytically cleaved into DSP and DPP, with the DSP sequence at the 5' end and the DPP at the 3' end

of the transcript (Fig 2). DSP and DPP are found primarily in dentin matrix [16]. The chemical features of DPP and DSP are remarkably different between the two proteins and their ratio is approximately 10:1 [11].

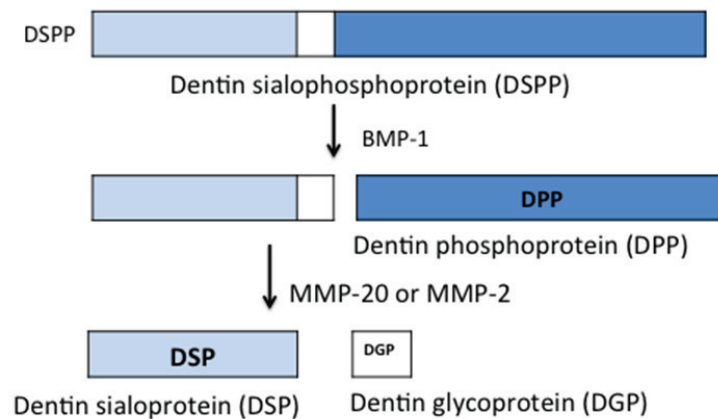


Figure 2: DSPP, DPP and DSP

Dentin sialophosphoprotein is cleaved to form dentin phosphoprotein and dentin sialoprotein.

DPP (dentin phosphoporyn) is a highly acidic protein and is the most abundant non-collagenous ECM component in dentin other than type I collagen [14]. DPP was initially believed to be directly involved in controlling the rate and site of dentin mineralization. The high levels of Asp and Pse make DPP a very polyanionic protein which binds to large amounts of calcium with a relatively high affinity [11]. DPP forms insoluble aggregates in the presence of Mg^{++} and Ca^{++} [17, 18]. *In vitro*

mineralization studies show that DPP is an important initiator and modulator of the formation and growth of hydroxyapatite crystals [19-21]. Upon synthesis and secretion by mature odontoblasts, DPP is transported to the mineralization front where DPP binds to collagen fibrils and assumes a structure promoting the initial formation of apatite crystals [22-24]. As mineralization proceeds predentin is converted to dentin and the mineral crystals will grow in an oriented fashion. DPP and other proteins bind to the growing HA and inhibit or slow crystal growth, which can influence the size and shape of the apatite crystals [23, 25].

DSP is a glycoprotein also found in dentin, but in low quantities. It contains little or no phosphate. The functions of DSP are currently undefined, but it has little to no effect on *in vitro* mineralization. Recent *in vivo* studies indicate that DSP may be involved in the initiation of dentin mineralization and not maturation [20, 26].

DSPP gene mutations have been identified in families with dentinogenesis imperfecta type II disorder. Dentinogenesis imperfecta (DI), is an autosomal dominant disorder which primarily affects dentin mineralization and results in abnormal dentin formation. It causes dentin hypomineralization and significant tooth decay. DSPP gene knockout experiments show DSPP-deficient mice have defects in mineralization of dentin and bone. Sreenath et al. reports that DSPP knockout mice have tooth anomalies

similar to human dentinogenesis imperfecta III with enlarged pulp chambers, increased width of predentin zone, hypomineralization, and pulp exposure [16, 27]. This indicates that DSPP is critical in dentin mineralization. Although the exact mechanism by which DSPP participates in biomineralization is unclear, it is hypothesized that the proteolytic processing of DSPP and other non-collagenous dentin matrix proteins (SIBLING proteins) is an activation step in dentin mineralization [28].

Genetic alterations in dentin matrix proteins result in dentinogenesis imperfecta. Dentinogenesis imperfect is classified into three subtypes [16]. Dentinogenesis imperfecta type I (DI-I) is always associated with one of the osteogenesis imperfectas, and are associated with collagens: COL1A1, COL1A2, COL5A1, and COL2A1. DI-II and DI-III are more severe and restricted to the dentin. DI type II presents with opalescent dentin with obliterated pulp chambers caused by a disorder in dentin mineralization. DI type III presents with a more abnormal phenotype that affects enamel, dentin, and pulp [14]. DI-III are referred to as 'shell teeth', in which dentin mineralization does not occur after mantle dentin is formed and radiographically the teeth have enlarged pulp chambers along with a high incidence of pulp exposures [16].

1.2.3 DSPP deficient, DPP deficient and DSP transgene mouse models

Dentin dysplasia, another human disorder of dentin mineralization, is similar to

DI-II and is caused by a Tyr6Asp protein transition mutation in the hydrophobic core sequence of the DSPP gene. This mutation prevents DSPP from entering the endoplasmic reticulum. In order to understand molecular events that control dentin mineralization during tooth development, Sreenath et al generated DSPP ^{-/-} null mice by deleting the DSPP coding region in embryonic stem cells. These null mice displayed an enlarged pulp cavity, widened pre-dentin zone, decreased dentin width, and high incidence of pulp exposures, similar to findings in DI-III. In addition, these mice also showed an increased accumulation of dentin proteoglycans biglycan and decorin within the widened pre-dentin and scalloped (void spaces) in the dentin. These findings correlate with defective mineralization [16].

Suzuki et al. reported the generation of conditional DPP knockout (DPPcKO) mice [26], in which only DSP was expressed in a DSPP null background. The results were that DPPcKO teeth showed a partial rescue of the DSP null phenotype with the pre-dentin width restored, an absence of irregular unmineralized areas in dentin, and a reduction in the frequency of pulp exposures. Micro-computed tomography (micro-CT) analysis of the DPPcKO molars confirmed the partial rescue with significant recovery in the dentin volume, but not in the dentin mineral density. These results indicate DSP and DPP have distinct roles in dentin mineralization, with DSP regulating initiation of dentin

mineralization, and DPP being involved in the maturation of mineralized dentin.

Paine et al [14] showed that while an overexpression of DSP results in an increased rate of enamel mineralization, the morphology of the enamel is not significantly altered. In the wild type mice, inclusion of DSP in the forming aprismatic enamel may have accounted for its increased hardness. In contrast, overexpressed DPP created chalky and pitted enamel of non-uniform thickness that is more prone to wear. DPP overexpressed mice also show a disruption in the prismatic enamel structure. These studies confirm that DSP and DPP have distinct functions in tooth formation and support the notion that dentin proteins expressed by presecretory ameloblasts contribute to the unique properties of the DEJ [14].

1.2.4 Wnt pathway

Wnt genes encode a family of secreted signaling proteins that specify cell lineage pathways in development. Wnt is an extracellular ligand that interacts with both a seven transmembrane protein in the Fzd family and also a single transmembrane protein in the LRP family (LDL-receptor-related protein 5/6 or Arrow) to efficiently transduce the signal from Wnt to an intracellular pathway to stabilize B-catenin. These proteins are important signaling molecules and regulate embryonic induction, generation of cell polarity, and the specification of mesenchymal cell fate (MCF) [10]. Wnt genes are a

family of glycoproteins that mediate autocrine and paracrine effects by binding to frizzled (Fzd) receptors and LDL-related protein 5/6 (LRP5/6) coreceptors. In the Wnt/B-catenin pathway, Wnt ligands mediate downstream effects by stabilizing β -catenin, a multifunctional protein involved in cell adhesion and transcriptional regulation. Regulation of β -catenin stability is mediated by a complex of proteins including Axin, glycogen synthetase kinase 3 (GSK3), GSK3-binding protein (GBP) and casein kinase 1 (CK1). In the absence of Wnt stimulation, cytoplasmic β -catenin localizing in a multi-protein 'destruction complex' is phosphorylated by CK1a and GSK-3B and degraded. Binding of Wnt ligands to Fzd and LRP5/6 promotes dissociation of the destruction complex and prevents β -catenin degradation. When β -catenin accumulates in the cytoplasm, it translocates into the nucleus. Once in the nucleus, β -catenin forms complexes with members of the LEF/TCF (lymphoid enhancer factor family of transcription factors family of transcription factors / T-cell factor) which mediate transcription of Wnt target genes [29-31]

18 Wnt ligands have been identified in humans and an equal number of receptors and co-receptors which are encoded in the Frizzled and low-density-related lipoprotein receptor 5/6 (LRP5/6) gene families. When Wnt binds to either Frizzled (Fzd) or a complex comprising Frizzled and LRP5/6, a signal is transduced to the phosphoprotein

Dsh in the cytoplasm. There are three Dsh proteins in mammals (Dsh-1, Dsh-2, Dsh-3). At the level of Dsh, the Wnt signal branches into three separate pathways, the canonical, non-canonical, or planar cell polarity (PCP), and Wnt-Ca²⁺ pathways. In all three pathways Dsh is a key transducer of the Wnt signal that operates at the plasma membrane or in the cytoplasm [29]. Canonical signaling mediates gene induction. Wnt signaling uses DIX and PDZ in domains of Dsh to induce β -catenin accumulation in the cytoplasm and subsequent translocation into the nucleus.

Wnt signaling by the β -catenin pathway plays crucial roles in animal development and tumorigenesis. Studies using *Drosophila* S2 cells and mammalian 293 cells to study transmission of a Wnt signal across the plasma membrane show that Wnt protein binds to extracellular domains of both LRP and Frizzled receptors, forming membrane associated hetero-oligomers that interact with both Disheveled (through the intracellular portions of Frizzled) and Axin (through intracellular domains on LRP). Wnt proteins act by forming hetero-oligomers of Frizzled and LRP. Mutations that disrupt the Frizzled signaling activity affect the ability of Frizzled to induce membrane translocation of Dvl and reduce the physical interaction between Dvl and Frizzled. This suggests that a physical interaction between Fzl and Dvl is required for the signaling. However, the exact molecular mechanism by which these two membrane receptors

transmit the Wnt signal across the plasma membrane to regulate the β -catenin degradation complex is unknown [31].

Defects in Wnt signaling are implicated in pathologies ranging from cancer to neural tube defects. Mutations in components of the Wnt signaling pathway, such as β -Catenin, have causative roles in colon cancer in humans, while mutations in Disheveled (Dsh) are associated with neural fold closure disorders.

1.2.5 Wnt and DSPP

Yamashiro et al [10] showed that Wnt10a is specifically associated with the differentiation of odontoblasts and shows co-localization with DSPP mRNA expression in fully differentiated secretory odontoblasts in mouse molars suggesting Wnt10a is an upstream regulatory molecule in the cell-matrix interaction for DSPP expression. It is specifically expressed in the primary and secondary enamel knots, the epithelial signaling centers regulating tooth development. *In situ* hybridization analysis reveals Wnt10a expression is intense in the odontoblast cell layer and maintained in secretory odontoblasts where it is co-expressed with DSPP. Wnt10a was not expressed in pulpal mesenchyme which indicates Wnt10a is induced when precursor cells start to differentiate into odontoblasts indicating that Wnt10a and cell/matrix interactions play an important role in odontoblast differentiation and that Wnt10a links morphogenesis and

the differentiation of odontoblasts [10].

Wnt10b is a regulator of mesenchymal stem cell fate and shows 64% homology with Wnt10a [32]. They are expressed in the enamel knot and the dental epithelium in early molar tooth development as well as growing incisor [33-35]. *In vivo* studies show Wnt10b inhibits adipogenesis and stimulates osteoblastogenesis, thereby impacting bone mass. The exact mechanism by which Wnt10b exerts its effects downstream is unclear. *In vitro* and *in vivo* studies by Cawthon et al suggested that Wnt6, Wnt10a, which forms a gene cluster with Wnt6, and Wnt10b, share similar downstream target influencing differentiation of mesenchymal precursors [30].

1.2.6 Dentin fluorosis

Increased fluoride intake and time of exposure results in more severe alterations in mineralized tissues, including enamel, dentin and bone. The severity of fluorosis is related to the concentration of fluoride in the serum [36]. Fluorosed dentin has long been known to have an increased fluoride content relative to fluorosis severity. Studies by Viera *et al.* [37][36] measured microhardness and dentin mineralization in tooth samples from Montreal and Toronto, Canada, and Fortaleza, Brazil where water fluoride levels were 0.2 ppm, 1 ppm, and 0.7 ppm, respectively. They found that dentin fluoride concentration correlated with dental fluorosis severity, enamel fluoride concentration

correlated with dentin microhardness and dentin mineralization, and dental fluorosis severity correlated with dentin microhardness. The teeth from Brazil exposed to 0.7 ppm fluoride in the drinking water, were harder and less mineralized and presented with higher dentin fluoride concentration values. The Montreal (0.2 ppm) teeth had lower levels of dental fluorosis when compared with teeth from Toronto (1 ppm) and Fortaleza (0.7 ppm).

Moreover numbers of studies have found that the severity of dentin fluorosis is related to fluoride levels in the drinking water, dentin fluoride concentration, and dentin microhardness [37-39]. Severely fluorosed deer [39] have a disrupted dentin crystal structure. The disrupted crystal formation may relate to the findings in a recent *in vitro* study by Waidyasekera and co-workers [40], who described crystallites in fluorosed human dentin as larger, but not homogenously arranged, and found a lower density of crystallites in fluorosed dentin. However it is not conclusive yet whether the effect of chronic exposure to excessive amount of fluoride on dentin mineralization is catabolic or not. [41].

1.3 Mouse incisor as a good model to study effect of fluoride on dentin

The effects of chronic fluoride exposure on human and rodent tooth formation have been compared by Fejerskov et al [42]. They reported that human dentin samples

collected from an area with chronic endemic dental fluorosis (with 3.5 ppm F in drinking water) exhibited inhibition of mineralization similar to the incisor dentin from rats receiving chronic high dose of fluoride (56.5 and 113 ppm F in drinking water for 1-3 months) The continuous eruption of rodent incisors provides constantly developing odontoblasts, which enables us to study the effect of fluoride at any stage of time during the animal's life.

2. AIMS OF THE STUDY

The representative and relatively few studies of the effects of ingested fluoride on dentin formation raise questions as to how fluoride affects dentin formation, and given the use of ingested fluoride for caries prevention, it is important to address these questions. The aim of this study was to determine whether fluoride ingestion during dentinogenesis could affect odontoblast cell function. Ultimately, this work enhances our basic understanding of the effects of excess amount of ingested fluoride on dentin formation using mouse incisor as an experimental model.

3. MATERIALS AND METHODS

3.1 Animal preparation

Everett and his group have been reported differences in dental fluorosis susceptibility and bone formation among 12 inbred mice strains defining: resistant strains (129P3/J, FVB/NJ, CBA/J, and DBA/1J), intermediate strains (SWR/J, BALB/ cByJ, C57BL/10J, and DBA/2J), and sensitive strains (A/J, SJL/J/ C3H/HeJ, and C57BL/6J) [43]. Amongst the fluoride sensitive strains, systemic fluoride exposure (50 and/or 100 ppm F in drinking water) resulted in increased osteoclastogenesis and/or reduction of bone quality in C3H/HeJ and A/J strains, while an anabolic effect on bone formation was observed in C57BL/6J strains under the same fluoride exposure level, indicating the possible opposite effect of fluoride on mesenchymal tissues of these strains [44-46]. Therefore, to confirm whether the effect of excessive amount of fluoride on odontoblasts (dentin/mesenchymal tissue forming cells) correlates with enamel fluorosis, I employed two enamel fluorosis susceptible strains, A/J and C57BL/6J, which show different effects on mesenchymal tissue formation/metabolism, .

Three week old A/J and C57BL/6J female mice were treated with either 0 (control) or 50 ppm fluoride (0.053 mM fluoride) supplemented deionized drinking water

for 4 weeks. All animal handling was properly performed according to the protocol approved by UCSF animal care committee.

3.2 Tissue preparation

After the fluoride treatment, the mice were euthanized by either perfuse-fixation or cardiac puncture, and the dentin was analyzed by histomorphology and immunohistochemistry.

3.2.1 Perfuse Fixed Sample

The mice were anesthetized with 240mg/kg 2,2,2-Tribromoethanol (Avertin (SIGMA-ALDRICH CO., St Louis, MO), and euthanized by perfuse-fixation with 4% paraformaldehyde in 0.06M Na-cacodylate buffer (pH 7.3). Briefly, the mice were first perfused with normal saline for 3 minutes using a peristaltic pump (Thermo Fisher Scientific) to flush out the blood and then with fixative for 10 minutes through a cannula inserted in the left ventricle up to the ascending aorta. Slow perfusion speed (approximately 2ml/min) was employed and an incision was made in the right auricle for drainage immediately before or concomitant with the onset of perfusion. After completion of perfusion fixation, the animal heads were decapitated, de-skinned and the isolated maxillae and mandibles were further immersed in the same fixative overnight at

4°C. After fixation, the samples were decalcified by 8% EDTA (pH 7.3) for 10 days at 4°C. Decalcified mouse samples were dehydrated through a series of ethanol and routinely embedded in paraffin then cut as 5 micrometer thick sections using a microtome (RM2255, Leica Microsystems).

3.2.2 Blood Collection and Fresh Freezing

Mice were anesthetized with 240mg/kg 2,2,2-Tribromoethanol (Avertin (SIGMA-ALDRICH CO., St Louis, MO)), and blood will be collected by cardiac puncture, followed by decapitation. The maxillae and mandibles were quickly dissected and immediately frozen in α -hexane cooled with dry ice and further embedded in 5% carboxymethylcellulose (CMC). Cryosectioning was by cryostat (CM3500 Leica Microsystems, Germany) according to Kawamoto's method at 8 μ m thick [47].

3.3 Serum fluoride measurement

Serum fluoride concentration was analyzed using the modified method of Taves [48-50]. Briefly, 2 ml of hexamethyldisiloxane presaturated 6N HCl was placed in a plastic petri dish with a test tube cap glued on it. The test tube cap was filled with 100 μ l of 1.65 M NaOH. The serum was mixed with the HCl and the petri dish was rapidly covered with a Vaseline sealed lid. Fluoride was diffused from the acidified samples

into the NaOH for 22 hrs. The NaOH and trapped fluoride was dried in a 65°C oven, and then was reconstituted to neutral pH with 500 µl of 0.66 M acetic acid. A fluoride ion-specific electrode (Mettler Toledo, Columbus, OH) was used to measure the fluoride concentration of the buffered solution contained in the cap, comparing the measurements of known standards. Fluoride concentrations (ppm) was averaged from the 3 mice from each treatment group and used to characterize the overall relative fluoride exposure for each animal group.

3.4 Measurement of dentin thickness

Fresh frozen undecalcified sections were prepared by Kawamoto's method [47], and alkaline phosphatase (ALPase) activity was detected by enzyme histochemically to visualize transitional stage of ameloblasts [51-53]. Under the microscope, thickness of dentin and predentin was measured at the position right underneath the transitional ameloblasts where ALPase activity started to appear in ameloblasts as shown in (Fig 3).

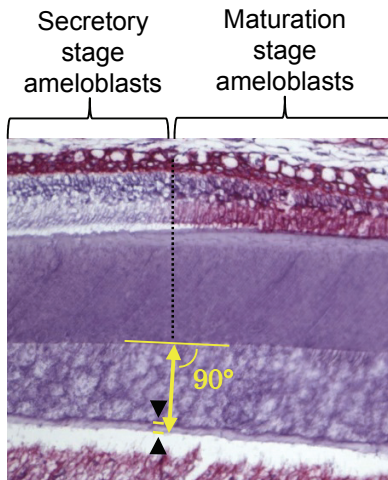


Figure 3: Dentin and pre-dentin measurement
 Sagittal section of a mouse incisor stained for ALPase at the junction between secretory and maturation stage ameloblasts (dotted line). Dentin underlying the enamel matrix is shown between the yellow double heads allows. Pre-dentin is the space in

3.5 Immunostaining

After deparaffinization, the sections of maxilla and mandible were incubated with 10% swine and 5% goat sera followed by incubation with primary antibodies (Table 1) overnight at room temperature. A biotinylated swine anti-rabbit IgG F(ab')₂ fraction (Dako Cytomation Inc., Carpinteria, CA) was used as the secondary antibody for 1 h at room temperature incubation followed by an incubation with alkaline phosphatase conjugated streptavidin (Vector Laboratories Inc., Burlingame, CA) for 30 min. For mouse monoclonal primary antibodies (Table 1), immunostaining was performed using M.O.M kit according to the manufacture's instruction followed by alkaline phosphatase conjugated streptavidin treatment.

Visualization was performed using the Alkaline Phosphatase Substrate Kit (Vector Laboratories, Inc., Burlingame, CA) according to manufacture instructions. 500ul of 10mM levamisole, as an inhibitor of endogenous tissue non-specific ALPase, was added to reagents just before use and mixed. 100 ul of the reaction reagent was applied to each section and incubated at room temperature for up to 1 hour. If no reaction was seen after 1 hour then it was assumed there was no reaction. The sections were then rinsed with dH₂O at room temperature and stored in 4°C. Counter-staining was performed with methyl green (DAKO #S1962) for 15-30 minutes then rinsed with dH₂O and allowed to air dry. Slides were placed in the 95% and 100% ethanol respectively then soaked in xylene. A cover slip was placed on the section using Vector® mounting medium (#H5000). Normal rabbit or mouse serum was used as negative controls.

Table 1: List of primary antibodies

Primary Antibody	Source	Dilution
Mouse anti-mouse β -catenin	BD Biosciences #61053, clone 14/B-Catenin	1 to 75
Rabbit anti-human Dkk-1	R&D Systems #MAB1765, clone 259631	1 to 50
Rabbit anti-human DSPP	Dr. Fisher (NIDCR/NIH), #LF-154	1 to 100
Rabbit anti-pPKA	Sigma-Ardrich #SAB4503969	1 to 50
Rabbit anti-human SOST	abcam #ab63097	1 to 50
Rabbit anti-Wnt10a	Norvus Biologicals #NBP1-69116	1 to 125
Rabbit anti-human Wnt10b	Thermo Sceitific #PA5-27933	1 to 100

3.6 Imaging

Cellular imaging was conducted using the light microscope Nikon E800 system (Nikon TMS, Melville, NY, USA) and photographed using QCapture Imaging software (Quantitative Imaging Corporation, Silicon Graphic, Inc) to compare the cellular morphology of control and fluorosed cells. Photoshop software (Adobe Systems Inc.) was used to minimally adjust image contrast.

3.7 Measurement of β -catenin expression

After immunostaining of β -catenin, sections were counterstained with Hoechst (Life Technologies Corporation) for 20 minutes at room temperature. The immunopositive reaction was observed under a Nikon Eclipse E800 fluorescent microscope and photographed using SimplePCI Version 5.3.1 software. Intensity of staining was quantified by densitometry function of Photoshop software.

3.7 Statistical Analysis

Student's t-test was used for statistical analysis of the ratio of the relative width of pre-dentin as compare to overall dentin and intensity of β -catenin immunostaining. A difference between experimental groups was be considered significant when the probability value will be less than 5.0%.

4. RESULTS

4.1 Serum fluoride level is elevated in both A/J and C57 strains

To investigate the effects of fluoride on dentin formation, I first evaluated the serum fluoride level in fluoride treated mice as compared to control mice. Serum fluoride concentrations increased in A/J mice from 2.09 μM (control) to 3.27 μM (fluoride treated) and in C57 mice from 1.64 μM (control) to 5.3 μM (fluoride treated) respectively (Table 2). These serum fluoride levels in fluoride exposed mice correspond to those of humans drinking 3-5 ppm fluoride supplemented water.

Table 2: serum fluoride concentration

	Control [μM]	Fluorosis [μM]
AJ	2.09 \pm 0.18	3.27 \pm 0.6 *
C57	1.64 \pm 0.69	5.3 \pm 0.43 **

* $p < 0.05$, ** $p < 0.01$

4.2 Pre-dentin is thicker in fluoride exposed mice

After confirming that the serum fluoride levels increased in the fluoride ingesting mice, I examined the relative thickness of dentin and pre-dentin in fluoride exposed mice as compared to controls using undecalcified frozen sections supported by plastic tape to avoid artificial shrinkage of tissue (Fig 4). Overall dentin thickness did not

change in any strains. In A/J, predentin thickness was significantly increased in fluoride treated mice (10.74 μm) compared to control mice (8.14 μm), hence percentage of pre-dentin in overall dentin significantly increased by 3.19% in fluoride exposed mice (Table 3). In C57 mice, pre-dentin thickness also significantly increased from 8.53 μm in control to 9.96 μm in fluorosed mice, resulting in a 1.43% increase in the percent of pre-dentin relative to the total pre-dentin and mineralized dentin layers (overall dentin) (Table 4).

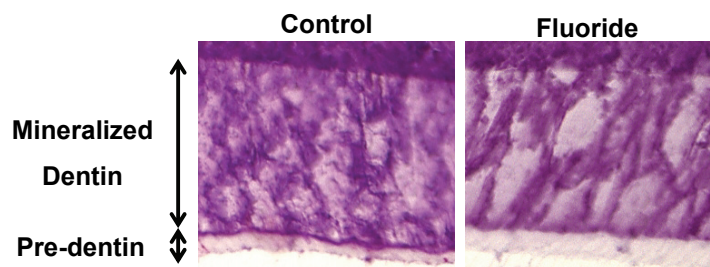


Figure 4: Light microscope images of dentin and pre-dentin

Hematoxylin stained section of control and fluoride treated dentin showing pre-dentin layer (light purple) clearly differentiated from the mineralized dentin (dark purple).

Table 4: Thickness of dentin, A/J mice

	Overall dentin [μm]	Pre-dentin [μm]	% of pre-dentin
control	102.70 ± 2.95	8.14 ± 1.10	7.94 ± 1.08
fluorosis	96.70 ± 3.64	10.74 ± 0.53	11.13 ± 0.94
P value	0.090	0.021*	0.018*

n=6, mean±SD

Table 3: Thickness of dentin, C57 mice

	Overall dentin [μm]	Pre-dentin [μm]	% of pre-dentin
control	103.79 ± 6.59	8.53 ± 0.59	8.22 ± 0.15
fluorosis	103.60 ± 11.17	9.96 ± 0.55	9.65 ± 0.57
P value	0.098	0.035*	0.014*

n=6, mean±SD

4.3 DSPP/DSP expression is downregulated in fluorosed dentin

The phenotype of widened pre-dentin layer in fluorosed mice resembles dentin sialophosphoprotein (DSPP) knockout mice reported by Sreenath et al. [16] with a widened pre-dentin zone and defective dentin mineralization. Because this phenotype is similar to human dentinogenesis imperfecta type III, I further examined expression of DSPP protein using anti-DSP/DSPP antibody.

Immunohistochemistry revealed DSP/DSPP protein expression level was downregulated both in early and mature stage odontoblasts, although staining in the

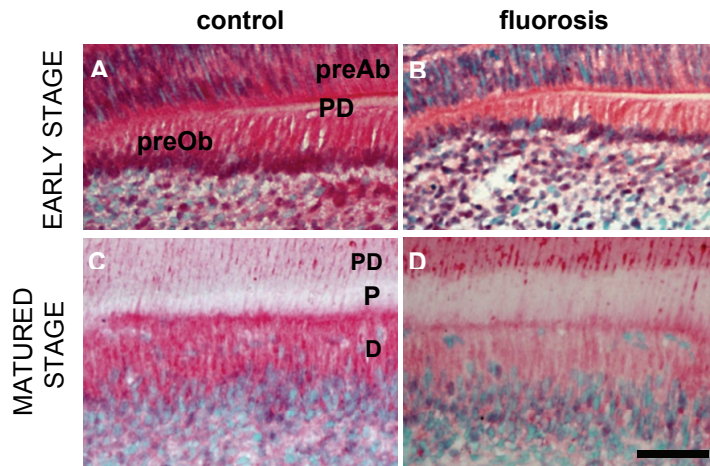


Figure 5: Immunohistochemistry of DSP/DSPP on odontoblasts

DSP/DSPP expression (in red) was downregulated in fluorosed odontoblasts at both early stage (pre-odontoblasts and odontoblasts) and matured stage as well as pre-ameloblasts. preAb: pre-ameloblast, preOb: pre-odontoblast, Ob: odontoblast, D: dentin, PD: pre-dentin. Green: as nuclear counterstaining

dentinal tubules was more intense in mature stage of fluorosed mice (Fig 5).

4.4 Wnt10a and Wnt10b expression is downregulated in fluorosed odontoblasts

Wnt10a has been shown to be an upstream regulatory molecule for DSPP expression specifically associated with the differentiation of odontoblasts in mouse

molars [10]. Therefore, to further explore the possible effects of fluoride on synthesis of DSPP, I examined Wnt10a expression by immunohistochemistry.

Wnt10a expression was downregulated in both early and mature stage odontoblasts, however the effect of downregulation was more obvious in early stages of odontoblast differentiation (Fig 6) and only slightly downregulated in mature stage odontoblasts. (Fig 6C and 6D). Downregulation was not only in odontoblasts, but also evident in the pulp tissues and inner enamel epithelium or preameloblasts. (Fig 6A and 6B).

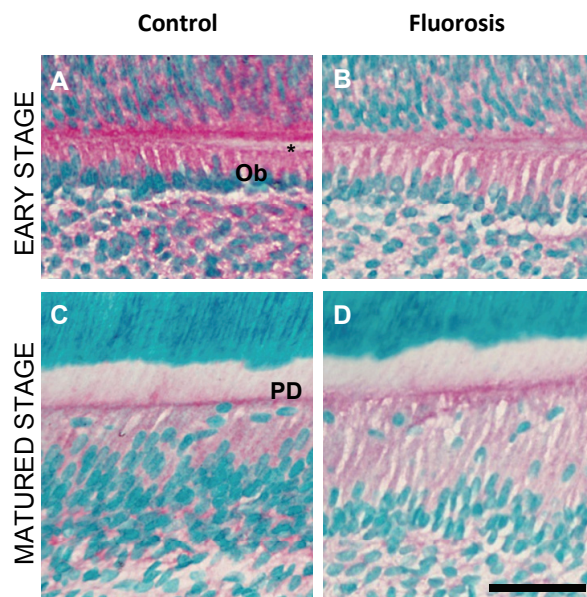


Figure 6: Wnt10a expression

Wnt10a expression is downregulated in fluorosed odontoblasts. preAb: pre-secretory ameloblast, preOb: pre-odontoblast, Ob: odontoblast, D: dentin, PD and *: pre-dentin

Wnt10a and its 64% homologue Wnt10b share similar downstream targets influencing differentiation of mesenchymal precursors [30, 32], and are expressed in the enamel knot and the dental epithelium in early molar tooth development as well as growing incisor [33-35]. To investigate the possibility that fluoride also affects Wnt10b expression as well, I further immunostained Wnt10b and found that Wnt10b expression was also downregulated in both early and mature stage odontoblasts (Fig 7). This data suggests the possibility that reduced levels of DSP/DSPP in fluorosed dentin may be related to suppression of Wnt10a expression and downregulation of Wnt10b in fluoride exposed odontoblasts may be correlated as well.

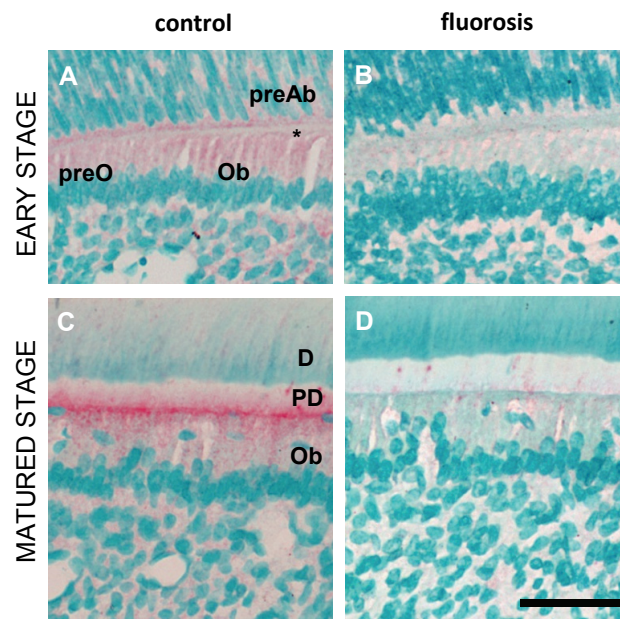


Figure 7: Wnt10b expression

Wnt10b expression is downregulated in fluorosed odontoblasts. preAb: pre-secretory ameloblast, preOb: pre-odontoblast, Ob: odontoblast, D: dentin, PD and * : pre-dentin

4.5 Cytoplasmic and nuclear β -catenin expression was downregulated in pre-odontoblasts.

Wnt ligand is a known upstream regulator for translocation of β -catenin into nucleus where it induces transcription of target genes as an active molecule. To further investigate the possible effects of fluoride on the Wnt/ β -catenin pathway activity, I hypothesized that nuclear translocation of β -catenin may also be down regulated in

fluoride exposed the odontoblasts. I immunostained β -catenin and measured its expression level as a percentage of nuclear β -catenin to cytoplasmic β -catenin. Consistent with this possibility, I found that the ratio of nuclear to cytoplasmic β -catenin in the early stage of odontoblasts from fluoride ingested mice was reduced, suggesting the suppression of Wnt pathway activity in odontoblasts under the exposure to fluoride (Fig 8). It is interesting to note that overall β -catenin expression was also downregulated in fluoride exposed mice (Fig 9).

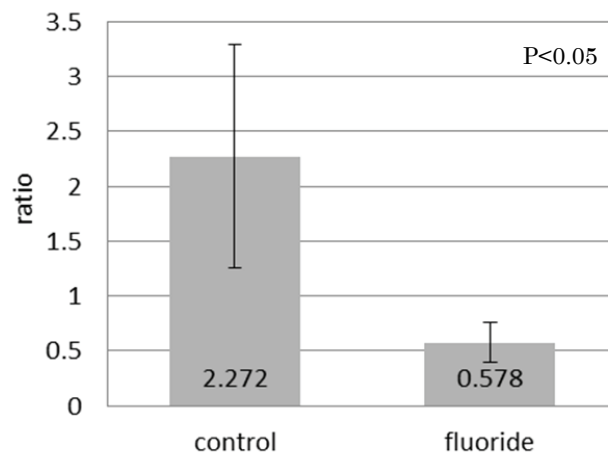


Figure 8: Ratio of nuclear vs. cytoplasmic β -catenin

The relative amount of nuclear β -catenin in the fluoride treated odontoblasts was significantly reduced as compared to controls.

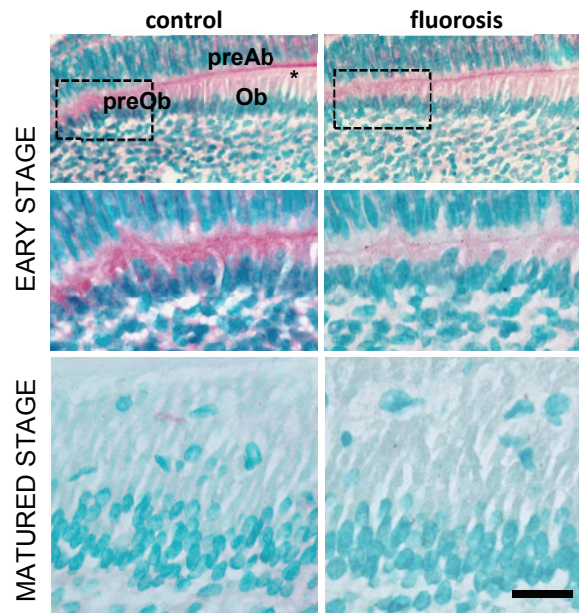


Figure 9: β -catenin staining

Both cytosolic and nuclear β -catenin was downregulated in fluorosed pre-odontoblasts. Fluoride exposure did not alter the expression level of β -catenin at the mature stage. preAb: pre-secretory ameloblast, preOb: pre-odontoblast, Ob: odontoblast, D: dentin, PD and * : pre-dentin

4.6 DKK-1 and SOST expression was slightly downregulated at the early stage,

however there was no change in the mature stage.

DKK-1 and SOST are known antagonist of the canonical Wnt/ β -catenin signaling pathway [54, 55]. To determine if the inhibition of Wnt/ β -catenin pathway activity was due to activated Wnt antagonist (DKK-1 and SOST), I immunostained DKK-1 and SOST. DKK-1 and SOST expression in fluoride exposed mice was slightly downregulated in the early stage pre-odontoblasts and early odontoblasts compared to control (Fig 10A and B and Fig 11A and B). However, in mature stage odontoblasts, there was no obvious difference in expression of both DKK-1 and SOST between control and fluoride exposed mice (Fig 10C and D and Fig 11C and D).

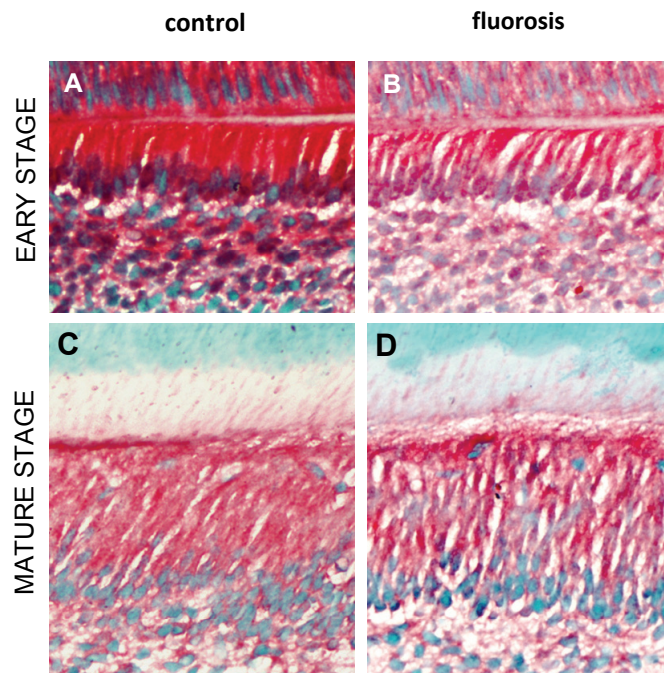


Figure 10: DKK-1 imunostaining

DKK-1 expression was downregulated in early stage, but did not change at the matured stage. preAb: pre-ameloblast, preOb: pre-odontoblast, Ob: odontoblast, D:

dentin, PD and * : pre-dentin, Bar 100 μm

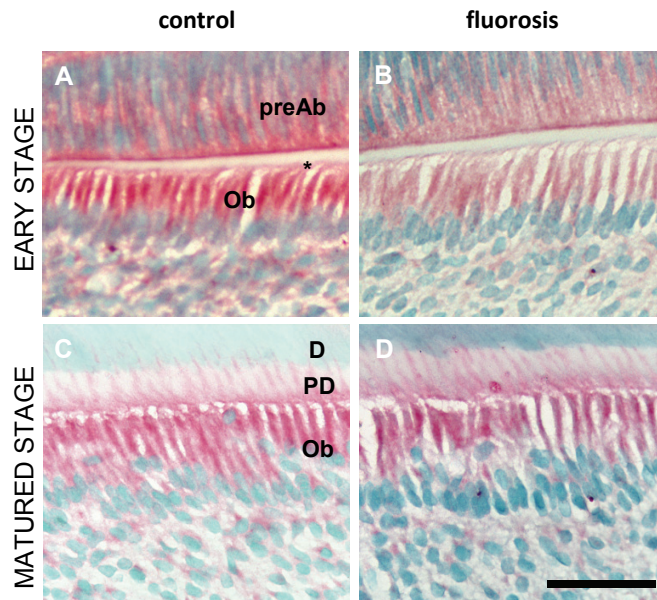


Figure 11: SOST expression

SOST expression was slightly downregulated at early stage but, did not change at the matured stage. preAb: pre-ameloblast, preOb: pre-odontoblast, Ob: odontoblast, D: dentin, PD and * : pre-dentin, Bar 100 μm

4.7 pPKA expression level was downregulated

Protein kinase A (pPKA) is known to be involved in the expression of dentin and enamel related genes. Narayanan et al reported odontoblasts treated with PKA inhibitor showed increased expression of DSPP mRNA [56]. Therefore I examined expression of active catalytic domain of PKA (phosphorylated PKA, pPKA). In fluoride exposed

odontoblasts, expression level of pPKA was downregulated in maturation stage (Fig 12):

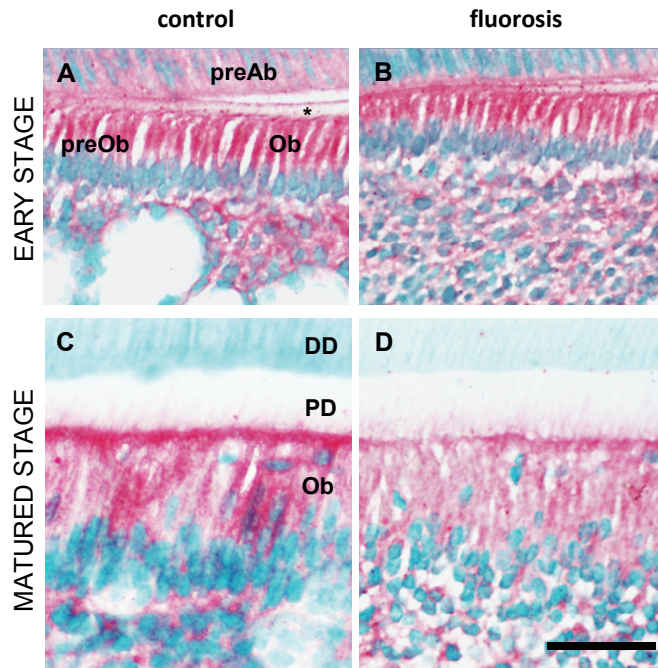


Figure 12: pPKA expression

Fluoride exposure downregulated the synthesis of pPKA at the mature stage. preAb:

pre-ameloblast, preOb: pre-odontoblast, Ob: odontoblast, D: dentin, PD and * :

pre-dentin, Bar 100 μ m

5. DISCUSSION

.5.1 Serum fluoride levels were similar to those of humans ingesting 3-5 ppm fluoride

It is known that rats given 100 ppm fluoride supplemented water for 6 weeks show 5-10 μM serum fluoride [57]. Serum fluoride level in mice given 50 ppm or 100 ppm fluoride in drinking water for 3 weeks elevated to 8 μM (by 50 ppm F water) and 15 μM (by 100 ppm F water) respectively [44]. These serum fluoride levels are equivalent to human plasma fluoride concentrations, 2 to 10 μM (caused by 2-10 ppm F in drinking water) is clinically relevant to dental fluorosis. In our study, serum fluoride concentrations increased in A/J mice from 2.09 μM (control) to 3.27 μM (fluoride treated) and in C57 mice from 1.64 μM (control) to 5.3 μM (fluoride treated) respectively. These serum fluoride levels in fluoride exposed mice correspond to those of humans drinking 3-5 ppm fluoride supplemented water.

It was interesting to note, although both A/J and C57 mice are categorized as the highest in fluoride sensitivity, the increase in serum fluoride concentration in A/J and C57 mice was different. Elevation of serum fluoride in A/J mice was less than two fold, whereas the C57 strain showed nearly three folds increase in serum fluoride, which agreed with a previous report by Yan and co-workers comparing the physical property of

12 inbred mice strains against fluoride ingestion [58].

5.2 Significance of downregulation of DSP/DSPP in fluoride exposed odontoblasts

DSPP is a highly phosphorylated matrix protein, known to be involved in early dentin mineralization [11]. Dentin phosphoprotein (DPP) and dentin sialoprotein (DSP) are the proteolytically cleavage products of dentin sialophosphoprotein (DSPP) and found significantly in the extracellular matrix (ECM) of dentin [11]. DSPP knockout mice show a phenotype similar to human dentinogenesis imperfecta type III with enlarged pulp chambers, increased width of pre-dentin layer, hypomineralized dentin, and pulp exposure indicating DSPP was critical in dentin mineralization [16, 27]. Generation of DPPcKO mice, in which only DSP is expressed in a DSPP null background, showed a partial rescue of the DSPP null phenotype with the predentin width restored, an absence of irregular unmineralized areas in dentin, and less frequent pulp exposure [26]. Micro-computed tomography (micro-CT) analysis of DPPcKO molars further confirmed this partial rescue with a significant recovery in the dentin volume, but not in the dentin mineral density [26]. These results indicate distinct roles of DSP and DPP in dentin mineralization, with DSP regulating initiation of dentin mineralization, and DPP being involved in the maturation of mineralized dentin.

The resemblance of the dentin phenotypes of two rodent models, DSPP ko mice and fluoride exposed mice, implies that excessive fluoride affected the dentin matrix proteins regulating the biomineralization of dentin, in addition to the direct modulation on mineral deposition. In this study, I investigated how fluoride ingestion affects DSPP protein synthesis, by immunostaining of DSPP/DSP, and found reduced DSPP/DSP in the fluorosed odontoblasts indicating the alteration in pre-dentin thickness attributed to downregulation of DSP/DSPP.

Further evidence from studies by Milan and co-workers [59] indicated altered phosphorylation of dentin matrix proteins in Wistar rats given 20 ppm fluoride in drinking water, which could account for the changes in dentin mineralization. The possible mechanism of this finding was suggested by their *in vitro* work [60] presenting the inhibition effect of fluoride on casein kinase II, a potent kinase phosphorylates the most abundant NCPs in dentin matrix (dentin phosphoprotein, DPP, a protein hydrolysis product of DSPP) [61, 62].

5.2 Significance of downregulation of Wnt10a and Wnt10b in fluoride exposed

odontoblasts

Downregulation of DSP/DSPP synthesis indicates the reduction of the upstream pathway activity regulating the DSPP transcription. Wnt10a has been shown to be an

upstream regulatory molecule for DSPP expression specifically associated with the differentiation of odontoblasts in mouse molars [10] (Fig 13).

In this study, I found that Wnt10a and its 64% homologue, Wnt10b, protein expression was downregulated in fluoride exposed odontoblasts. However, expression of antagonists for Wnt receptor (DKK-1 and SOST) were not upregulated, but in fact were rather slightly suppressed in pre-odontoblasts, suggesting the possibility that reduced levels of DSP/DSPP in fluorosed dentin may be caused reduced Wnt10a and Wnt10b expression.

Immunostaining in this study showed reduction of nucleus translocation of β -catenin in early stage of odontoblasts from fluoride ingested mice, further supporting the possibility that fluoride can suppress Wnt pathway activity in odontoblasts. We do not know how fluoride affects β -catenin synthesis. Reduced level of β -catenin in fluoride exposed odontoblast also participates in the inhibition of Wnt/ β -catenin pathway activity in fluoride exposed mice.

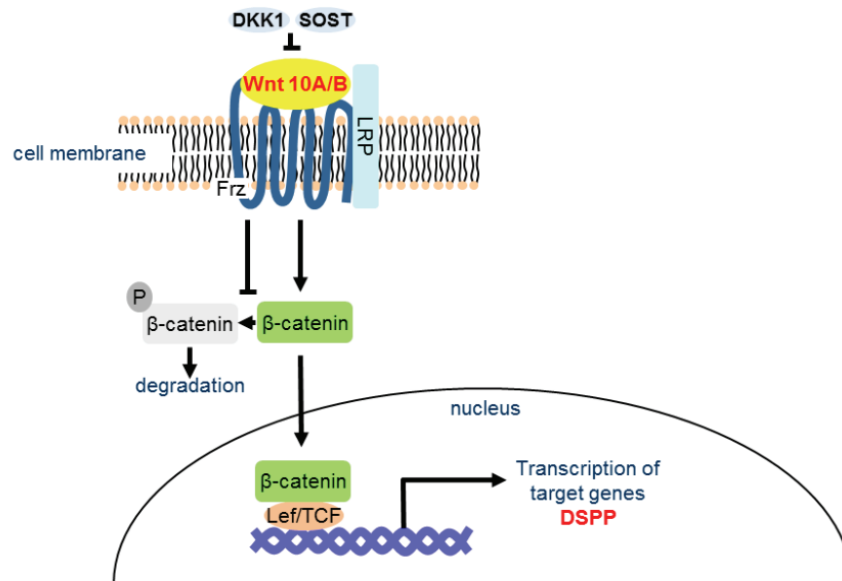


Figure 13: Wnt/ β -catenin pathway

5.3 NF κ B pathway is a potential upstream pathway to regulate Wnt10a and Wnt10b expression

NF κ B nuclear complex, a downstream transcriptional factor of tumor necrosis factor α (TNF- α) pathway, is known to stimulate transcription of Wnt10a and Wnt10b [63, 64]. The NF κ B family of transcription factors is composed of homo- and heterodimers of Rel proteins; NF κ B 1(p50); NF κ B 2(p52), RelA (p65), RelB, and c-Rel (Rel). The post-translational modifications play an important role and regulate different functions of NF- κ B. Kinases including protein kinase A catalytic subunit (PKAc), protein kinase C ζ (PKC ζ), casein kinase-II (CK II), IKK and mitogen- and stress-activated kinase 1 (MSK1), phosphorylate p65 in response to signaling events. Phosphorylated p65 is

further acetylated at specific sites by CREB-binding protein (CBP) and p300 acetyltransferases [65], resulted in full transcriptional activity of the NFκB (Fig 14). In vitro study by Milan et al. reported the CKII activity by fluoride [60]. In this study, I also found less PKA (phospho-PKA) in mature odontoblasts exposed to fluoride as compared to controls, possibly suggesting that downregulation of Wnt10a and Wnt10b is due to inhibition of PKA activity.

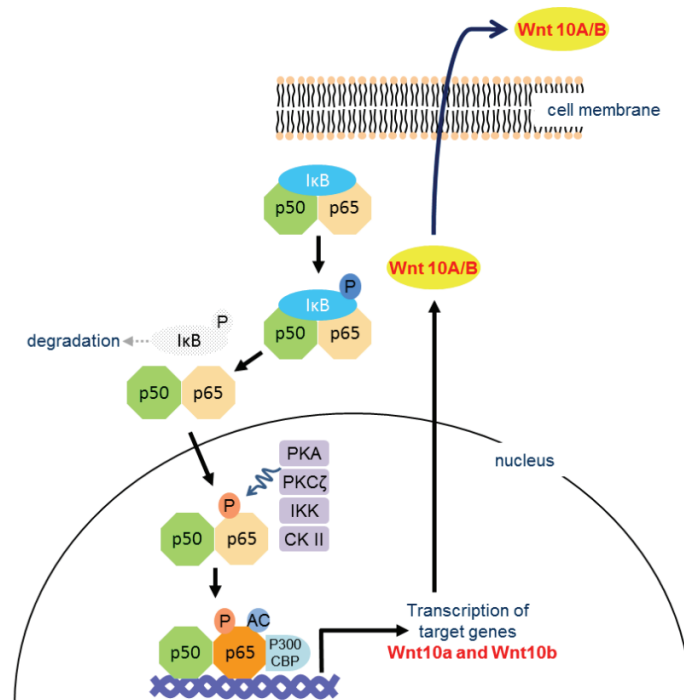


Figure 14: NFκB pathway and Wnt transcription

5.4 Clinical Relevance

5.4.1 Hidden Caries

Our observation that fluoride can increase the pre-dentin layer and alter synthesis of DSP, lead us to consider the possibility that increased fluoride exposure may contribute to what has been referred to as hidden caries. In the 1980s the term Hidden Caries was introduced to dentistry. Hidden caries is a sub-type of occlusal pit and fissure type caries. It is used to describe carious lesions seen in the dentin on a bitewing radiograph, where clinically the occlusal enamel appears intact or is minimally demineralized [66] (Fig 15).



Figure 15: Hidden caries

Radiolucency in the dentin (arrow) under an intact enamel surface suggests hidden caries.

Cariogenic bacteria penetrate into the enamel through a minimal opening in the enamel surface, but once reaching the less mineralized dentin, caries progresses at an increased rate. Meanwhile the fluoridated enamel undergoes remineralization while caries progresses at an increased rate in the dentin. Hidden carious lesions present a challenge to dentists in diagnosis, treatment planning, and research, due to their ‘hidden’ nature.

As a result, dentists are likely to misdiagnose dental caries [67].

Weerheijm et al clinically evaluated first and second permanent molars on 359 patients between six and eighteen years old at the Department of Academic Centre for Dentistry Amsterdam (ACTA) [66, 68]. Despite thorough examination, 15 percent of the teeth that showed no clinical signs of enamel caries, showed caries in the dentin on bitewing radiographs. Their studies show the absence of clinical signs of occlusal enamel caries does not guarantee sound dentition.

Another study conducted by Weerheijm et al in 1997 investigated the influence of water fluoridation on the occurrence of hidden caries in clinically sound enamel in 515 15 year old children. Data was collected in 1968 and 1969. Participants were divided into two groups. The Tiel group (F) was exposed to artificially fluoridated drinking water at a concentration of 1.1 ppm from birth until the day of data collection. The second group in Culemborg (NF) were exposed to water containing 0.1 ppm. Clinical data of 270 inhabitants of Tiel (F) and 245 of Culemborg (NF) were compared with the radiographic findings. Their investigations show a proportional reduction of surfaces with hidden caries in the areas receiving water fluoridation compared to the control subjects. [69].

The widespread use of fluorides can interfere with the prevalence of clinically

undetected occlusal caries. Hashizume et al examined the role of public water fluoridation and fluoride dentifrice on the prevalence of hidden caries in 8-10 year old children. Clinical and radiographic data was collected on children in Porto Alegre, Brazil at two time periods, in 1975 (n=228) and 1996 (n=213). Only children who were examined in 1996 had the benefit of fluoridated water since birth and also had regular access to fluoride dentifrice. The criteria applied for hidden caries was clinical sound surfaces that also presented with radiolucent zone of dentin when data was compared between 1975 and 1996. The prevalence of clinically sound surfaces and percentage of hidden caries in 1975 was 0.51 and 26.4% as opposed to 2.67 and 12.9% in 1996. The prevalence of hidden caries between the two periods was statistically significant ($p < 0.05$). These results show that the use of fluoride through drinking water and dentifrices results in a decrease in the prevalence of hidden caries [70]. However, it is possible that at increasing levels of fluoride exposure, which would result in enamel fluorosis, and serum levels such as were found in my study dentin may be more caries susceptible.

5.4.2 Clinical significance of Dentin Fluorosis

Fluorosed dentin has long been known to have an increasing fluoride content relative to enamel fluorosis severity. Viera and colleagues [37] reported that mildly fluorosed human enamel had reduced dentin microhardness in human teeth. As the

severity of fluorosis increases, dentin crystals become disorganized [39, 40, 71, 72]. The differences in reported dentin crystal formation in mild and more severe dentin may relate to the findings in a recent *in vitro* study by Waidyasekera and colleagues that showed moderately fluorosed human enamel to be more caries resistant, whereas mildly and moderately fluorosed dentin is more caries susceptible [73].

Further understanding of dentin fluorosis will effectively address questions of potential clinical significance for dentin structure formation, which link to potential alterations in caries susceptibility, dentin structure relevant to bonding of composite based dental materials, the capability of dentin to remineralize, secondary dentin formation, and dentin bridge formation. As a pediatric dentist I will consider the caries progression especially the case of hidden caries progression in the fluorosed dentin caused by the exposure to excess amount of fluoride.

6. CONCLUSION

Tooth formation is regulated by signaling molecules and how they interact with their downstream targets. Noncollagenous proteins are prominent components of the extracellular matrix and are important in cell-cell and cell-matrix communication, and in dentin mineralization. The goal of this study was to help elucidate the effects of systemic fluoride exposure on dentin formation. The *in vivo* results using mice model reported in this thesis show a correlation between fluoride overexposure and alteration in progress of dentin mineralization. I found that fluoride causes downregulation of Wnt10a, Wnt10b, β -catenin, and DSPP resulting in wider predentin thickness. The results suggest that fluoride affects dentin mineralization and odontoblasts cell activity. Additional work is needed to elucidate the exact mechanism regulating dentin mineralization.

7. FUTURE WORK

7.1 Detection of Wnt/ β -catenin and NF κ B pathway activity in vivo

The problem of reliably accessing the activity of Wnt and NF κ B signaling in vivo is compounded by difficulties in spatiotemporal detection of active parameters for those pathways. Hitherto, Wnt pathway and NF κ B activity in the animal can be visualized by reporter transgenes that respond to the signals in intact animals and therefore reflect endogenous Wnt or NF κ B signaling. The TOP-GAL transgenic mice (Tg(Fos-lacZ)^{34Efu/J}) are a reporter strain that express β -galactosidase in the presence of the LEF/TCF mediated signaling pathway and activated β -catenin. The β -catenin-activated transgene driving expression of nuclear β -galactosidase reporter (BAT-GAL) transgene mice (Tg(BAT-lacZ)^{3Picc}) are an another strain expressing the lacZ gene under the control of β -catenin binding to TCF elements, which can be used as a general read-out of β -catenin activity.

The B10.Cg-H2k Tg(NF κ B/Fos-luc)^{26Rinc/J} mice) have the luciferase gene driven by two copies of the NF-kappaB (NF-kB or NF κ B) regulatory element. The presence of nuclear NF-kB DNA binding activity is consistent with luciferase reporter activity in any tissue. If fluoride ingested from drinking water changes the pathway

activities, we will see the difference in the localization and intensity of β -galactosidase (for Wnt pathway) and luciferase (for NF κ B pathway) in odontoblasts.

7.2 Mineralization Analysis

To access the quality of dentin matrix mineralization following analysis could be done in future. A) micro CT for quantification of mineralization, B) electron probe microanalysis for analyzing the distribution and quantification of elements like Ca, P and F in the dentin matrix, C) mechanical stress analysis for understanding the mechanical property of fluoride exposed dentin

7.3 DMP1 and DSPP

Dentin mineralization requires multiple transcriptional mechanisms working together to induce gene expression, the purpose of which is the progressive development of the odontoblast [15]. Narayanan et al studies showed that DMP1 (a non-collagenous protein expressed in mineralized tissues) is localized in the nucleus during early odontoblast differentiation. It is an acidic protein rich in aspartic acid, glutamic acid, and serine residues. DMP1 is acts an important regulatory molecule in the biomineralization process by initiating hydroxyapatite nucleation at the mineralization front. Narayanan showed overexpressed DMP1 binds specifically with the DSPP

promoter to activate DSPP transcription. Their findings provide a foundation to understand how DMP1 regulates DSPP gene expression [15]. If DSPP is upregulated, then expression of DMP1 should also be increased. Factors that downregulate DSPP promoter activity is transforming growth factor β 1 (TGF β 1), CAAT/enhancer-binding protein B, and Nrfl. Odontoblast-specific overexpression of TGF β 1 in the transgenic mouse model showed a reduced expression of DSPP indicating that TGF β 1-mediated signaling pathway down-regulates DSPP expression. CAAT/enhancer-binding protein B and Nrfl interact and down-regulate the expression of DSPP in odontoblasts. RUNX2 has been shown to differentially regulate the DSPP promoter activity during differentiation of odontoblasts. At the cellular level, RUNX2 down-regulates DSPP promoter activity in preodontoblasts while increasing its expression during terminal differentiation. At the molecular level, DSPP gene undergoes several post-transcriptional modifications, a common one being alternative splicing.

We should therefore consider the possibility of these molecules altering the DSPP expression in fluoride exposed odontoblasts.

7.4 Cell Culture System

An additional in vitro study using an odontoblast cell culture system could be performed to further explore these pathways and related molecule analyses. Gene

expression will be analyzed by qPCR and subcellular localization of proteins and its quantification will be accessed by western blot. Using these methods we may also explore the effects of fluoride on other molecules including BMP2, Runx2, NF- κ B and DMP1, which may be involved in regulation of DSPP [11].

8. REFERENCES

1. Denbesten, P. and W. Li, *Chronic fluoride toxicity: dental fluorosis*. Monogr Oral Sci, 2011. **22**: p. 81-96.
2. Levine, R.S., *Fluoride and caries prevention: 1. Scientific rationale*. Dent Update, 1991. **18**(3): p. 105-6, 108-10.
3. Slooff, W., et al., *Basisdocument fluoriden*. 1988: Rijksinstituut voor Volksgezondheid en Milieuhygiëne.
4. Giambro, N.J., K. Probst, and P.K. Den Besten, *Characterization of fluorosed human enamel by color reflectance, ultrastructure, and elemental composition*. Caries Res, 1995. **29**(4): p. 251-7.
5. Janssen, P.J.C.M., et al., *Integrated Criteria Document Fluorides: Effects*. 1989: National Institute of Public Health and Environmental Protection.
6. Jolly, S.S., et al., *Epidemiological, clinical, and biochemical study of endemic dental and skeletal fluorosis in punjab*. Br Med J, 1968. **4**(5628): p. 427-9.
7. Choubisa, S.L., et al., *Fluorosis in some tribal villages of Dungarpur district of Rajasthan, India*. Fluoride, 1997. **30**(4): p. 223-228.
8. Murray, J.J., et al., *Appropriate use of fluorides for human health*. 1986, Geneva Albany, NY: World Health Organization ;

WHO Publications Center USA distributor. vi, 131 p.

9. Centers for Disease Control (U.S.) and Centers for Disease Control and Prevention (U.S.), *Morbidity and mortality weekly report : MMWR, in Populations Receiving Optimally Fluoridated Public Drinking Water —United States, 1992–2006* 2008, U.S. Dept. of Health, Education, and Welfare, Public Health Service: Atlanta, Ga. p. 737.
10. Yamashiro, T., et al., *Wnt10a regulates dentin sialophosphoprotein mRNA expression and possibly links odontoblast differentiation and tooth morphogenesis.* Differentiation, 2007. **75**(5): p. 452-62.
11. Prasad, M., W.T. Butler, and C. Qin, *Dentin sialophosphoprotein in biomineralization.* Connect Tissue Res, 2010. **51**(5): p. 404-17.
12. Fisher, L.W., et al., *Flexible structures of SIBLING proteins, bone sialoprotein, and osteopontin.* Biochem Biophys Res Commun, 2001. **280**(2): p. 460-5.
13. Fujisawa, R. and M. Tamura, *Acidic bone matrix proteins and their roles in calcification.* Front Biosci, 2012. **17**: p. 1891-903.
14. Paine, M.L., et al., *Dentin sialoprotein and dentin phosphoprotein overexpression during amelogenesis.* J Biol Chem, 2005. **280**(36): p. 31991-8.
15. Narayanan, K., et al., *Dentin matrix protein 1 regulates dentin*

- sialophosphoprotein gene transcription during early odontoblast differentiation. J Biol Chem*, 2006. **281**(28): p. 19064-71.
16. Sreenath, T., et al., *Dentin sialophosphoprotein knockout mouse teeth display widened predentin zone and develop defective dentin mineralization similar to human dentinogenesis imperfecta type III. J Biol Chem*, 2003. **278**(27): p. 24874-80.
 17. Kuboki, Y., et al., *Calcium-specific precipitation of dentin phosphoprotein: a new method of purification and the significance for the mechanism of calcification. J Dent Res*, 1979. **58**(9): p. 1926-32.
 18. Marsh, M.E., *Self-association of calcium and magnesium complexes of dentin phosphophoryn. Biochemistry*, 1989. **28**(1): p. 339-45.
 19. Linde, A., *Dentin matrix proteins: composition and possible functions in calcification. Anat Rec*, 1989. **224**(2): p. 154-66.
 20. Boskey, A.L., et al., *Concentration-dependent effects of dentin phosphophoryn in the regulation of in vitro hydroxyapatite formation and growth. Bone Miner*, 1990. **11**(1): p. 55-65.
 21. Saito, T., et al., *Mineral induction by immobilized phosphoproteins. Bone*, 1997. **21**(4): p. 305-11.

22. MacDougall, M., M. Zeichner-David, and H.C. Slavkin, *Production and characterization of antibodies against murine dentine phosphoprotein*. *Biochem J*, 1985. **232**(2): p. 493-500.
23. Weinstock, M. and C.P. Leblond, *Radioautographic visualization of the deposition of a phosphoprotein at the mineralization front in the dentin of the rat incisor*. *J Cell Biol*, 1973. **56**(3): p. 838-45.
24. Rabie, A.M. and A. Veis, *An immunocytochemical study of the routes of secretion of collagen and phosphophoryn from odontoblasts into dentin*. *Connect Tissue Res*, 1995. **31**(3): p. 197-209.
25. Butler, W.T., J.C. Brunn, and C. Qin, *Dentin extracellular matrix (ECM) proteins: comparison to bone ECM and contribution to dynamics of dentinogenesis*. *Connect Tissue Res*, 2003. **44 Suppl 1**: p. 171-8.
26. Suzuki, S., et al., *Dentin sialoprotein and dentin phosphoprotein have distinct roles in dentin mineralization*. *Matrix Biol*, 2009. **28**(4): p. 221-9.
27. Verdelis, K., et al., *DSPP effects on in vivo bone mineralization*. *Bone*, 2008. **43**(6): p. 983-90.
28. Qin, C., O. Baba, and W.T. Butler, *Post-translational modifications of sibling proteins and their roles in osteogenesis and dentinogenesis*. *Crit Rev Oral Biol*

- Med, 2004. **15**(3): p. 126-36.
29. Habas, R. and I.B. Dawid, *Dishevelled and Wnt signaling: is the nucleus the final frontier?* J Biol, 2005. **4**(1): p. 2.
 30. Cawthorn, W.P., et al., *Wnt6, Wnt10a and Wnt10b inhibit adipogenesis and stimulate osteoblastogenesis through a beta-catenin-dependent mechanism.* Bone, 2012. **50**(2): p. 477-89.
 31. Cong, F., L. Schweizer, and H. Varmus, *Wnt signals across the plasma membrane to activate the beta-catenin pathway by forming oligomers containing its receptors, Frizzled and LRP.* Development, 2004. **131**(20): p. 5103-15.
 32. Qurrat Ul, A., et al., *Integrative analyses of conserved WNT clusters and their co-operative behaviour in human breast cancer.* Bioinformatics, 2011. **7**(7): p. 339-46.
 33. Dassule, H.R. and A.P. McMahon, *Analysis of epithelial-mesenchymal interactions in the initial morphogenesis of the mammalian tooth.* Dev Biol, 1998. **202**(2): p. 215-27.
 34. Sarkar, L. and P.T. Sharpe, *Expression of Wnt signalling pathway genes during tooth development.* Mech Dev, 1999. **85**(1-2): p. 197-200.
 35. Suomalainen, M. and I. Thesleff, *Patterns of Wnt pathway activity in the mouse*

- incisor indicate absence of Wnt/beta-catenin signaling in the epithelial stem cells.*
- Dev Dyn, 2010. **239**(1): p. 364-72.
36. Angmar-Mansson, B., Y. Ericsson, and O. Ekberg, *Plasma fluoride and enamel fluorosis.* Calcif Tissue Res, 1976. **22**(1): p. 77-84.
37. Vieira, A., et al., *How does fluoride affect dentin microhardness and mineralization?* J Dent Res, 2005. **84**(10): p. 951-7.
38. Vieira, A.P., et al., *Is fluoride concentration in dentin and enamel a good indicator of dental fluorosis?* J Dent Res, 2004. **83**(1): p. 76-80.
39. Kierdorf, U., H. Kierdorf, and O. Fejerskov, *Fluoride-induced developmental changes in enamel and dentine of European roe deer (Capreolus capreolus L.) as a result of environmental pollution.* Arch Oral Biol, 1993. **38**(12): p. 1071-81.
40. Waidyasekera, K., et al., *Why does fluorosed dentine show a higher susceptibility for caries: an ultra-morphological explanation.* J Med Dent Sci. **57**(1): p. 17-23.
41. Rojas-Sanchez, F., et al., *Dentin in severe fluorosis: a quantitative histochemical study.* J Dent Res, 2007. **86**(9): p. 857-61.
42. Fejerskov, O., J.A. Yaeger, and A. Thylstrup, *Microradiography of the effect of acute and chronic administration of fluoride on human and rat dentine and enamel.* Arch Oral Biol, 1979. **24**(2): p. 123-30.

43. Everett, E.T., et al., *Dental fluorosis: variability among different inbred mouse strains*. J Dent Res, 2002. **81**(11): p. 794-8.
44. Everett, E.T., *Fluoride's effects on the formation of teeth and bones, and the influence of genetics*. J Dent Res, 2011. **90**(5): p. 552-60.
45. Yan, D., et al., *Genetic background influences fluoride's effects on osteoclastogenesis*. Bone, 2007. **41**(6): p. 1036-44.
46. Mousny, M., et al., *The genetic influence on bone susceptibility to fluoride*. Bone, 2006. **39**(6): p. 1283-9.
47. Kawamoto, T., *Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole-animals, insects and plants*. Arch Histol Cytol, 2003. **66**(2): p. 123-43.
48. Taves, D.R., *Determination of submicromolar concentrations of fluoride in biological samples*. Talanta, 1968. **15**(10): p. 1015-23.
49. Taves, D.R., *Separation of fluoride by rapid diffusion using hexamethyldisiloxane*. Talanta, 1968. **15**(9): p. 969-74.
50. Bawden, J.W., et al., *Effect of an acute maternal fluoride dose on fetal plasma fluoride levels and enamel fluoride uptake in guinea pigs*. J Dent Res, 1989. **68**(7): p. 1169-72.

51. Gomez, S. and A. Boyde, *Correlated alkaline phosphatase histochemistry and quantitative backscattered electron imaging in the study of rat incisor ameloblasts and enamel mineralization*. *Microsc Res Tech*, 1994. **29**(1): p. 29-36.
52. Symons, N.B., *Alkaline phosphatase activity in the developing teeth of the rat*. *J Anat*, 1955. **89**(2): p. 238-45.
53. Burstone, S., *Enzyme histochemistry, and its application in the study of neoplasms*. 1962: Academic Press. 275-276.
54. Chen, L., et al., *Structural insight into the mechanisms of Wnt signaling antagonism by Dkk*. *J Biol Chem*, 2008. **283**(34): p. 23364-70.
55. Liu, X.L., et al., *The influence of fluoride on the expression of inhibitors of Wnt/beta-catenin signaling pathway in rat skin fibroblast Cells*. *Biol Trace Elem Res*, 2012. **148**(1): p. 117-21.
56. Narayanan, K., et al., *The CCAAT enhancer-binding protein (C/EBP)beta and Nrfl interact to regulate dentin sialophosphoprotein (DSPP) gene expression during odontoblast differentiation*. *J Biol Chem*, 2004. **279**(44): p. 45423-32.
57. DenBesten, P.K., et al., *Effects of fluoride on rat dental enamel matrix proteinases*. *Arch Oral Biol*, 2002. **47**(11): p. 763-70.
58. Yan, D., et al., *Phenotypic variation of fluoride responses between inbred strains*

- of mice*. Cells Tissues Organs, 2011. **194**(2-4): p. 261-7.
59. Milan, A.M., R.J. Waddington, and G. Embery, *Altered phosphorylation of rat dentine phosphoproteins by fluoride in vivo*. Calcif Tissue Int, 1999. **64**(3): p. 234-8.
60. Milan, A.M., R.J. Waddington, and G. Embery, *Fluoride alters casein kinase II and alkaline phosphatase activity in vitro with potential implications for dentine mineralization*. Arch Oral Biol, 2001. **46**(4): p. 343-51.
61. Dimuzio, M.T. and A. Veis, *Phosphoryns-major noncollagenous proteins of rat incisor dentin*. Calcif Tissue Res, 1978. **25**(2): p. 169-78.
62. Butler, W.T., *The chemistry and biology of mineralized tissues: proceedings of the Second International Conference on the Chemistry and Biology of Mineralized Tissues, held in Gulf Shores, Alabama, September 9-14, 1984*. 1985, [S.l.: s.n.]. 436 p.
63. Katoh, M. and M. Katoh, *API- and NF-kappaB-binding sites conserved among mammalian WNT10B orthologs elucidate the TNFalpha-WNT10B signaling loop implicated in carcinogenesis and adipogenesis*. Int J Mol Med, 2007. **19**(4): p. 699-703.
64. Li, J., et al., *Novel NEMO/IkappaB kinase and NF-kappa B target genes at the*

- pre-B to immature B cell transition*. J Biol Chem, 2001. **276**(21): p. 18579-90.
65. Chen, L.F., et al., *NF-kappaB RelA phosphorylation regulates RelA acetylation*. Mol Cell Biol, 2005. **25**(18): p. 7966-75.
66. Weerheijm, K.L., *Occlusal 'hidden caries'*. Dent Update, 1997. **24**(5): p. 182-4.
67. Zadik, Y. and R. Bechor, *[Hidden occlusal caries lesion--a diagnostic challenge]*. Refuat Hapeh Vehashinayim, 2008. **25**(1): p. 34-9, 83.
68. Weerheijm, K.L., R.J. Gruythuysen, and W.E. van Amerongen, *Prevalence of hidden caries*. ASDC J Dent Child, 1992. **59**(6): p. 408-12.
69. Weerheijm, K.L., E.A. Kidd, and H.J. Groen, *The effect of fluoridation on the occurrence of hidden caries in clinically sound occlusal surfaces*. Caries Res, 1997. **31**(1): p. 30-4.
70. Hashizume, L.N., et al., *Effect of the widespread use of fluorides on the occurrence of hidden caries in children*. Int J Paediatr Dent, 2013. **23**(1): p. 72-6.
71. Nelson, D.G., et al., *Proton microprobe determination of fluorine profiles in the enamel and dentine of erupting incisors from sheep given low and high daily doses of fluoride*. Arch Oral Biol, 1989. **34**(6): p. 419-29.
72. Yaeger, J.A., *The effects of high fluoride diets on developing enamel and dentin in the incisors of rats*. Am J Anat, 1966. **118**(2): p. 665-83.

73. Waidyasekera, P.G., et al., *Caries susceptibility of human fluorosed enamel and dentine*. J Dent, 2007. **35**(4): p. 343-9.

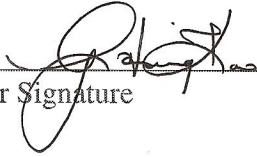
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