A Correlation between Wnt/Beta-catenin Signaling and the Rate of Dentin Secretion

Yuan Zhao DDS, PhD[1,2], Xue Yuan PhD[2], Teresita Bellido PhD[3,] Jill A. Helms DDS, PhD[1,2].

1. Department of Cariology and Endodontology, School of Dentistry, Lanzhou University, Lanzhou, China
2. Division of Plastic and Reconstructive Surgery, Department of Surgery, Stanford School of Medicine, Stanford, California
3. Departments of Anatomy and Cell Biology and Medicine, Division of Endocrinology, Indiana University School of Medicine and Roudebush Veterans Administration Medical Center, Indianapolis, Indiana

ABSTRACT

INTRODUCTION

Odontoblasts produce dentin throughout life and in response to trauma. The purpose of this study was to identify the roles of endogenous Wnt signaling in regulating the rate of dentin accumulation.

METHODS

Histology, immunohistochemistry, vital dye labeling, and histomorphometric assays were used to quantify the rate of dentin accumulation as a function of age. Two strains of Wnt reporter mice were used to identify and follow the distribution and number of Wnt-responsive odontoblasts as a function of age. To show a causal relationship between dentin secretion and Wnt signaling, dentin accumulation was monitored in a strain of mice in which Wnt signaling was aberrantly elevated.

RESULTS

Dentin deposition occurs throughout life, but the rate of accumulation slows with age. This decline in dentin secretion correlates with a decrease in endogenous Wnt signaling. In a genetically modified strain of mice, instead of tubular dentin, aberrantly elevated Wnt signaling resulted in accumulation of reparative dentin or osteodentin secreted from predontoblasts.

CONCLUSIONS

Wnt signaling regulates dentin secretion by odontoblasts, and the formation of reparative or osteodentin is the direct consequence of elevated Wnt signaling. These preclinical data have therapeutic implications for the development of a biologically based pulp capping medicant.

KEY WORDS: Aging, odontoblasts, pulp, Wnt signal

SIGNIFICANCE

Our study revealed Wnt signaling regulates dentin secretion by odontoblasts, which has therapeutic implications for the development of a biologically based pulp capping medicant.

Bone turnover is maintained by osteoclasts that resorb bone matrix and osteoblasts that deposit new matrix. With aging, the balance between osteoblast and osteoclast function is lost, and the result is osteoporosis, a low bone mass disease[1], [2]. On the other hand, the dental pulp only houses dentin-producing odontoblasts; consequently, dentin accumulates with age[3].

In response to activities of daily living including exercise that can cause microcracks, osteoclasts resorb mineralized matrix, and osteoblasts deposit new osteoid. This remodeling activity restores the structural integrity of the skeleton. The dental pulp is also capable of mounting a response to subacute trauma (eg, extreme temperature changes or acid production by bacteria), but this is distinctive from bone because there is no remodeling activity. Instead, odontoblasts can only increase their rate of dentin secretion and, in doing so, create a thicker protective barrier between the insult and the pulp[4]. In acute trauma, other cells in the pulp (eg, preodontoblasts) can be recruited to rapidly secrete a mineralized matrix that protects the pulp. This mineralized tissue does not have a tubular structure because the cells producing it lack processes[5], [6] and is called osteoidlike dentin or osteodentin[7].

A key regulator of the balance between osteoblast and osteoclast function is the Wnt pathway[8], [9], [10]. Wnt signals operate at multiple levels in the programs of osteoblastogenesis and osteoclastogenesis, but, in general, the effects of this pathway can be summarized as having a pro-osteogenic effect[11]. For example, the genetic gain in Wnt function mutations causes high bone mass conditions[12], [13], whereas the loss of Wnt function leads to low bone mass diseases including osteoporosis[14]. Studies have shown that the Wnt pathway is also operational in the dental pulp[15], [16], [17], [18], [19], [20].

In the present study, we sought to better understand the role of Wnt signaling in age-related changes to the pulp, specifically how these changes might contribute to the ability

of the pulp to respond to subacute traumas resulting from the activities of daily living. We used 4 transgenic mouse strains: in the *Axin2^LacZ/+* strain of mice, the *LacZ* gene is under control of the promoter for the Wnt-dependent target gene *Axin2*[21]; consequently, Wnt-responsive cells can be identified by visualization of X-gal staining[15]. In the *Axin2^CreERT2/+*; *R26R^mTmG/+* strain of mice, the *Cre* gene is under control of the *Axin2* promoter; when presented with tamoxifen, cells expressing *Cre* undergo a recombination event and then become permanently labeled with green fluorescent protein (GFP)[16, 19]. Thus, Wnt-responsive cells and all their progeny express GFP[15]. Finally, in the *DMP1-8kb-Cre^+/−*; *Catnb^lox(ex3)/lox(ex3)* (also referred to as daβcat^Ot) or *DMP1^GFP* mice, the *Cre* or *GFP* gene is under control of the *DMP1* promoter; *DMP1* is expressed in odontoblasts[22]. Therefore, in daβcat^Ot mice, beta-catenin is stabilized, leading to elevated Wnt signaling in *DMP1*-expressing cells.

Collectively, analyses of tissues from these mice showed that

1. odontoblasts are sensitive to an endogenous Wnt signal;

2. endogenous Wnt signaling in the pulp decreases with age along with the rate of dentin secretion; and

3. genetically amplifying Wnt signaling in the pulp leads to a massive and rapid accumulation of dentin that, because of its rate of accrual, resembles osteodentin.

MATERIALS AND METHODS

ANIMALS

Experimental procedures were approved by the Stanford Committee on Animal Research (#13146) and the Institutional Animal Care and Use Committee of Indiana University School of Medicine. Four strains of transgenic mice were used: *Axin2^LacZ/+*, *Axin2^CreERT2/+*; *R26R^mTmG/+*, *DMP1-8kb-Cre^+/−*; *Catnb^lox(ex3)/lox(ex3)* (daβcat^Ot), and *DMP1^GFP*. For detailed information on the individual strains as well as breeding, please see the Supplemental Materials and Methods (available online at www.jendodon.com).

VITAL DYE LABELING AND QUANTIFICATION OF MINERAL APPOSITION RATE

Vital dye labeling was previously described[23]. Mice were intraperitoneally injected with 20 mg/kg calcein (Sigma-Aldrich, St Louis, MO) and 30 mg/kg alizarin red (Sigma-Aldrich) with a 10-day interval. Two days after alizarin red injection, the mice were sacrificed and harvested. Samples were fixed in 4% paraformaldehyde overnight, dehydrated with 30% sucrose overnight, and then processed for hard tissue embedding and sectioning using Kawamoto's method[24]. Mineral apposition rates were calculated by measuring the distance between the calcein-labeled bone (green line) and the alizarin red–labeled bone (red line); this measurement was made at 8 distinct, randomly chosen sites around the maxillary first molars in 3 separate mice. The mineral apposition rate was then calculated as the distance in micrometers per interval of time between when the labels were injected.

SAMPLE PREPARATION, PROCESSING, HISTOLOGY, X-GAL STAINING, AND IMMUNOHISTOCHEMISTRY ASSAYS

Maxillae were harvested, fixed, sectioned at a thickness of 8 μm, and processed using established procedures[18]. Aniline blue and Movat pentachrome staining were performed as described previously[18, 25]. For aniline blue staining, sections were submerged in 1% aniline blue (B8563, Sigma-Aldrich) for 3 minutes until a dark blue color developed. Slides were then washed in 1% acetic acid (A38-212; Fisher Scientific, Waltham, MA) for 5 minutes and repeated twice until appropriately discolored. The Movat pentachrome method uses alcian blue (A3157, Sigma-Aldrich), sodium thiosulfate (14518, Alfa Aesar), crocein scarlet (210757, Sigma-Aldrich) acid fuchsin (F8129, Sigma-Aldrich), phosphotungstic acid (P4006, Sigma-Aldrich), acetic acid, and alcoholic saffron (3801, Sigma-Aldrich). Movat pentachrome staining results in nuclei that are black; collagen and reticulin that are yellow; glycosaminoglycans that are light blue; and muscle, cytoplasm, and keratin that are red[26]. Dentin and alveolar bone were stained green/yellow, whereas the periodontal ligament and dental pulp stain red.

To detect beta-galactosidase activity, X-gal staining was performed (Supplemental Materials and Methods is available online at www.jendodon.com). Immunostaining was performed using standard procedures[25]. For the information of primary and secondary

antibodies, please see the <u>Supplemental Materials and Methods</u> (available online at <u>www.jendodon.com</u>).

STATISTICAL ANALYSES

All data were presented as mean ± standard deviation of each group. One-way analysis of variance was used to quantify differences using Prism 7 (GraphPad Software, San Diego, CA). A P value ≤ .05 was considered significant.

RESULTS

DENTIN DEPOSITION RATE DECLINES WITH AGE

Similar to humans, we found that mice exhibited an accumulation of dentin throughout life, and, as a consequence, the volume of the pulp cavity was gradually reduced (<u>Fig. 1</u>*A*–*C*, quantified in <u>Fig. 1</u>*D*). This increase in dentin volume occurred even as the rate of dentin accumulation slowed in older mice (<u>Fig. 1</u>*D*); for example, the average rate of dentin deposition in 1-month-old mice was between 2.1~8.4 µm/d (<u>Fig. 1</u>*E*), whereas in 6-month-old mice, the rate of deposition was below the limit of resolution (<u>Fig. 1</u>*F*).
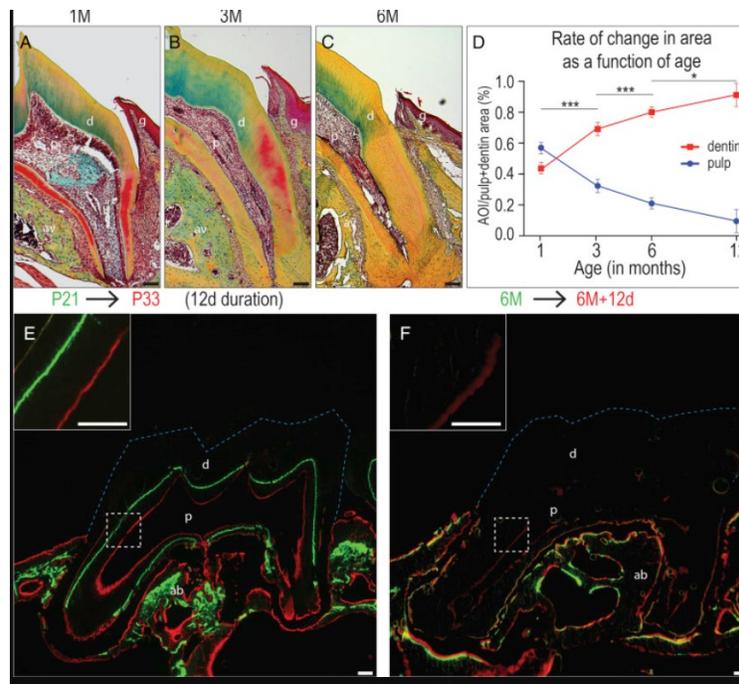
Figure 1. The dentin deposition rate declines with age. Representative Movat pentachrome–stained maxillary molar sections showing the dentin thickness and pulp

volume in the (*A*) 1-month-old (1M), (*B*) 3-month-old (3M), and (*C*) 6-month-old (6M) groups. In Movat pentachrome, dentin and alveolar bone stain green to yellow, and the periodontal ligament and dental pulp stain red. (*D*) Quantification of the pulp area and dentin area over the total pulp + dentin area from 1M, 3M, 6M, and 12M mice (*n* = 3 for each group). Vital dye labeling showing the new dentin formation in mice around (*E*) 1 month and (*F*) 6 months old in a 10-day interval. Calcein (*green*) was first injected, alizarin red (*red*) was injected 10 days later, and mice were harvested 2 days after the last injection. The *blue dotted lines* indicate the crown. d, dentin; p, pulp; ab, alveolar bone; g, gingiva. Scale bars = 100 μm. Data are expressed as the mean ± standard deviation. *P < .05; ***P < .0001.

THE DECLINE IN DENTIN DEPOSITION IS PARALLELED BY REDUCED WNT RESPONSIVENESS

Does Wnt signaling regulate the rate of dentin secretion? In *Axin2$^{LacZ/+}$* reporter mice, cells that are responsive to an endogenous Wnt signal can be identified by their X-gal^{+ve} status. In 1-month-old mice, abundant X-gal^{+ve} cells lined the dentin/pulp interface (Fig. 2*A*). In 3-month-old mice, a single layer of X-gal^{+ve} Wnt-responsive odontoblasts was evident (Fig. 2*B*). In 6-month-old mice, only a small portion of odontoblasts remained X-gal^{+ve} (Fig. 2*C*). Thus, within the pulp, there was a significant decline in the number of X-gal^{+ve} cells as a function of age (Fig. 2*D*).
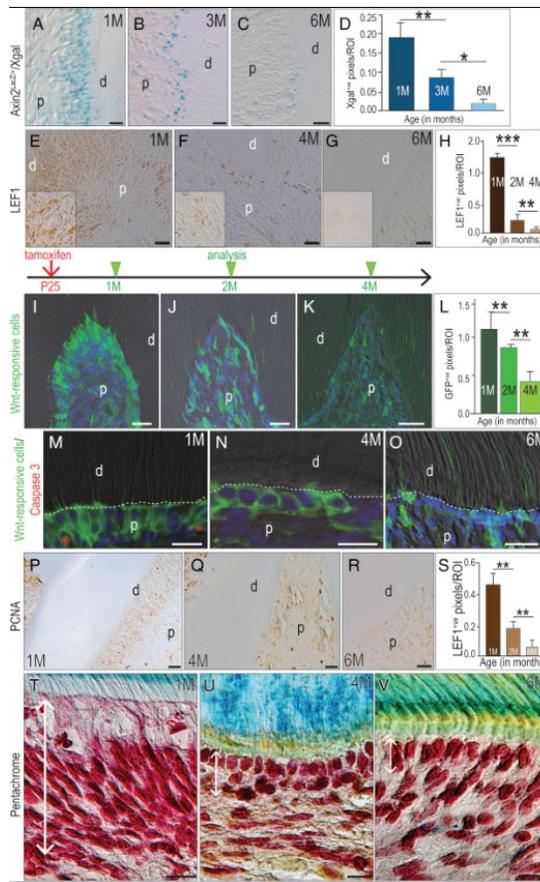
Figure 2. The decline in dentin deposition is paralleled by a decline in the population of Wnt-responsive odontoblasts. X-gal^{+ve}, Wnt-responsive cells in the maxillary first molars at the age of (*A*) 1 month (1M), (*B*) 3 months (3M), and (*C*) 6 months (6M). (*D*) Quantification of X-gal^{+ve} pixels in the pulp by age. Microscopic images showing Lef1 expression in odontoblasts in the (*E*) 1M, (*F*) 4M, and (*G*) 6M groups. (*H*) Quantification of Lef1^{+ve} pixels in the pulp by age. (*I*) GFP^{+ve}, Wnt-responsive cells in the maxillary first molar dental pulp of 1M-old mice 5 days after tamoxifen treatment. GFP^{+ve}, Wnt-responsive cells were examined with (*J*) 1-month tracing and (*K*) 3-month tracing. (*L*) Quantification of GFP^{+ve}, Wnt-responsive cells in the pulp area. Coimmunostaining of apoptosis marker caspase 3 (*red*) with Wnt-responsive cells (*green*) in odontoblasts of (*M*) 1M, (*N*) 4M, and (*O*) 6M mice. Immunostaining for the proliferation marker PCNA in odontoblasts in (*P*) 1M, (*Q*) 4M, and (*R*) 6M mice. (*S*) Quantification of PCNA^{+ve} pixels in the pulp by age. Pentachrome staining showing the odontoblasts in the maxillary first molar in (*T*) 1M, (*U*) 4M, and (*V*) 6M mice. *Double-headed arrows* indicate the odontoblast

layer. d, dentin; LEF1, lymphoid enhancer binding factor 1; p, pulp; PCNA, proliferating cell nuclear antigen. Scale bars = 25 µm. Data are expressed as the mean ± standard deviation. *P < .05; **P < .01; ***P < .0001

We used a second method to determine if endogenous Wnt signaling declined with age. In 1-month-old mice, cells at the dentin/pulp interface abundantly expressed the Wnt transcription factor lymphoid enhancer-binding factor-1 (Lef1) (Fig. 2E). Lef1 immunostaining was reduced in 4-month-old mice (Fig. 2F), and by 6 months of age, Lef1^{+ve} cells were no longer detectable in the pulp (Fig. 2G), showing a progressive decline in Wnt signaling in the pulp as a function of age (quantified in Fig. 2H).

The gradual loss in Wnt-responsive odontoblasts was confirmed using a lineage tracing strain *Axin2$^{CreERT2/+}$*; *R26R$^{mTmG/+}$* mice. The Wnt-responsive population and its descendants were identifiable by virtue of their GFP expression. In 1-month-old mice, most odontoblasts were GFP^{+ve} (Fig. 2I), indicating that they were responsive to an endogenous Wnt signal. In 2-month-old mice, the number of GFP^{+ve} cells had declined significantly (Fig. 2J), indicating that descendants of the initial Wnt-responsive population had been lost. In 4-month-old mice, GFP^{+ve} descendants of the initial Wnt-responsive population had diminished still further (Fig. 2K, quantified in Fig. 2L).

How were the GFP^{+ve} cells lost? Coimmunostaining of GFP and the apoptosis marker caspase 3 was performed at the 1, 4, and 6-month time points. Only the 1-month old mice showed evidence of GFP/caspase 3 coexpression (Fig. 2M); in 4- and 6-month old mice, caspase 3 expression was undetectable (Fig. 2N and O). Therefore, some reduction in the Wnt-responsive population appeared to be caused by apoptosis. Coupled with the gradual reduction in mitotic activity as shown by proliferating cell nuclear antigen immunostaining (Fig. 2P–S), it became readily apparent that the density of Wnt-responsive cells at the dentin-pulp interface was reduced as a function of age (Fig. 2T–V).

WNT SIGNALING REGULATES THE RATE OF DENTIN SECRETION

Thus far, data showed a correlation between Wnt signaling and the rate of dentin deposition. To establish a causal relationship between Wnt signaling and the rate of

dentin deposition, we genetically engineered odontoblasts to always express a stabilized form of the Wnt pathway intermediate, beta-catenin. To achieve this, we used a strain of mice in which *Cre* was expressed in odontoblasts. Odontoblasts and pulp cells expressed *DMP1* (Fig. 3*A*), suggesting that the *DMP1-Cre* strain of mice could serve as a means to activate Wnt signaling in odontoblasts.
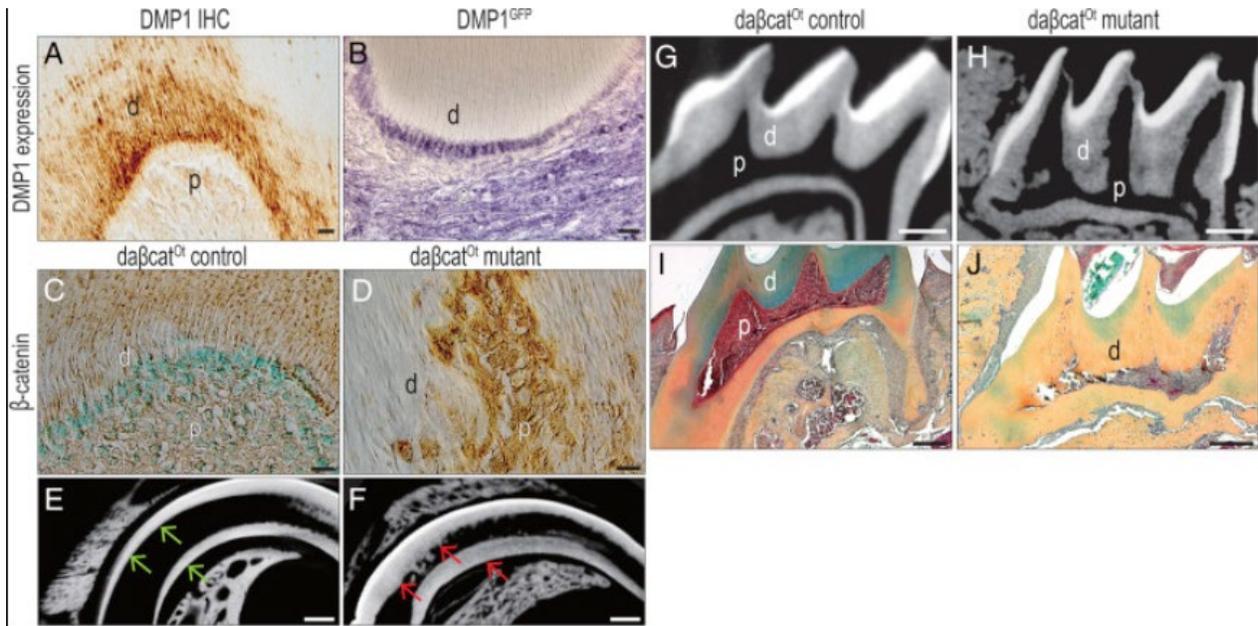
Figure 3. Wnt signaling regulates the rate of dentin secretion. (*A*) Immunohistochemistry identifies DMP1 expression (*brown*) in a maxillary first molar. (*B*) Immunohistochemistry confirmed the GFP expression (*purple*) in a maxillary first molar in *DMP1^GFP* mice. Immunohistochemistry for beta-catenin (*brown*) in odontoblasts in (*C*) control and (*D*) daβcat^Ot mutant mice (42 days old). The sections were counterstained with fast green. Representative micro–computed tomographic sagittal sectional reconstructed images of an incisor from (*E*) control and (*F*) daβcat^Ot mutant mice (42 days old). Representative micro–computed tomogrpahic sagittal sectional reconstructed images of the maxillary first molar from (*G*) control and (*H*) daβcat^Ot mutant mice (42 days old). Pentachrome staining of the maxillary first molar from (*I*) control and (*J*) daβcat^Ot mutant mice (42 days old). Dentin stains green to yellow, with the more mature dentin staining yellow. d, dentin; p, pulp. Scale bars = (*A–D*) 25 μm and (*E–J*) 200 μm.

We confirmed that *DMP1* was expressed in odontoblasts and pulp cells by following the expression of GFP in *DMP1*^{*GFP*} reporter mice. In these transgenic mice, GFP is under control of the *DMP1* promoter, and as expected, odontoblasts were positively labeled (Fig. 3*B*). We then proceeded with crossing *DMP1-Cre* mice with a strain of mice expressing the stabilized form of beta-catenin. These mice, referred to as daβcat^{Ot}, exhibited amplified Wnt/beta-catenin signaling in *DMP*-expressing cells (Fig. 3*C* and *D*[27]).

Compared with age-matched littermates (Fig. 3*E*), significantly more dentin accumulated in daβcat^{Ot} mutant teeth in both the incisors (Fig. 3*F*) and the molars (compare Fig. 3*G* with *H*). The dentin accumulation nearly eliminated the pulp cavity in the crown region (Fig. 3*G* and *H*). Histologic analyses confirmed micro–computed tomographic imaging (compare Fig. 3*I* with *J*). Cumulatively, these data showed that amplified Wnt signaling in odontoblasts led to a dramatic increase in dentin accumulation.

ACCELERATING DEPOSITION CONVERTS A TUBULAR DENTIN INTO OSTEODENTIN

The nature of the dentin was examined in daβcat^{Ot} mutant mice. Although in control animals the tubular structure of dentin was obvious and the pulp cavity was densely cellular (Fig. 4*A*), the dentin of daβcat^{Ot} mutant had no tubular arrangement, and cells were trapped within the mineralized matrix (Fig. 4*B*). In controls, aniline blue staining highlighted the ordered nature of the odontoblasts and the tubular dentin (Fig. 4*C*); in daβcat^{Ot} mutants, the odontoblasts were disorganized, and the dentin had an osteoidlike appearance (Fig. 4*D*).
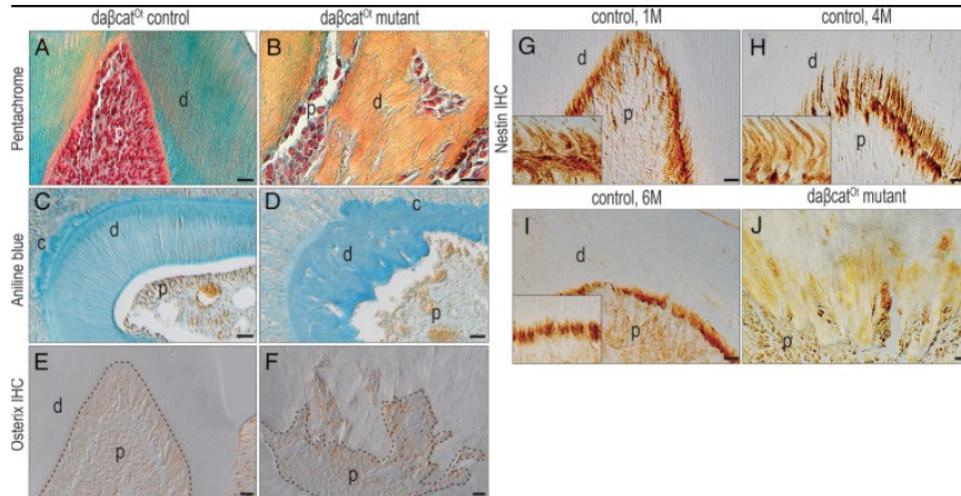
Figure 4. Accelerating deposition converts a tubular dentin into osteodentin. Representative pentachrome staining of a maxillary first molar in (*A*) control and (*B*) daβcat^{Ot} mutant mice. A representative cross section of aniline blue staining of the maxillary first molar in (*C*) control and (*D*) daβcat^{Ot} mutant mice. Immunostaining of osterix of the maxillary first molar in (*E*) control and (*F*) daβcat^{Ot} mutant mice. Immunostaining of nestin in (*G*) 1-month (1M), (*H*) 4-month (4M), and (*I*) 6-month (6M) control mice. (*J*) Immunostaining of nestin in daβcat^{Ot} mutant mice. d, dentin; p, pulp. Scale bar = 25 μm.

Osterix expression was undetectable in the adult pulp of control mice (Fig. 4*E*). In contrast, osterix was strongly expressed in the adult pulp of daβcat^{Ot} mutant mice (Fig. 4*F*), indicating the preodontoblasts, instead of odontoblasts, were presented in daβcat^{Ot} mutant. Nestin is expressed by mature, fully differentiated odontoblasts as shown by analyses of 1-, 4-, and 6-month-old pulps (Fig. 4*G*–*I*[28, 29]). In daβcat^{Ot} mutants, nestin expression was abolished and in its place were only preodontoblasts secreting atubular osteodentin (Fig. 4*J*). These data supported our hypothesis that aberrantly elevated Wnt signaling triggered osteodentin production by preodontoblasts.

DISCUSSION

WNT SIGNALS MAINTAIN DENTIN SECRETION

Here we provide evidence that the survival of odontoblasts and their continued production of dentin is ensured in part by endogenous Wnt signaling. This interpretation is strongly supported by the literature[30]. For example, using a loss-of-function approach, Han et al[31] showed that beta-catenin was required for odontoblast differentiation, and in a murine preclinical study, Yoshioka et al[32] showed that Wnt signaling and beta-catenin are both up-regulated in response to subacute pulp trauma. These data and others[19, 33] firmly establish the Wnt/beta-catenin pathway as a critical regulator of odontoblast function. We extend these observations by showing that the age-related decline in dentin production is mirrored by a decline in Wnt signaling (Fig. 1) and a diminishment in the population of Wnt-responsive cells in the pulp (Fig. 2).

Not all published data comport with our model. For example, Amri et al[34] showed that rather than activation, inhibition of Wnt/beta-catenin signaling by *Msx2* controls the formation of osteodentin. It is not clear at this time how these data can be reconciled with findings shown here. Others have shown using *in vitro* analyses that *Msx2* enhances Wnt signaling and promotes mineralization[35]. Some of these differences may be caused by timing or the duration of the Wnt signal. Indeed, constitutive activation or constitutive repression of the Wnt/beta-catenin pathway can lead to similar outcomes in mineralized tissues.

THE REPAIR RESPONSE OF THE PULP IS ORCHESTRATED BY WNT SIGNALS

In patients and animal models, the pulp shows an extraordinarily vigorous repair response to trauma. Variously referred to as reactionary, reparative, and tertiary dentin, the mineralized matrix produced in response to injury tends to be atubular and rather than being secreted by fully differentiated odontoblasts, it is produced by preodontoblastic progenitor cells in the pulp[6, 36]. This "backup" system appears to be ideally suited for a relatively quiescent tissue that must survive for an entire lifetime. How this quiescent status is maintained and how it is reversed upon injury are still open for debate. In tissues that do not turn over, adult stem cells exist in a mitotically arrested state with a low

metabolic demand[37]. However, in response to injury, this stem cell pool is capable of proliferating and then differentiating to aid in tissue repair[37]. Applying this theory to the pulp seems reasonable; it is a tissue with a very low turnover rate but fully capable of mounting a robust repair response to trauma. In many tissues, Wnt signals maintain adult stem cells in a quiescent state[38], and we propose the pathway plays a similar role in the adult pulp.

CLINICAL IMPLICATIONS

If primary odontoblasts respond to trauma and secrete copious amounts of new dentin, then the pulp generally retains its vitality. When that repair response is attenuated with age, the result is typically chronic inflammation followed by pulp necrosis, which necessitates a root canal. The vast majority of endodontic cases begin with subacute, chronic trauma to the pulp (eg, chronic pulpitis that is not adequately repaired by the pulp's natural defense strategy). Our goal is to devise new biologically based strategies to enhance this natural repair response that has become attenuated as a result of aging.

We envision a therapeutic strategy to stimulate the repair potential of an aged pulp by enhancing Wnt signaling via delivery of a liposomal formulation of the WNT protein. We showed the feasibility of such an approach[15] with one important caveat. In our studies, the dentin injury was produced in an otherwise healthy tooth. This characteristic does not match with most endodontic pulp cases in which the pulp is inflamed and potentially infected at the time of treatment. Consequently, it will be critically important to test a WNT-based strategy to enhance dentin secretion in a model that replicates this clinical scenario.

ACKNOWLEDGMENTS

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SUPPLEMENTAL METHODS AND MATERIALS

ANIMALS

Axin2^{LacZ/+} (#11809809) and *Axin2^{CreERT2/+}*; *R26R^{mTmG/+}* (#018867 and #007576) mice were obtained from Jackson Labs (Bar Harbor, ME). To induce *Cre* expression in *Axin2^{CreERT2/+}*; *R26R^{mTmG/+}* mice, tamoxifen (4 mg/25 g body weight) was delivered intraperitoneally for 3 consecutive days; animals were then sacrificed at the indicated time points. In this strain, delivery of tamoxifen instigates a recombination event wherein cells that are responsive to endogenous Wnt signaling start to express green fluorescent protein (GFP). Subsequently, any descendants arising from this initial population of Wnt-responsive cells are also labeled with GFP. This strategy allowed us to unambiguously determine whether Wnt-responsive cells in the dentin-pulp complex declined with age.

Daβcat^{Ot} mice were generated by crossing *dentin matrix acidic phosphoprotein 1 (DMP1)-8kb-Cre* mice with *Catnb^{lox(ex3)}* mice in which LoxP sites flank exon 3 that encodes for beta-catenin degradation[1], [2]. *DMP1-8kb-Cre^{+/−}* mice were crossed with *Catnb^{lox(ex3)/lox(ex3)}* mice to generate *Catnb^{lox(ex3)/+}*; *DMP1-8kb-Cre^{+/−}* (daβcat^{Ot} mutant mice) and *Catnb^{lox(ex3)/+}* mice (daβcat^{Ot} control mice). The daβcat^{Ot} control mice are indistinguishable from the wild-type C57BL/6 mice and used as littermate controls in the

current study. Mice expressing GFP in osteocytes (*DMP1*[GFP])[3], [4] were also used in this study.

X-gal Staining

To detect beta-galactosidase activity, sections were fixed with 0.2% glutaraldehyde/phosphate-buffered saline (PBS) for 15 minutes and then washed 3 times with wash buffer containing 0.005% Nonidet P-40 (Sigma-Aldrich, St. Louis, MO), 0.01% sodium deoxycholate, and 2 mmol/L $MgCl_2$/PBS. Tissue sections were stained overnight at 37°C in a staining solution containing 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, 2 mmol/L $MgCl_2$, and 1 mg/mL X-gal (Thermo Fisher Scientific, Waltham, MA). Sections were rinsed 2 times in PBS, dehydrated in a graded ethanol series, and cleared in CitriSolv (Fisher Scientific, Pittsburgh, PA) and then mounted with Permount (Fisher Scientific)[5], [6].

Micro–computed Tomographic Analyses

Micro-computed tomographic imaging was performed using a SkyScan 1176 scanner (SkyScan, Bruker, Belgium) at a 5-μm resolution. Scanning was done at 45 kV, 556 mA. Samples were reconstructed and segmented with ScanIP (Synopsys, Mountain View, CA). CT Analyzer software (version 1.02, SkyScan) was used for morphometric quantification.

Immunohistochemistry

Primary antibodies and their dilutions are as follows: anti-osterix (ab22552; Abcam, Cambridge, UK), anti-CTNNB1 (â-catenin) antibody (BD Biosciences, East Rutherford, NJ), antinestin (ab6142, Abcam), anti-Lef1 (2230S; Cell Signaling Technology, Danvers, MA), anti-GFP (2956, Cell Signaling Technology), and anti-DMP1 (ab103203, Abcam). In all cases, the negative controls were performed at the same time using PBS to substitute primary antibody. Secondary antibodies are biotinylated goat antirabbit immunoglobulin G antibody (BA-1000; Vector Lab, Burlingame, CA) and biotinylated horse antimouse immunoglobulin G antibody (BA-2000, Vector Lab). The staining was visualized using the ABC peroxidase standard staining kit (32020, Thermo Fisher Scientific) and the DAB peroxidase substrate kit (SK4100, Vector Lab). For GFP staining, nickel solution was

added to the DAB substrate to enhance the signaling; therefore, the GFP^{+ve} cells were dark purple. For â-catenin staining, fast green was used for counterstaining. Tissue sections were photographed using a digital imaging system (Leica, Wetzlar, Germany).

Histomorphometry

Histomorphometric measurements were performed to measure the areas of dentin and pulp. From 4 separate time points, 3 mice were analyzed, and from each mouse, 6 tissue sections were chosen for analysis. The dentin, the pulp, and the dentin + pulp areas were calculated using ImageJ (National Institutes of Health, Bethesda, MD) on each tissue section. The area of interest was specified as either the dentin or the pulp, and this value was then divided by the total area occupied by dentin + pulp.

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