ORIGINAL ARTICLE

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Wnt Acts as a Prosurvival Signal to Enhance Dentin Regeneration

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ABSTRACT

Wnt proteins are lipid-modified, short-range signals that control stem cell self-renewal and tissue regeneration. We identified a population of Wnt responsive cells in the pulp cavity, characterized their function, and then created a pulp injury. The repair response was evaluated over time using molecular, cellular, and quantitative assays. We tested how healing was impacted by wound environments in which Wnt signaling was amplified. We found that a Wnt-amplified environment was associated with superior pulp healing. Although cell death was still rampant, the number of cells undergoing apoptosis was significantly reduced. This resulted in significantly better survival of injured pulp cells, and resulted in the formation of more tertiary dentin. We engineered a liposome-reconstituted form of WNT3A then tested whether this biomimetic compound could activate cells in the injured tooth pulp and stimulate dentin regeneration. Pulp cells responded to the elevated Wnt stimulus by differentiating into secretory odontoblasts. Thus, transiently amplifying the body's natural Wnt response resulted in improved pulp vitality. These data have direct clinical implications for treating dental caries, the most prevalent disease affecting mankind. © 2015 American Society for Bone and Mineral Research.

KEY WORDS: REPARATIVE DENTIN; RODENT MODEL; TOOTH INJURY; WNT/BETA CATENIN/LRP; DENTAL BIOLOGY

Introduction

Most toothaches are the result of chronic bacterial infections that cause inflammation of the connective, vascular, lymphatic, and nervous tissues occupying a chamber in the center of the tooth. When these tissues, collectively referred to as the pulp, become chronically inflamed they must be removed in a procedure known as a root canal treatment. Even when bacterial infections do not penetrate into the pulp chamber, a root canal may be required because bacterial byproducts can diffuse through the remaining tooth structure and cause chronic pulp inflammation.[1] In an effort to treat these conditions, a century-old procedure called pulp capping is often used.[2] Pulp capping consists of placing a material such as calcium hydroxide on the remaining tooth structure, which creates a high pH, antimicrobial environment.[3] When the inflammatory insult is acute, and the extent of trauma is mild, then the pulp itself can sometimes mount a repair response.

Odontoblasts are similar to osteoblasts that line the surfaces of bones and also secrete an extracellular matrix that undergoes mineralization.[4,5] There is, however, one unique feature of considerable importance: odontoblasts are trapped within the pulp chamber and thus any repair attempts must be made from the inside out. Consequently, the precept, "cavities cannot be healed" still stands.[6]

A chief goal of regenerative dental medicine is to stimulate from stem/progenitor cells residing in the pulp cavity the generation of dentin with the same structural and biological

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properties of native dentin.[7] In doing so, the vitality and function of the existing teeth can be preserved.[8] Many investigators have focused on the role of Wnt signaling in the development, morphogenesis, and maintenance of dental tissues (reviewed in Thesleff and Tummers[9]). In recent years, however, the Wnt pathway has been implicated as a prosurvival/antiapoptotic signal, especially for stem cells in a healing environment.[10–12] Here, we asked whether enhancing Wnt signaling in the context of an acute pulp injury would induce a superior repair response.

Materials and Methods

Animals

The Stanford Committee on Animal Research and the Animal Care Committee of the University Paris Descartes (agreement CEEA34.CC.016.11, Comité d'éthique pour l'expérimentation animale n°34, Paris, France) approved all experimental procedures. Rats were purchased from Janvier Labs (Saint Berthevin Cedex, France). *Axin2*$^{LacZ/LacZ}$ (#11809809) and *Axin2*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ (#018867 and #007576, respectively) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). For *Axin2*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice, tamoxifen was delivered i.p. (4.0 mg/25 mg body weight) for 5 consecutive days.

Animal surgeries

Adult male mice (3 to 5 months old) were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (16 mg/kg). In total, 72 mice (36 *Axin2*$^{LacZ/+}$ and 36 *Axin2*$^{LacZ/LacZ}$ mice) were used. A cavity was created with a Ø 0.3-mm-diameter round bur (E0123; Dentsply Maillefer, Ballaigues, Switzerland); a #6 k-file was used to expose the dental pulp. Glass ionomer cement (3M) was used to cap the injury. Mice were euthanized at the time points indicated.

In rats, a cavity was created with a Ø 0.2-mm-diameter round bur; a root-canal-shaping rotary nickel-titanium file system (Protaper, Dentsply) was used to expose the dental pulp. After pulp exposure, beads treated either with a liposomal formulation of WNT3A (L-WNT3A) ($n = 18$) or a liposomal formulation of PBS (L-PBS) ($n = 18$) were implanted into the pulp chamber using a blunt steel probe (see Preparation and Delivery of L-WNT3A for details on bead preparation). Biodentine cement (Septodont, Saint-Maur des Fossés, France) was used to cap the injury. Rats were euthanized at the time points indicated.

Preparation and delivery of L-WNT3A

Purified recombinant human WNT3A protein was incubated with liposome vesicles as described.[13] L-WNT3A (10 ng, see Zhao and colleagues[14]) or L-PBS ($n = 18$ for each condition) was delivered on Affi-Gel agarose beads (Bio-Rad Laboratories, Hercules, CA, USA) that had been soaked overnight at 37 °C in the relevant solutions.[15]

Sample preparation, processing, histology, histomorphometrics, and cellular assays

Maxillae were harvested, the skin and outer layers of muscle were removed, and the tissues were fixed. Tissues were sectioned at a thickness of 8 μm and processed using established procedures.[16] Histologic staining was performed as described.[16] A minimum of six sections were used to quantify the amount of new dentin. Histomorphometric measurements were performed as described.[17]

X-gal staining was performed as described.[18] TUNEL staining was performed as described by the manufacturer (In Situ Cell Death Detection Kit; Roche Diagnostics, Mannheim, Germany). Immunostaining was performed using standard procedures.[10] For cell proliferation analysis, BrdU labeling reagent (Invitrogen, Carlsbad, CA, USA) was either injected i.p., or added to culture media according to the manufacturer's instructions; animals were euthanized 12 hours later and both bone marrow–derived stem cells and dental pulp stem cells were fixed 12 hours later.

Primary antibodies and their dilutions were as follows: anti-biotinylated BrdU (1:200), anti-Nestin (1:300), anti-Ki67 (1:200), anti–proliferating cell nuclear antigen (PCNA) (1:1000), and anti-dentin sialoprotein (DSP) (1:1000).

Dental pulp stem cells and bone marrow treatments

Human dental pulp stem cells were isolated as described,[19] in accordance with the Ethics committee of the Pirkanmaa Hospital District, Tampere, Finland (R06009). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing Nutrient Mixture F-12 with 10% fetal bovine serum. Cells were treated with L-PBS or L-WNT3A (effective concentration = 0.06 μg/mL) at 37 °C for 6, 12, and 24 hours. RNA was isolated afterward and analyzed by qRT-PCR (see Quantitative RT-PCR) and BrdU incorporation (see Preparation and Delivery of L-WNT3A).

Bone marrow was harvested from the femurs and tibias of adult mice, aliquotted to produce similar-sized samples. DNA content was measured to ensure that variation between samples was <10%.[20] Each aliquot was incubated with 20 μL of DMEM with 10% fetal bovine serum containing L-PBS or L-WNT3A (effective concentration = 0.15 μg/mL) at 37 °C for 4 hours. RNA was isolated afterward and analyzed by qRT-PCR, or the tissues were fixed in 4% paraformaldehyde (PFA) at 4 °C then processed into 22-oxacalcitrol (OCT) for cryosectioning. TUNEL activity and Ki67 expression were analyzed using 10-μm sections (see Preparation and Delivery of L-WNT3a).

Quantitative RT-PCR

Total RNA was extracted using TRIzol (Invitrogen). cDNA was synthesized by using SuperScript III First-Strand Synthesis Kit (Invitrogen) according to the manufacturer's instructions. RT-PCR and quantitative PCR (ABI Prism 7900 HT Sequence Detection System; Applied Biosystems, Inc., Foster City, CA, USA) were performed as described.[10] All reactions were performed in triplicate.

The following primer sets were used: *Axin2*, 5′-ACCCTGGGCCACTTTAAAG-3′ (sense) and 5′-CCTTCATACATCGGGAGCAC-3′ (antisense); *Axin2* exon 1, 5′-TCAGTAACAGCCCAAGAACC-3′ (sense) and 5′-GAGCCTCCTCTCTTTACAGC-3′ (antisense); *CASP3*, 5′-GCACTGGAATGTCATCTCGCT-3′ (sense) and 5′-GGCCCATGAATGTCTCTCTGAG-3′ (antisense); *Lef1*, 5′-ACACCCTGATGAAGGAAAGC-3′ (sense) and 5′-GACCCATTTGACATGTACGG-3′ (antisense); *PCNA*, 5′-CTTGGAATCCCAGAACAGGA-3′ (sense) and 5′-CAGCATCTCCAATGTGGCTA-3′ (antisense); *Nestin*: 5′-CTCGGGAGAGTCGCTTAGAG-3′ (sense) and 5′-CACAGCCAGCTGGAACTTT-3′ (antisense); *Dentin sialophosphoprotein* (*DSPP*): 5′-GGAATGGAGAGAGGACTGCT-3′ (sense) and 5′-AGGTGTTGTCTCCGTCAGTG-3′ (antisense); *Osteocalcin*: 5′-TGTGACGAGCTATCAAACCAG-3′ (sense) and 5′-GAGGATCAAGTTCTGGAGAGC-3′ (antisense); and *Collagen type I*: 5′-AAGGACAAGAGGCACGTCTG-3′ (sense) and 5′-CGCTGTTCTTGCAGTGGTAG-3′ (antisense).

Dentin volume and mineral density μCT analysis

Microcomputed topographies of the maxillae were performed using a SkyScan 1176 scanner (SkyScan, Bruker, Belgium) at a 5-μm resolution. Scanning was done at 45 kV, 556 mA. Reconstruction of sections was achieved using a modified Feldkamp cone-beam algorithm with beam hardening correction set to 50%. CTAnalyzer software (version 1.02; SkyScan) was employed for morphometric quantification.

Reparative dentin histomorphometry

Sections from rat molars were examined morphometrically at a constant magnification (×250) with a semiautomatic image analyzer coupling the microscope to a video camera and a computer.[21] Six sections per sample ($n = 6$ molars per group) were taken at the center of the pulp exposure site. At day 14, we determined the porosity of the dentin bridge on Masson's trichrome-stained sections by measuring the percentage of space containing cells within the reparative dentin.

Statistical analyses

Results are presented as mean ± SEM values of independent replicates. Student's t-test was used to quantify differences described in this article. A p value ≤ 0.05 was considered significant.

Results

Odontoblasts are Wnt responsive

Odontoblasts are distinguished from pulp cells by expression of the intermediate filament protein Nestin[22] (see Fig. 1*C*) and DSP[23] (see Fig. 1*A–D*). Nestin^{+ve}, DSP^{+ve} odontoblasts are also Wnt responsive: X-gal staining of tissues from *Axin2*$^{LacZ/+}$ mice, in which the promoter of the Wnt target gene *Axin2* drives *LacZ* expression,[24,25] showed that odontoblasts lining the inner surface of the pulp cavity were Wnt responsive (Fig. 1*E*). A second, inducible *Axin2* reporter strain (*Axin2*$^{CreERT2/+}$; *R26R*$^{mTmG/+}$) verified that odontoblasts respond to an endogenous Wnt signal; following tamoxifen delivery, the GFP signal in tissues from *Axin2*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice was localized to the odontoblast body and processes (Fig. 1*F*). Analyses of embryonic and early postnatal dental tissues (Supporting Fig. 1) showed that these young odontoblasts were also Wnt responsive. Collectively, these data demonstrate that odontoblasts maintain a Wnt-responsive status throughout their lifetime.

Deletion of *Axin2* does not affect the dentin/pulp complex

In *Axin2*$^{LacZ/LacZ}$ mice the negative Wnt regulator *Axin2* is deleted[24,25]; consequently, endogenous Wnt responsiveness is elevated.[10,26] We evaluated teeth from *Axin2*$^{LacZ/+}$ and *Axin2*$^{LacZ/LacZ}$ mice to determine whether amplified Wnt signaling affected the gross morphology of the dentition. We found no significant differences in the size of the pulp cavities, or the thickness and density of the alveolar bone; furthermore, the size of the pulp chambers was unaffected (Fig. 2*A–F*). The dentin volume (Fig. 2*G*), and the mineral densities of enamel and dentin were also equivalent in *Axin2*$^{LacZ/+}$ and *Axin2*$^{LacZ/LacZ}$ mice (Fig. 2*H*). Histologic examination showed that the *Axin2*$^{LacZ/LacZ}$ pulp was indistinguishable from that of heterozygous and wild-type littermates (Fig. 2*I*, *J*; $n \geq 20$ for each genotype). The distribution of X-gal^{+ve} cells in *Axin2*$^{LacZ/LacZ}$ and *Axin2*$^{LacZ/+}$ mice was unchanged; the only difference of note was the intensity of X-gal staining in *Axin2*$^{LacZ/LacZ}$ mice, which is expected because homozygous mice carry two copies of the *LacZ* gene (Fig. 2*K*, *L*).

Axin2 is a ligand-dependent inhibitor of Wnt signaling; consequently, it is anticipated that in the absence of a Wnt stimulus, *Axin2*$^{LacZ/LacZ}$ mice should show baseline Wnt signaling, equivalent to that seen in *Axin2*$^{LacZ/+}$ and wild-type mice.[10,24] qRT-PCR verified that baseline Wnt signaling as measured by *Lef1* and *Axin2* (exon 1) expression was equivalent in *Axin2*$^{LacZ/+}$ and *Axin2*$^{LacZ/LacZ}$ mice (Fig. 2*M*). Markers of cell proliferation (Fig. 2*M*), and the odontogenic proteins Nestin (Fig. 2*N*, *O*), DSPP, Osteocalcin, and Collagen type I (Fig. 2*P*) all showed no significant differences in expression levels between *Axin2*$^{LacZ/+}$ and *Axin2*$^{LacZ/LacZ}$ mice.

Axin2$^{LacZ/LacZ}$ mice exhibit a superior reparative response following acute pulp exposure

In bone and central nervous system injury models, *Axin2*$^{LacZ/LacZ}$ mice showed an enhanced repair response[10,27] that is attributed to elevated Wnt signaling at the site of trauma.[20] Therefore, the response of *Axin2*$^{LacZ/LacZ}$ mice and their *Axin2*$^{LacZ/+}$ control littermates to an acute pulp exposure was tested. By postoperative day 14, the pulp cavities in *Axin2*$^{LacZ/+}$ mice were largely necrotic ($n = 6$; Fig. 3*A*). A distinctly different response was observed in *Axin2*$^{LacZ/LacZ}$ mice, in which instead of necrotic pulp tissue, the cavity was occupied by reparative dentin ($n = 6$; Fig. 3*B*; quantified in Fig. 3*C*). The organization of the matrix was examined using Picrosirius red staining and visualization under polarized light. In *Axin2*$^{LacZ/+}$ controls, no organized collagenous network was evident in the injury site (Fig. 3*D*); in contrast, in *Axin2*$^{LacZ/LacZ}$ mice a dense and packed collagen fiber network formed a dentin bridge (Fig. 3*E*). In *Axin2*$^{LacZ/LacZ}$ mice but not in controls, secretory odontoblasts[28,29] were immunopositive for DSP (Fig. 3*F*, *G*) and Nestin (Fig. 3*H*, *I*).

We examined these immunopositive cells in the injured pulp from *Axin2*$^{LacZ/LacZ}$ mice more closely. Higher magnification images demonstrated that some of the DSP signal was seen in the extracellular matrix of the dentinal tubules[30] (Fig. 3*J*), whereas other DSP immunostaining detected odontoblasts (Fig. 3*K*). Within the newly formed dentin, DSP appeared to be accumulated in the pulp extracellular matrix, on the pulp tissue side of the mineralization front (Fig. 3*L*; see also Suzuki and colleagues[31]). Some Nestin^{+ve} cells were also odontoblasts (Fig. 3*M*) but others, such as those in the new dentin, appeared to be differentiating odontoblasts (Fig. 3*N*) based on the work of others.[32]

On postoperative day 4, granulation tissue filled the pulp chambers in *Axin2*$^{LacZ/+}$ controls ($n = 6$; Fig. 3*O*). *Axin2*$^{LacZ/LacZ}$ mice showed minimal granulation tissue ($n = 6$; Fig. 3*P*). qRT-PCR revealed that the endogenous Wnt response, as measured by *Axin2 exon1* expression, was significantly elevated in *Axin2*$^{LacZ/LacZ}$ mice compared to controls (Fig. 3*Q*).

Exposure of the pulp causes extensive cellular necrosis[33]; there is also a period of latent apoptosis when pulp cells damaged by the injury can either die or recover.[34] In *Axin2*$^{LacZ/+}$ controls, abundant TUNEL staining identified these dying cells (Fig. 3*R*). In *Axin2*$^{LacZ/LacZ}$ mice, very few TUNEL^{+ve} cells were evident, even on postoperative day 4 (Fig. 3*S*). Apoptosis is largely controlled by caspase activity[35] and, as anticipated by

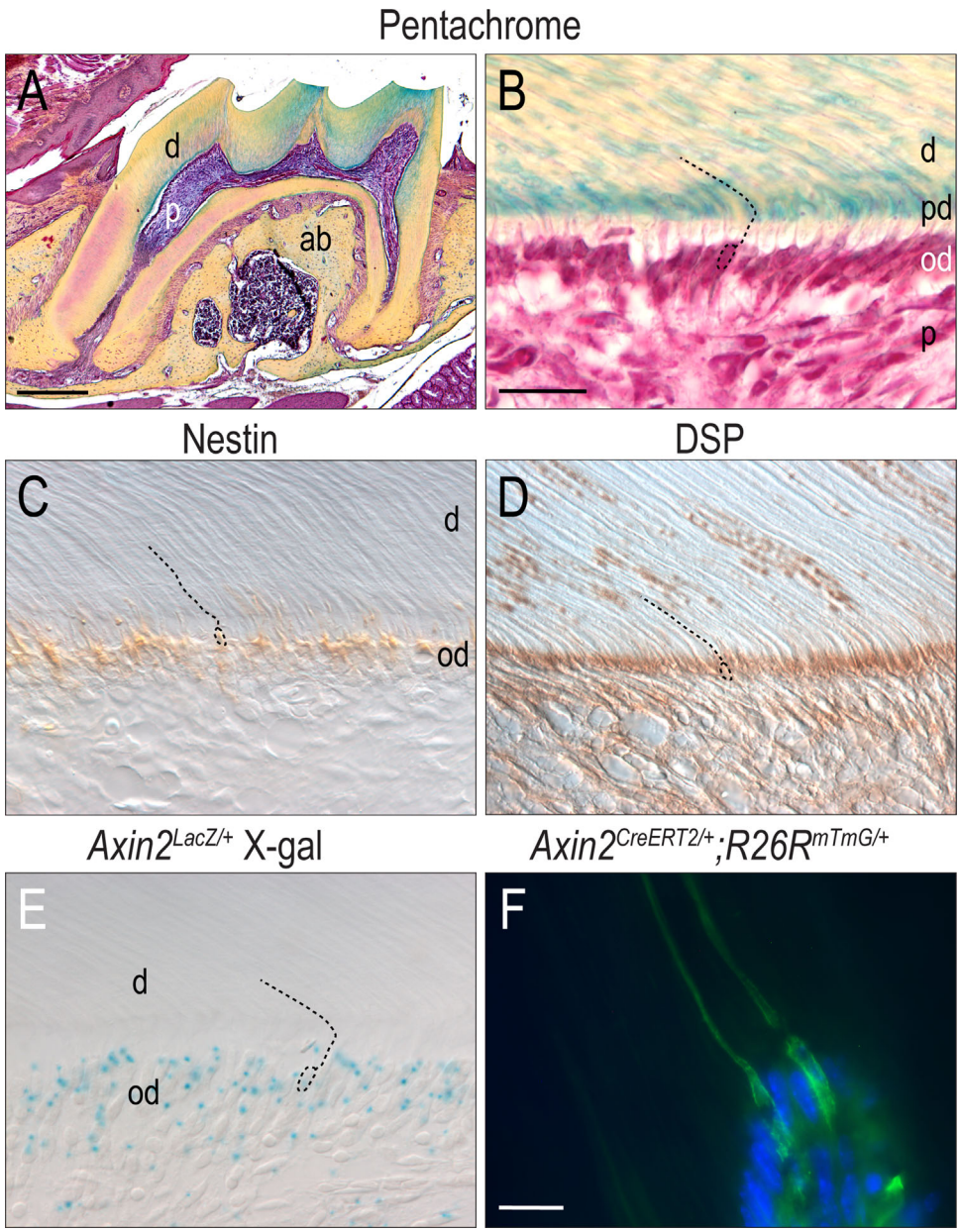

Fig. 1. Odontoblasts are Wnt responsive. (*A*) In skeletally mature mice, pentachrome staining identifies dentin (yellow to yellow-green), pulp (purple), and alveolar bone (yellow). (*B*) Higher magnification of the pulpal-dentin complex illustrates the organization of pulp cells and odontoblasts (pink; outlined) juxtaposed to the pre-dentin and dentin (blue and blue-yellow). (*C*) In the pulp cavity only polarized, secretory odontoblasts (outlined) are positive for Nestin immunostaining. (*D*) These polarized, secretory odontoblasts (outlined) express DSP. (*E*) X-gal staining and (*F*) GFP fluorescence, respectively in adult *Axin2*$^{LacZ/+}$ and *Axin2*$^{CreERT2/+}$;R26R$^{mTmG/+}$ mice, demonstrates that polarized, secretory odontoblasts and pulp cells are Wnt responsive. In *Axin2*$^{CreERT2/+}$;R26R$^{mTmG/+}$ animals, tamoxifen was delivered for 5 days after which the animals were euthanized. Scale bars: 400 μm (*A*), 25 μm (*B–E*), 10 μm (*F*). ab = alveolar bone; d = dentin; od = odontoblast; p = pulp; pd = pre-dentin.

the TUNEL staining, *Casp8* expression in *Axin2*$^{LacZ/LacZ}$ mice was significantly lower than its expression in *Axin2*$^{LacZ/+}$ controls (Fig. 3*T*). Cell proliferation, as indicated by Ki67 immunostaining, was greater in *Axin2*$^{LacZ/LacZ}$ mice compared to *Axin2*$^{LacZ/+}$ controls (Fig. 3*U*, *V*). Thus, in response to an acute pulp injury that caused a significant elevation in endogenous Wnt signaling, *Axin2*$^{LacZ/LacZ}$ mice fared better than their heterozygous littermates. The elevated Wnt environment was correlated with reduced cell death, enhanced cell proliferation, and an overall improvement in the repair response of the pulp.

Wnt signaling regulates apoptosis and proliferation in dental pulp stem cells

We tested whether a Wnt stimulus alone was sufficient to reduce cell death and enhance cell proliferation in pulp cells. Dental

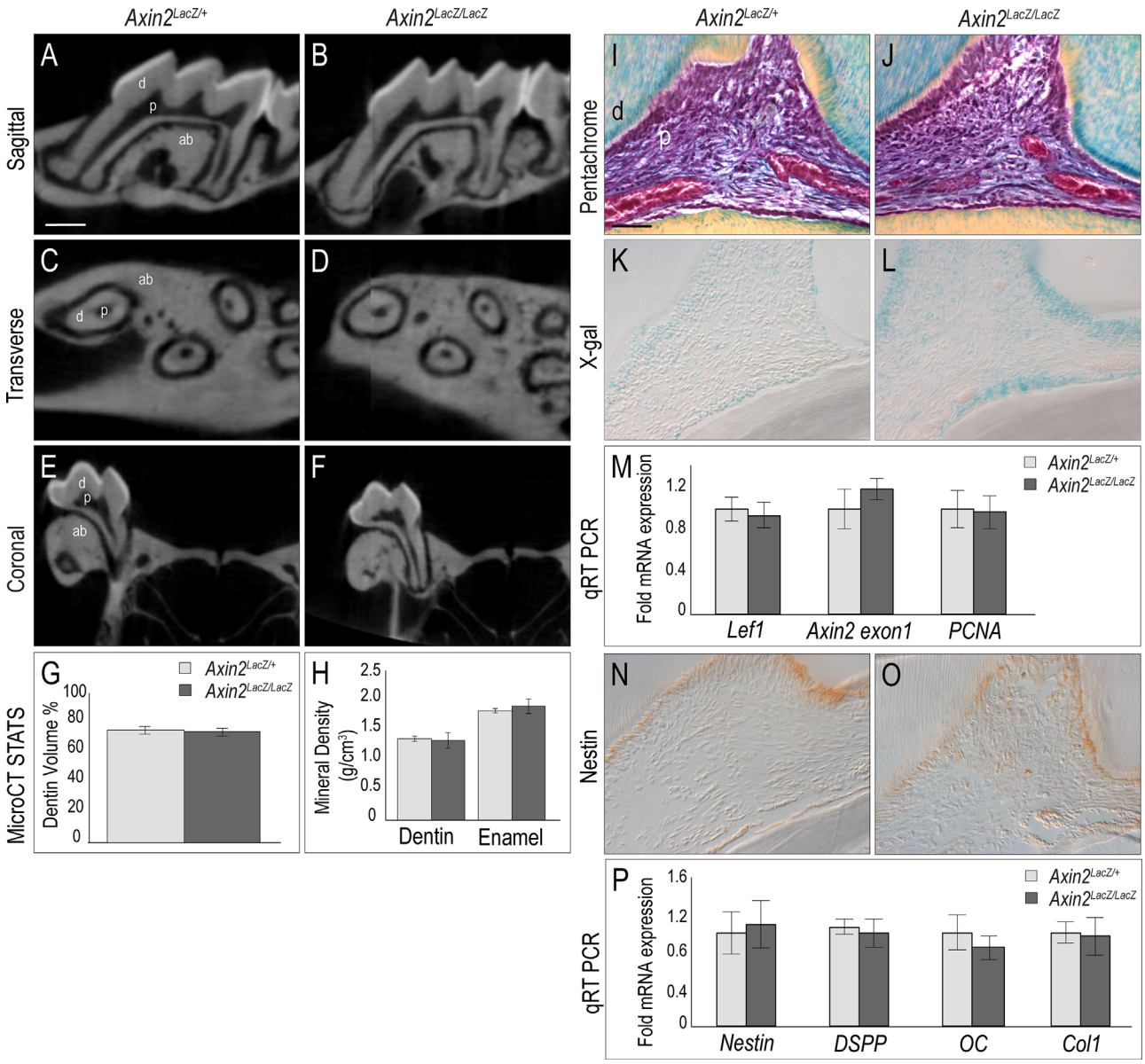

Fig. 2. Axin2 deletion does not disrupt odontogenesis or pulpal-dentin homeostasis. (*A–F*) μCT reconstructions of the molar region in skeletally mature, male (*A*, *C*, *E*) *Axin2*$^{LacZ/+}$ and (*B*, *D*, *F*) *Axin2*$^{LacZ/LacZ}$ mice. Quantified μCT demonstrate no differences in (*G*) dentin volume, or (*H*) dentin and enamel mineral density in molars from age-matched, sex-matched, adult *Axin2*$^{LacZ/+}$ and *Axin2*$^{LacZ/LacZ}$ mice. Pentachrome staining indicates the cellularity and organization of the pulp cavities from (*I*) *Axin2*$^{LacZ/+}$ and (*J*) *Axin2*$^{LacZ/LacZ}$ mice. (*K*) X-gal staining in odontoblasts and sub-odontoblasts in *Axin2*$^{LacZ/+}$ and (*L*) *Axin2*$^{LacZ/LacZ}$ mice; the stronger staining in *L* is due to *Axin2*$^{LacZ/LacZ}$ mice carrying two copies of the *LacZ* gene. (*M*) Quantitative RT-PCR analyses of pulp tissues from *Axin2*$^{LacZ/+}$ and *Axin2*$^{LacZ/LacZ}$ mice, evaluated for the relative expression levels of *Lef1*, *Axin2 exon1*, and *PCNA*. (*N*) Nestin immunostaining in *Axin2*$^{LacZ/+}$ and (*O*) *Axin2*$^{LacZ/LacZ}$ mice. (*P*) Quantitative RT-PCR analyses of pulp tissues from *Axin2*$^{LacZ/+}$ and *Axin2*$^{LacZ/LacZ}$ mice, evaluated for expression of *Nestin*, *DSPP*, *OC*, and *Col1*. Scale bars: 500 μm (*A–F*), and 100 μm (*I–L*, *N–O*). μCT = micro–computed tomography; ab = alveolar bone; d = dentin; p = pulp.

pulp stem cells were isolated from human teeth[19] and analyzed first for their responsiveness to WNT3A protein.[13] Within 6 hours of treatment, dental pulp stem cells exhibited a 4.8-fold increase in *Axin2* expression that persisted for at least 24 hours (Fig. 4*A*). The mitotic activity of dental pulp stem cells was significantly increased by L-WNT3A treatment (Fig. 4*B*). Human CASPASE 3 expression was significantly reduced by L-WNT3A treatment (Fig. 4*C*).

In their undifferentiated state, pulp and bone marrow have been considered to be equivalent tissues.[36,37] We tested whether freshly harvested bone marrow responded to L-WNT3A in a manner similar to the human dental pulp stem cells. Whole bone marrow from mice was harvested and treated with L-WNT3A or L-PBS and within 24 hours a significant increase in Wnt responsiveness was detected (Fig. 4*D*). The elevation in Wnt responsiveness occurred simultaneously with an increase in cell

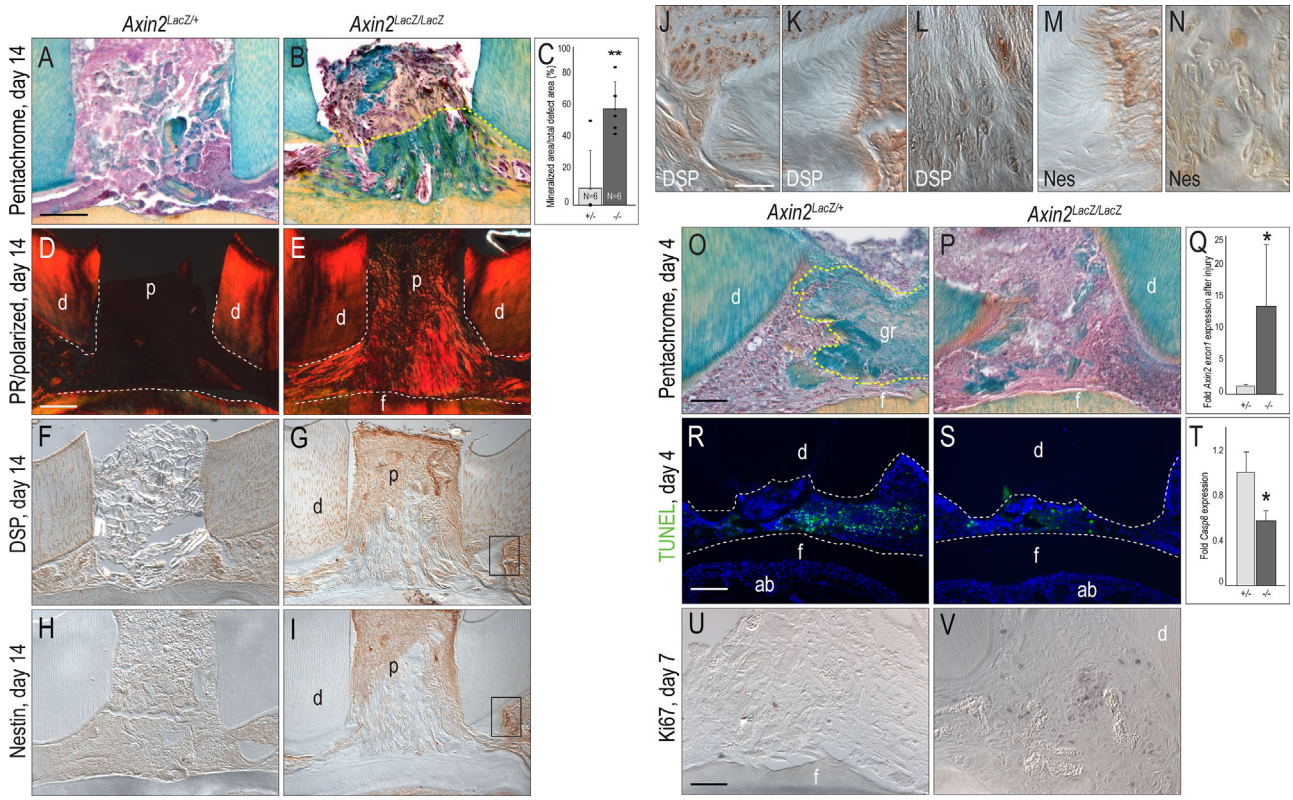

Fig. 3. Injury response to an acute pulp exposure in Axin2$^{LacZ/LacZ}$ mice. (*A*) In *Axin2*$^{LacZ/+}$ mice on day 14, pentachrome staining identifies a pink-colored, acellular granulation tissue that occupies the pulp injury site. (*B*) In *Axin2*$^{LacZ/LacZ}$ mice, the pulp injury site is occupied by a green-yellow mineralized matrix and a dense infiltrate of cells. (*C*) Quantification of histomorphometric analyses, demonstrating pulp injury sites in *Axin2*$^{LacZ/LacZ}$ mice. Under polarized light, Picrosirius red staining of (*D*) *Axin2*$^{LacZ/+}$ injury sites and (*E*) *Axin2*$^{LacZ/LacZ}$ injury sites. In tissues from *Axin2*$^{LacZ/+}$ and *Axin2*$^{LacZ/LacZ}$ mice, respectively, immunostaining for (*F*, *G*) DSP, and (*H*, *I*) Nestin. (*J*–*L*) Immunostaining for DSP and (*M*, *N*) Nestin. Pentachrome staining of pulp injuries on postinjury day 4 in (*O*) *Axin2*$^{LacZ/+}$ and in (*P*) *Axin2*$^{LacZ/LacZ}$ mice, (*Q*) quantified where *Axin2 exon1* expression was measured. TUNEL staining indicates programmed cell death in (*R*) *Axin2*$^{LacZ/+}$ and (*S*) *Axin2*$^{LacZ/LacZ}$ mice. (*T*) Quantitative RT-PCR for *CASP8* expression. On postinjury day 7, Ki67 immunostaining in (*U*) Axin2$^{LacZ/+}$ and (*V*) *Axin2*$^{LacZ/LacZ}$ mice. Scale bars: 25 μm (*J*–*N*), 50 μm (*O*, *P*, *U*, *V*), 100 μm (*A*–*I*, *R*, *S*). **p* <0.05; ***p* <0.01. Error bars = SEM. ab = alveolar bone; d = dentin; f = furcation; gr = granulation tissue; p = pulp.

proliferation (Fig. 4*E*) and a reduction in cell death (Fig. 4*F*). Thus, exposure to a WNT stimulus is sufficient to activate Wnt signaling, enhance mitotic activity, and reduce apoptosis in two stem cell populations.

L-WNT3A treatment preserves pulp vitality after an acute pulp exposure

Given the ability of L-WNT3A to reduce apoptosis and promote cell proliferation in vitro, we next tested whether a liposomal formulation of human WNT3A protein (L-WNT3A) could elicit similar effects in pulp tissue after an acute injury. Acute pulp exposures were generated in wild-type rats and treated with L-WNT3A or an equivalent liposomal formulation of PBS (L-PBS) then sealed to prevent bacterial contamination. Histological analyses verified that the size and extent of the injury was equivalent between the treatment groups ($n = 6$ for both treatment groups; Fig. 5*A*, *B*).

By postoperative day 4, L-PBS controls exhibited extensive pulp necrosis and apoptosis ($n = 6$; Fig. 5*C*, *C′*); in L-WNT3A–treated cases, TUNEL staining was minimal ($n = 6$; Fig. 5*D*). The TUNEL staining that was observed in the L-WNT3A–treated samples was generally restricted to the roof of the pulp cavity, near the site of exposure ($n = 6$; Fig. 5*D′*).

In an elevated Wnt environment such as is observed in *Axin2*$^{LacZ/LacZ}$ mice,[10] cell proliferation is significantly elevated after an injury (Fig. 3); this suggests a more vigorous repair response. We observed the same effect after L-WNT3A treatment of the injured pulp: relative to L-PBS–treated pulp exposures, PCNA immunostaining was much more extensive in the L-WNT3A–treated samples (compare Fig. 5*E*, *F*).

Reduced apoptosis and increased proliferation in the L-WNT3A–treated pulps culminated in a superior repair response. In L-PBS cases, the pulp was largely occupied on postoperative day 14 by a amorphous, bone-like tissue, called atubular osteodentin[38] (Fig. 5*G*), whereas in L-WNT3A–treated cases the pulp cavities were filled with a highly organized, tubular dentin matrix (Fig. 5*H*). Similar to previous quantitative analyses (Fig. 3*C*), the dentin appeared to be denser in the L-WNT3A–treated samples compared to the L-PBS controls (Fig. 5*I*).

The reparative dentin matrix, as visualized by Picrosirius red staining, was distinctly different between the two groups : in

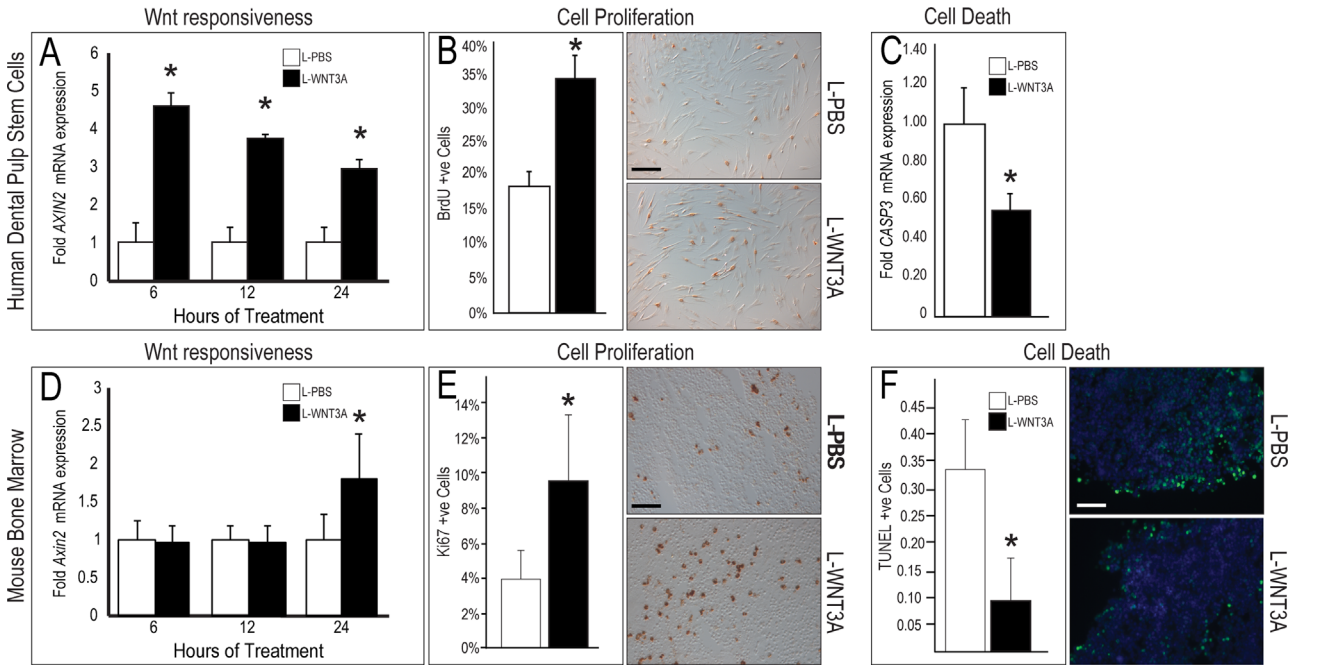

Fig. 4. WNT3A stimulates proliferation and survival of hDPSCs and mouse bone marrow–derived stem cells. (*A*) Quantitative RT-PCR analyses after 6, 12, and 24 hours of L-PBS or L-WNT3A treatment of human dental pulp stem cells. (*B*) Twelve hours posttreatment, the proliferative capacity of hDPSCs was assayed using the BrdU incorporation. (*C*) Quantitative RT-PCR for *CASP3* expression. (*D*) Quantitative RT-PCR analyses after 6, 12, and 24 hours of L-PBS or L-WNT3A treatment of whole bone marrow cells. (*E*) Twelve hours after whole bone marrow cells were exposed to L-WNT3A and L-PBS Ki67 expression and (*F*) TUNEL activity were evaluated. Scale bars: 100 μm (*B*, *E*, *F*). *$p < 0.05$. Error bars = SEM. hDPSC = human dental pulp stem cell.

L-PBS samples the collagenous matrix exhibited a basket-weave pattern, characteristic of bone (Fig. 5*J*); in L-WNT3A samples, the collagenous matrix had a linear organization, suggestive of tubular orthodentin (Fig. 5*K*).

Differentiated odontoblasts express Nestin;[39] few Nestin^{+ve} cells were detected in L-PBS–treated samples compared to the L-WNT3A samples (Fig. 5*L*, *M*). Differentiated odontoblasts also express the extracellular matrix protein DSP and once again, DSP^{+ve} cells were largely absent from the L-PBS–treated pulp compared to the L-WNT3A–treated pulp (Fig. 5*N*, *O*).

Discussion

When confronted with noxious stimuli the human pulp is capable of mounting a robust repair response—at least in young patients.[40,41] In older individuals, the pulp responds to the same noxious stimuli by undergoing necrosis.[40] If provided with appropriate cell survival cues, immature pulp cells can adopt an odontoblast phenotype and can secrete copious amounts of dentin[42] that ultimately serve to insulate the pulp from further onslaught.

We reasoned that providing a developmental signal such as WNT might stimulate an equivalent repair response in the adult pulp. This concept is not without precedent: investigators have demonstrated an ability to modulate odontoblast secretion in human teeth using extracellular matrix molecules,[43] and many groups have used embryonic events as a template for understanding dentin regeneration[44–47] (Supporting Fig. 1).

Such an approach, however, is predicated on odontoblasts maintaining a dependency on Wnt signaling into adulthood. Previous reports indicated that after postnatal day 15, molar odontoblasts and odontoblasts at the incisor tip lose their Wnt responsiveness in vivo.[48] We revisited this question of whether polarized, secretory odontoblasts maintain their dependence upon a Wnt signal into adulthood. We used two separate approaches: first, cryosectioned tissues from adult *Axin2*$^{LacZ/+}$ Wnt reporter mice were analyzed and both polarized odontoblasts and pulp cells were found to be X-gal^{+ve} (Figs. 1*E*, 2*K*). Second, tissues from adult *Axin2*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ Wnt reporter mice showed that polarized odontoblasts were GFP^{+ve} (Fig. 1*F*). From these in vivo studies we conclude that adult odontoblasts and pulp cells maintain a Wnt-responsive status in adulthood. An outstanding question is the source of the endogenous Wnt signal. Of the 19 Wnts, expression patterns for only two ligands have been demonstrated in the pulp and/or in odontoblasts.[49,50] The lack of antibodies that can localize the proteins makes identifying the source of Wnt ligands a challenge. Nonetheless, abundant data indicate the importance of this pathway in maintaining pulp homeostasis.[16]

In the absence of an in vivo Wnt stimulus, *Axin2*$^{LacZ/LacZ}$ cells behave the same as wild-type cells.[10] Because Axin2 represses Wnt signaling in a ligand-dependent manner,[24,25] the removal of *Axin2* results in an amplified Wnt response in vivo.[10,24] We and others have demonstrated that injury activates the endogenous Wnt pathway[51–54] and *Axin2*$^{LacZ/LacZ}$ mice mount a much stronger repair response compared to heterozygous and wild-type controls.[16,26,55] The response to pulpal injury is

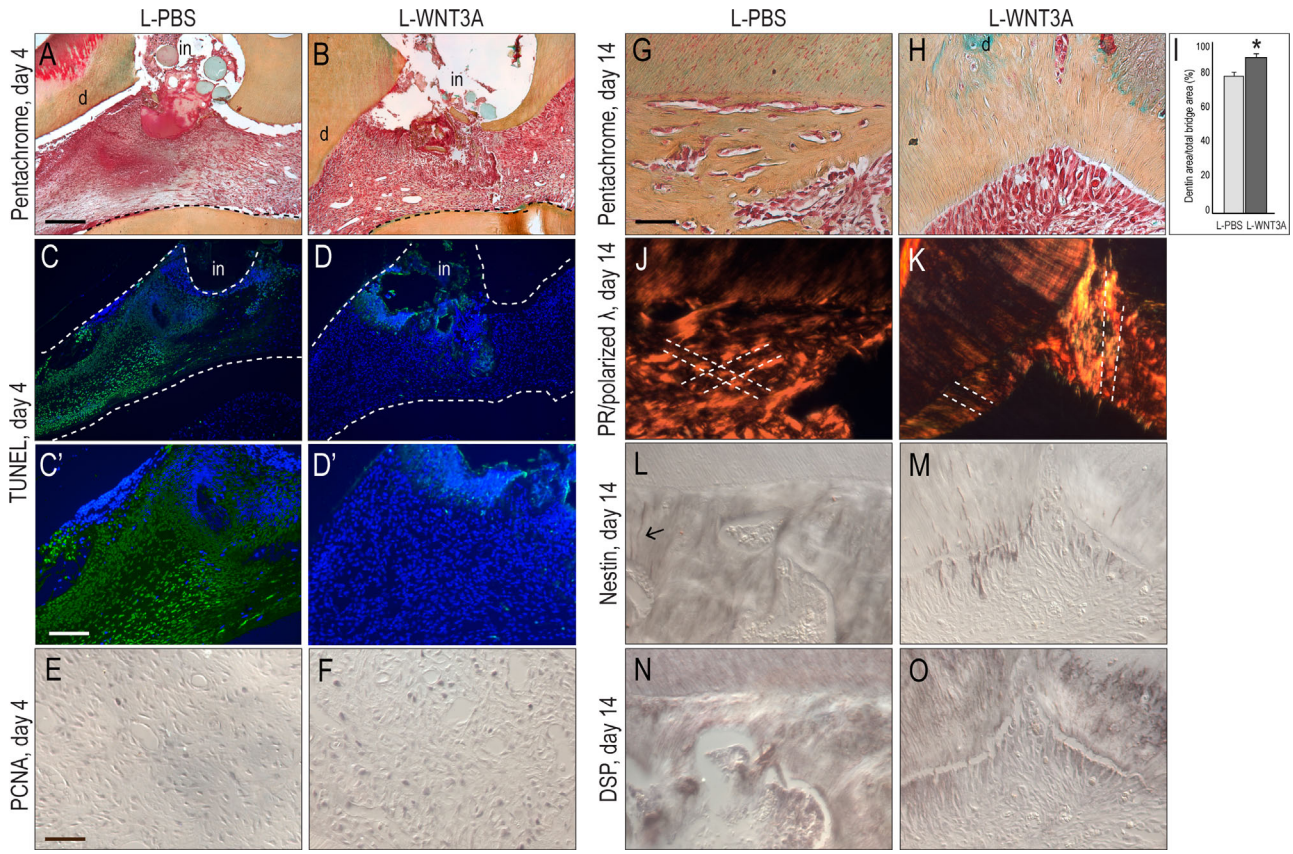

Fig. 5. L-WNT3A treatment induces dentin regeneration. Pentachrome staining of pulp injuries on postinjury day 4 in (*A*) L-PBS–treated rats and in (*B*) L-WNT3A–treated rats. Postsurgical day 4, TUNEL staining in the pulp cavities of (*C*, *C′*) L-PBS–treated and (*D*, *D′*) L-WNT3A–treated rats. On postsurgical day 4, PCNA expression in the pulp cavities of (*E*) L-PBS–treated and (*F*) L-WNT3A–treated rats. Pentachrome staining of (*G*) L-PBS–treated and (*H*) L-WNT3A–treated rats 14 days after injury. (*I*) Quantification of reparative dentin matrix. Under polarized light, Picrosirius red staining of pulp injuries on postinjury day 14 in (*J*) L-PBS–treated and (*K*) L-WNT3A–treated injury sites. On postsurgical day 14, Nestin expression in (*L*) L-PBS–treated and (*M*) L-WNT3A–treated rats, and DSP expression in (*N*) L-PBS–treated and (*O*) L-WNT3A–treated rats. Scale bars = 200 μm (*A*–*D*), 100 μm (*C′*, *D′*), 50 μm (*E*, *F*), 25 μm (*G*–*O*), 50 μm. $^{*}p < 0.01$. Error bars = SEM. d = dentin; in = injury site.

similar: elevating Wnt signaling by either removing a negative Wnt regulator (Fig. 3) or by providing exogenous WNT3A protein (Fig. 5) is sufficient to significantly improve the pulp cavity's repair response.

The mechanism of WNT action in the pulp may be due in part to the response of stem/progenitor cells within this tissue. Human dental pulp stem cells respond to human WNT3A protein by strongly up-activating the Wnt pathway, by becoming mitotically active, and by downregulating caspase activity (Figs. 4, 5), an enzyme that mediates the execution phase of apoptosis.[56] Collectively, these biological responses may prove to be especially valuable in therapeutic strategies that seek to improve a healing response.

In addition to these biological responses, a difference was noted in the type of reparative mineralized tissue that formed after L-PBS and L-WNT3A treatment (Fig. 5). In L-PBS–treated samples, a bonelike mineralized matrix, osteodentin, forms. Compared to dentin, osteodentin is porous and consequently its appearance in the injured pulp represents a suboptimal healing response. In L-WNT3A cases the reparative matrix resembled native dentin (Fig. 5*H*, *K*). This dentin matrix was produced by native DSP^{+ve} secretory odontoblasts (Fig. 5*M*) and it formed a dentin bridge that effectively separated the viable pulp cavity from the external environment. No such dentin bridge was evident in controls.

In light of new knowledge about the role of Wnt signaling in pulp development and homeostasis (Figs. 1, 2), we tested an alternative approach to root canal therapy that exploited the reliance of pulp cells on endogenous Wnt signaling (Figs. 3, 4). A liposome-reconstituted form of WNT3A protein effectively protected pulp cells from death and stimulated proliferation of undifferentiated cells in the pulp, which together significantly improved pulp healing. The strategy of activating endogenous stem cells via L-WNT3A to improve healing represents a viable means to achieve pulp regeneration in humans.

Disclosures

All authors state that they have no conflicts of interest.

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