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The role of bone morphogenetic proteins 2 and 4 in mouse dentinogenesis

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ABSTRACT

Objective: The bone morphogenetic proteins (BMPs) play crucial roles in tooth development. However, several BMPs retain expression in the dentin of the fully patterned and differentiated tooth. We hypothesized that BMP signaling therefore plays a role in the function of the differentiated odontoblast, the job of which is to lay down and mineralize the dentin matrix.

Design: We generated mice deficient in *Bmp2* and *4* using a dentin matrix protein 1 (*Dmp1*) promoter-driven cre recombinase that was expressed in differentiated odontoblasts.

Results: The first and second molars of these *Bmp2* and *Bmp4* double conditional knockout (DcKO) mice displayed reduced dentin and enlarged pulp chambers compared to cre-negative littermate controls. DcKO mouse dentin in first molars was characterized by small, disorganized dentinal fibers, a wider predentin layer, and reduced expression of dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1), and bone sialoprotein (BSP). DcKO mouse odontoblasts demonstrated increased type I collagen mRNA production, indicating that the loss of BMP signaling altered the rate of collagen gene expression in these cells. *Bmp2* and *Bmp4* single *Dmp1*-cre knockout mice displayed no discernable dentin phenotype.

Conclusions: These data demonstrate that BMP signaling in differentiated odontoblasts is necessary for proper dentin production in mature teeth.

1. Introduction

The mammalian tooth begins as an invagination of embryonic ectoderm into the adjacent mesenchyme. Reciprocal signals between these layers guide their development through the bud, cap, and bell stages to form the mature tissues of the tooth (Tummers & Thesleff, 2009). Crucial among these signals are the bone morphogenetic protein (BMP) members of the transforming growth factor beta (TGFß) superfamily. BMP 2 and BMP4 secretion by the early lamina stage ectoderm induces mesenchymal expression of the Msx1 and 2 homeodomain transcription factors that are essential for progression to the bud stage (Neubuser, Peters, Balling, & Martin, 1997; Nie, Luukko, & Kettunen, 2006). Furthermore, BMP4 secretion specifies the identity of the presumptive tooth as an incisor in the embryonic mandible, and inhibition of BMP signaling by appropriately-timed application of Noggin protein changes tooth fate from incisor to molar (Tucker, Matthews, & Sharpe, 1998). Beginning with the bud stage, BMP4 expression appears in the mesenchyme as well, and inactivation of the BMP receptor Bmpr1a in epithelial or mesenchymal layers arrests development between the bud and cap stages, highlighting the essential role of BMP signaling in early tooth progression (Åberg, Wozney, & Thesleff, 1997; Vainio, Karavanova, Jowett, & Thesleff, 1993).

BMP expression continues past the cap stage to tooth maturity, and several studies of cell- and stage-specific BMP deletions in mice have demonstrated their importance in dentin and enamel formation after initial tooth patterning. Feng, et al. reported that conditional cre/lox-mediated deletion of *Bmp2* in ameloblasts using an osterix-cre leaves thin and hypomineralized enamel (Feng et al., 2011). Yang and colleagues then demonstrated that *Bmp2* ablation in differentiating odontoblasts and osteoblasts using the 3.6*Col1aI*-cre results in severe decreases in root and crown dentin (Yang et al., 2012). This cre causes recombination of the *Bmp2* loxP allele after specification to the odontoblast lineage, but before terminal differentiation, and results in deficient expression of osterix, collagen, and dentin sialophosphoprotein (DSPP). *Bmp4* knockout in dentin and bone using the same 3.6*Col1aI* cre also leads to reduced dentin and enlarged pulp chambers caused by reduced expression of key odontoblast differentiation transcription factors (Gluhak-Heinrich et al., 2010). Together, these data demonstrate that BMPs direct cell differentiation and gene expression in the fully patterned tooth.

Dentinogenesis is an ongoing process accomplished by differentiated odontoblasts once tooth patterning is complete. Odontoblasts

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must form the primary dentin of the tooth crown, and then generate secondary dentin throughout life and tertiary dentin in response to injury. BMPs 2, 4, and 7 continue to be expressed in dentin after birth (Butler, Mikulski, Urist, Bridges, & Uyeno, 1977; Nakashima, Nagasawa, Yamada, & Reddi, 1994), and we hypothesized that BMP signaling plays an ongoing role in dentin production by the differentiated odontoblast. Although BMP7 has distribution and expression patterns similar to those of BMP2 and 4, its ablation in mice appears to have little consequence for tooth development, possibly because of functional redundancy with other BMP members or related growth factors (Dudley & Robertson, 1997; Helder et al., 1998). We therefore designed the present study to define the function of BMP2 and 4 in differentiated odontoblasts *in vivo*. We generated a mouse model in which the *Bmp2* and *4* alleles were deleted in the presence of cre recombinase under the control of the *Dmp1* promoter (*Dmp1*-Cre) (Lu et al., 2007). The striking dentin phenotype seen in these double conditional knockout mice demonstrates that BMP signaling plays a pivotal role in dentin synthesis and maturation.

2. Materials and methods

2.1. Generation of Dmp1-cre;$Bmp2^{f/f}$;$Bmp4^{f/f}$ (double conditional knockout, DcKO) mice

The generation of transgenic mice expressing cre recombinase under the control of a 9.6-kb *Dmp1* promoter + 4 kb of intron 1 fragment (*Dmp1-Cre*) was described previously (Lu et al., 2007). Mice harboring conditional alleles of the *Bmp2* or *Bmp4* gene (*Bmp2*-floxed or *Bmp4*-floxed mice) were also described previously and were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) (Liu et al., 2004; Ma & Martin, 2005). To study the inactivation of *Bmp2* and *4* in differentiated odontoblasts, these three lines were mated to generate *Dmp1*-cre;$Bmp2^{f/f}$;$Bmp4^{f/f}$ experimental group animals (referred to as double conditional knockout, DcKO mice) and $Bmp2^{f/f}$;$Bmp4^{f/f}$ littermate controls. Tail biopsies were analyzed by polymerase chain reaction (PCR) with primers recommended by The Jackson Laboratory. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Texas A&M University College of Dentistry.

2.2. Quantitative real time polymerase chain reaction (qPCR)

Total RNA was extracted from the molars of three-week-old DcKO and control mice, treated with DNase I (Promega, Madison, WI), and purified with the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). RNA (1 μg/ml per sample) was transcribed into cDNA with SuperScript III reverse transcriptase (Invitrogen, San Diego, CA). qPCR reactions were performed using the Brilliant SYBR Green QPCR Master Mix (Applied Biosystems; Foster City, CA) and the CFX-96 Real-Time PCR Detection System (Bio-Rad; Hercules, CA). Primers for relative (qPCR) of *Bmp2* and *Bmp4* were those used previously by Wang, et al. (Wang et al., 2010). The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as the internal control. The ΔΔ Ct method was used to calculate gene expression levels normalized to GAPDH value. Reactions were performed in triplicate on samples from three separate experiments and expressed as a relative fold change in gene expression compared to the control.

2.3. Plain X-ray radiography and microcomputed tomography (μCT)

Mandibles from one-month and three-month-old mice from both groups were dissected, fixed for 48 h in 4% formaldehyde and stored in 70% ethanol at 4C. The mandibles were then analyzed with a Faxitron MX-20 specimen radiography system (Faxitron X-ray Corp., Buffalo Grove, IL). For the μ-CT analyses, 3-months old gender matched mandibles were scanned using a μCT35 imaging system (Scanco Medical, Basserdorf, Switzerland). The whole mandible was scanned in 7.0 μm slice increments at medium resolution to assess the overall shape and structure. Following this evaluation, a high-resolution scan of the molar region in 3.5 μm slice increments was made for quantification purposes. (n = 4 each for wild type control and DcKO groups). For quantification, we used the Scanco MicroCT evaluation software. The tooth bearing area of the mandibles were scanned in a vertical position with long axis of the mandible perpendicular to the floor, which would generate the cross sections of the teeth in each slice. Contour lines were drawn around the first and second molars to define the region of interest. Once the entire molar was defined, the slices were submitted for analyses to generate the total volume (TV), dentin volume (DV), pulp volume (PV), DV/TV and PV/TV ratios and the apparent and mean densities.

2.4. Tissue preparation and histology evaluation

Under anesthesia, the control and DcKO mice at postnatal one and three months were perfused from the ascending aorta with 4% formaldehyde in 0.1 M phosphate-buffered saline. The mandibles were dissected and further soaked in the same fixative for 48 h, followed by demineralization in 14% EDTA (pH 7.4) at 4C for 2 weeks. The tissues were processed for paraffin embedding, and serial 5 μm sections were prepared. The sections were stained with hematoxylin and eosin (H&E) for histological analyses. Picrosirius Red staining (Junqueira, Bignolas, & Brentani, 1979) was performed to assess the morphology and organization of the collagen fibrils.

For the immunohistochemistry analyses, anti-DSP-2C12.3 monoclonal antibody was used at a concentration of 2.05 μg/ml. Anti-DMP1 monoclonal antibody that recognizes the C-terminal region of DMP1 was used at a concentration of 4.7 μg/ml. Anti-BSP monoclonal antibody 10D9.2 was used at a concentration of 4.5 μg/ml.

All the IHC experiments were carried out using the mouse on mouse kit for monoclonal antibodies (Vector Laboratories, Burlingame, CA). The 3, 3′-diaminobenzidine (DAB) kit (Vector Laboratories) was used for color development according to the manufacturer's instructions. Methyl Green was used as the counterstain.

2.5. In situ hybridization

In situ hybridization was performed as described previously to assess *Col1a1* mRNA levels in the molars of six-week-old mice for each group (Jani et al., 2016). The RNA probes were labeled with digoxigenin using a RNA labeling kit (Roche, Indianapolis, IN) and were detected by an enzyme-linked immunoassay with a specific anti-digoxigenin-alkaline phosphatase antibody conjugate and alkaline phosphatase substrate (Roche), following the manufacturer's instructions. Nuclear fast red was used for counterstaining.

2.6. Statistical analysis

Statistical evaluations of the data were conducted by independent Student's *t*-test to validate the differences between two groups. $P < 0.05$ was considered statistically significant. The data were presented as mean ± SD.

3. Results

3.1. Reduced BMP2 and 4 expression in Dmp1-cre double knockout mice

We employed *Dmp1*-cre transgenic mice in which cre activity correlates with endogenous DMP1 expression in osteocytes and odontoblasts. Cre is expressed in these mice at low levels in teeth before embryonic day 18.5 and then at high levels in odontoblasts after birth and is also found in odontoblastic precursor cells in the pulp (Lu et al., 2007). To ablate *Bmp2* and *4* selectively in odontoblasts, we therefore generated mice that were homozygous for *Bmp2* and *Bmp4* loxP alleles