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Bmp2 Is Required for Odontoblast Differentiation and Pulp Vasculogenesis

ABSTRACT

Using the Bmp2 floxed/3.6Col1a1-Cre (Bmp2-cKO[od]) mouse model, we have observed severe defects in odontogenesis and dentin formation with the removal of the Bmp2 gene in early-polarizing odontoblasts. The odontoblasts in the Bmp2-cKO[od] do not mature properly and fail to form proper dentin with normal dentinal tubules and activate terminal differentiation, as reflected by decreased Osterix, Col1a1, and Dspp expression. There is less dentin, and the dentin is hypomineralized and patchy. We also describe an indirect effect of the Bmp2 gene in odontoblasts on formation of the vascular bed and associated pericytes in the pulp. This vascular niche and numbers of CD146+ pericytes are likely controlled by odontogenic and Bmp2-dependent VegfA production in odontoblasts. The complex roles of Bmp2, postulated to be both direct and indirect, lead to permanent defects in the teeth throughout life, and result in teeth with low quantities of dentin and dentin of poor quality.

KEY WORDS: bone morphogenetic protein 2, blood vessels, dentinogenesis, dental pulp stem cells, odontogenesis, pericytes.

INTRODUCTION

The Bmp2 gene is expressed in post-natal odontoblasts and ameloblasts during tooth cytodifferentiation from birth to approximately 20 days after birth (Aberg *et al*., 1997). Previous studies have determined key roles for Bmp2 and Bmp4 during embryonic tooth development, but little is known about the specific role of Bmp2 during post-natal tooth cytodifferentiation (Maas and Bei, 1997). Several genes have been linked to dentinogenesis imperfecta (DGI) in humans and several mouse models (Sreenath *et al*., 2003). One of the most highly expressed odontoblast-specific terminal differentiation genes is dentin sialophosphoprotein (Dspp). Mutations in this gene have been linked to DGI type III. Studies have shown that Dspp in odontoblasts is regulated by Bmp signaling *in vitro* and *in vivo* (Chen *et al*., 2008, 2009; Cho *et al*., 2010). Defects in odontogenic collagen type 1a (Col1a1) production are also linked to dentin defects of various degrees in both humans and mice (Hart and Hart, 2007). It is known that dentin-derived Bmp2 drives dental stem cells from exfoliated deciduous teeth (SHED) into mature dentin-producing odontoblasts (Huang *et al*., 2009; Casagrande *et al*., 2010). CD146+ and Stro-1 perivascular cells have the capability of differentiating into odontoblasts *in vivo* (Shi and Gronthos, 2003). These types of cells in human bone marrow have been shown to have the characteristics of skeletal stem cells and organize around blood vessels (Sacchetti *et al*., 2007). Analysis of recent data, with a lineage-tracing method for NG2-marked pericytes, demonstrated that as much as 15% of the pericytes can become odontoblasts in an incisor model of tissue repair (Feng *et al*., 2011). One other likely region for a dental stem cell niche is the apical papilla at the base of the root (Huang *et al*., 2008).

We demonstrate how the Bmp2 gene controls the terminal differentiation of odontoblasts in post-natal animals and, at the same time, the formation of the vascular bed in the dental pulp and associated pericytes on the pulp blood vessels.

MATERIALS & METHODS

Animals

All mice were used in this study in compliance with the UTHSCSA institutional Animal Care and Committee guidelines. Mice deficient in Bmp2 in early odontoblasts and osteoblasts were generated by crossing Bmp2 floxed (Bmp2-fx/fx) with a 3.6Col1a1-Cre mouse (Liu *et al*., 2004; Singh *et al*., 2008). These mice are referred to as Bmp2-cKO[od]. Specific deletion of Bmp4 in teeth (odontoblasts), in the 3.6Col1a1-Cre model, has recently been described and demonstrates odontoblast specificity with a Rosa 26-GFP
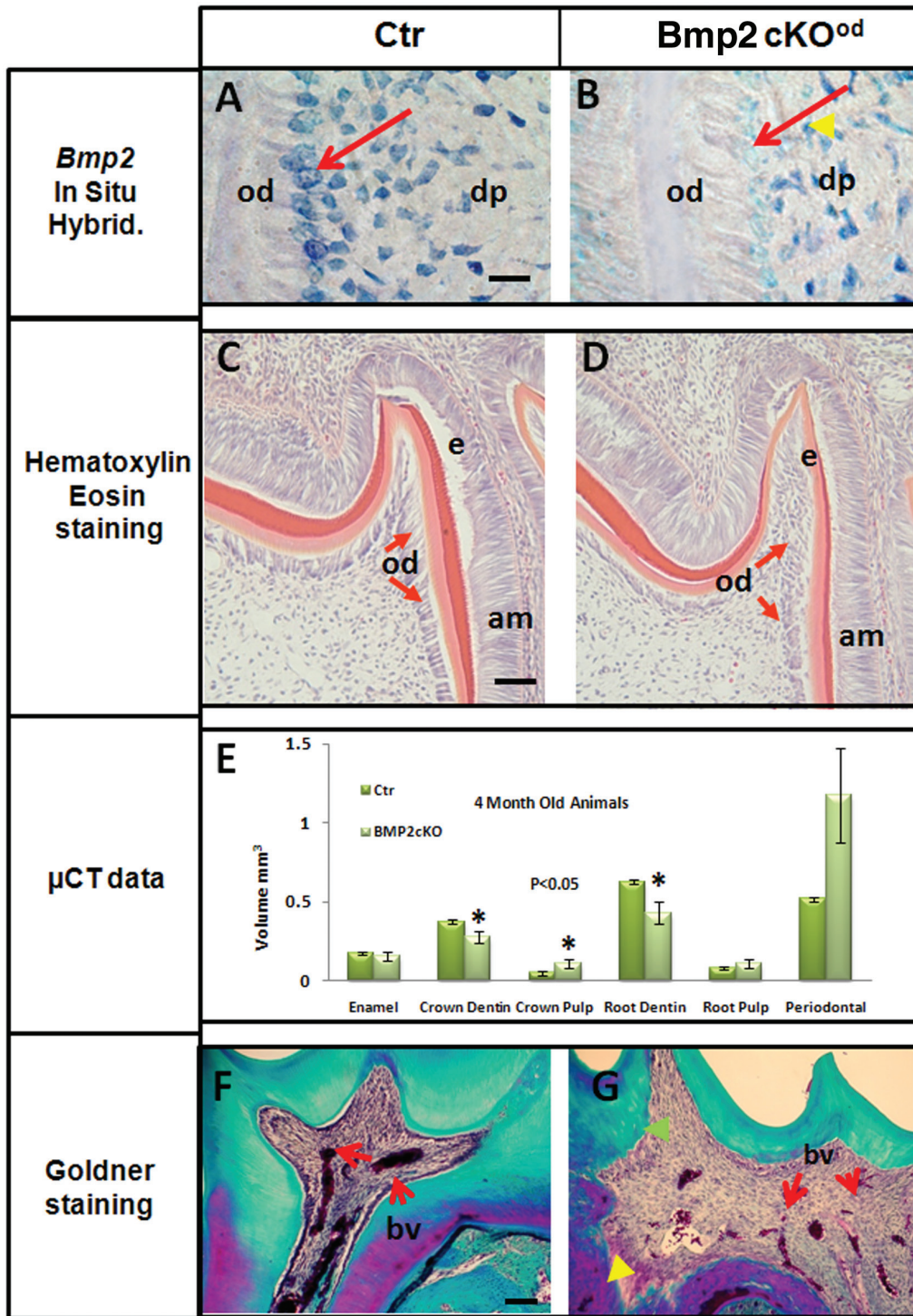
Figure 1. Characterization of tooth phenotype after Bmp2 deletion in a 3.6Col1a1Bmp2Cre-cKO (Bmp2-cKO^{od}) model. (Panel **A**) Control, Ctr. (Panel **B**) Bmp2-cKO^{od}. Bmp2 mRNA Exon3 (region deleted by Cre recombination) *in situ* hybridization (blue) of 5-day-old Bmp2-cKO^{od} mice was reduced about 90% in odontoblasts compared with control-WT mice (red arrows). Little change of Bmp2 Exon3 signal in the pulp was noted (yellow arrowhead). Bar = 20 μm. (Panel **C**) Control, Ctr. (Panel **D**) Bmp2-cKO^{od}. Hematoxylin & eosin staining of 5-day-old Bmp2-cKO^{od} mice. 1st molar shows 50% ($P < 0.001$, n = 3) thinner pre-dentin compared with that in control-Wt. Note the disorganized odontoblasts (red arrows) in the Bmp2-cKO^{od} animals, compared with the well-organized and polarized odontoblasts in the Ctr. Bar = 100 μm. (Panel **E**) μCT analysis: Four-month-old Bmp2-cKO^{od} mice displayed a significant ($P < 0.05$) decrease in crown dentin and root dentin volume and also displayed a significant ($P < 0.05$) increase in crown pulp volume (n = 3 in each group). (Panel **F**) Control, Ctr. (Panel **G**) Bmp2-cKO^{od}. Goldner staining of 1st molars of 4-month-old control and Bmp2-cKO^{od} animals demonstrated permanent post-natal quantitative and structural changes in the dentin, with extensive poorly mineralized and "patchy" dentin (purple and noted by yellow arrowhead in Panel G, Bmp2-cKO^{od}), as well as disorganized tubules (green arrowhead) with fewer and smaller blood vessels in the dental pulp (marked bv and red arrows). Bar = 40 μm. Ameloblasts (am), odontoblasts (od), dentin-pulp (dp), and blood vessels (bv).
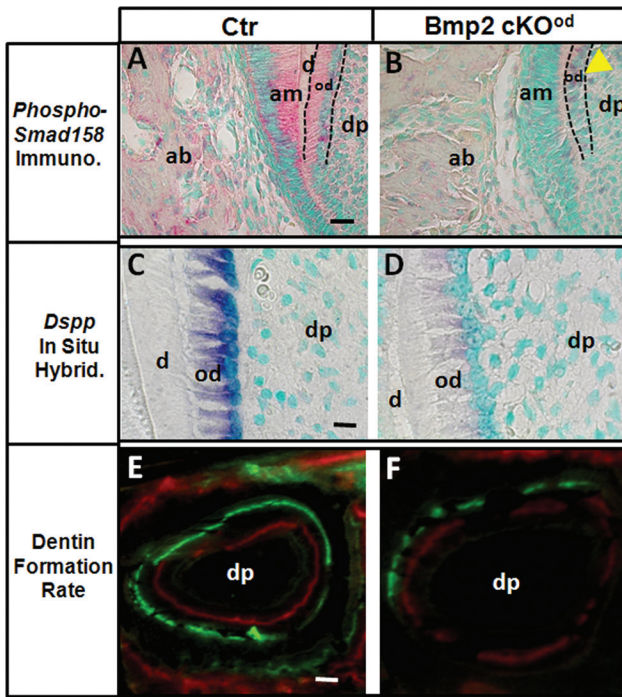
Figure 2. Bmp Signaling, Dspp expression, and dentin formation rate (DFR) of the post-natal teeth in the Bmp2-cKO^{od} model. (Panel **A**) Control, Ctr. (Panel **B**) Bmp2-cKO^{od}. Phospho-Smad 1/5/8 immunoreactivity was reduced 80% in odontoblasts (yellow arrowhead) and in the dental pulp and ameloblasts of the 1-day-old Bmp2-cKO^{od} compared with Ctr. Bar = 20 μm. (Panels **C, D**) *In situ* hybridization with Dspp probe shows a 90% reduced hybridization numerical estimated signal in odontoblasts of Bmp2-cKO^{od} compared with Ctr, 5-day-old animals. Bar = 20 μm. DFR analysis shows a 45% reduced dynamic dentin formation rate, as measured at 25 days, in Bmp2-cKO^{od} mice compared with Ctr (Panels **E, F**) (n = 3 animals *per* group, P < 0.05). Bar = 100 μm. Alveolar bone (ab), ameloblasts (am), odontoblasts (od), dental pulp (dp).

reporter in which the GFP cassette is activated after Cre recombination (Gluhak-Heinrich *et al.*, 2010).

In this study, we used a total of 86 animals: 29 Bmp2-cKO^{od}, 26 heterozygotes (Hets) (Bmp2 fx/+), and 31 wild types (Wt) with the following ages: 1, 5, and 14 days, 1 mo, 4 mos, and 8 mos. At least 3 independent littermates were used in each of these studies. Control mice, representing heterozygotes and wild types, did not show significant differences in tooth or bone phenotype and were pooled for statistical evaluation. For initial x-ray studies, all mice were evaluated with age and group size 4-20. For μCT, 3 animals *per* group (genotype) were used for statistical evaluation by a simple unpaired *t* test. For quantitation of the CD146+ cells, the cells were counted in 2 independent littermates from multiple sections, and significance was determined by simple unpaired *t* test. For most *in situ* hybridization and immunological studies, multiple sections from a given mouse were first evaluated and then from 2 or 3 independent littermates, and data from the 2-3 different animals were combined for statistical evaluation with a simple unpaired *t* test for significance. ANOVA analysis was also carried out for most studies with the 2 groups.

X-ray Analysis and μCT

Radiographic images of mandibles and associated teeth were obtained with image capture in a digital Faxitron radiograph unit (Model 8050-020, Field Emission Corporation, Inc. Tokyo, Japan) and analyzed with AnalySIS software measuring tooth parameters of the 1st and 2nd molars, as described in Gluhak-Heinrich *et al.* (2010). μCT analysis of 1st and 2nd molars, by Numira, Inc. (www.numirabio.com), shows volumetric evaluation of the enamel, crown dentin, crown pulp, root dentin, root pulp, and the periodontium with at least 2 independent litters of 1 and 4 mos of age. Male mice were used.

Tissue Preparation and Analysis

Tissues were fixed, demineralized, and processed RNase-free for *in situ* hybridization as described previously (Gluhak-Heinrich *et al.*, 2008). [A detailed description of the *in situ* hybridization protocol and our method for numerical estimate of expression signal (blue) and corrected background signals is given in the Appendix.] For dentin formation rates, mice injected with alizarin red at 14 days and calcein at 22 days were sacrificed at 25 days. We used plastic-embedded sections after double-labeling as described previously (Ye *et al.*, 2004). We used Col1a1, Dspp (Gluhak-Heinrich *et al.*, 2010), Bmp2 exon3 (region deleted by Cre), Osterix-Sp7, and VegfA probes. For numerical evaluation of expression signal hybridization, several parameters must be carefully followed. First, the mandibles are fixed with fresh DEPC-treated cold 4% paraformaldehyde for 48 hrs at 4°C with rotation at 30 rpm. The tissue is washed with RNase-free PBS and decalcified for 6 wks at 4°C, rotating 30 rpm with 15% EDTA–RNase-free solution and changed 2× *per* wk. This procedure ensures complete decalcification; leaving the fixative in during the 6-week decalcification is neither desirable nor necessary. Next, the digoxigenin probes are checked for an approximate linear relationship of RNA and digoxigenin signal (see Appendix). The *in situ* hybridization signal with the BCIP/NBT is blue to blue-purple, and the counterstain is light green. Consecutive sections were used for histological hematoxylin-eosin (H&E), immunocytochemistry, and Goldner staining (Intini *et al.*, 2007). Immunocytochemistry was carried out as previously described (Gluhak-Heinrich *et al.*, 2010) to detect Phospho-Smad1/5/8 (Cell Signaling Technology, Inc. #9511S, Boston, MA USA), CD31 (Abcam#28364, San Francisco, CA, USA), CD146 (Abcam#75769), and VegfA (Abcam#46154). With the Fast Red substrate for alkaline phosphatase used in the immunocytochemistry, the signal is always red, and a good contrast with the light green counterstain is achieved.

A full list of the ages of animals used in each method applied and the numbers of animals used for each method is given in the Appendix Table.

RESULTS

We determined that the 3.6Col1a1-Cre model deleted the Bmp2 gene in odontoblasts (Figs. 1A, 1B). The Bmp2 (Exon3) signal was reduced 90% in Bmp2-cKO^{od} odontoblasts (red arrow) of 1st molar teeth at 5 days of age (n = 3 animals *per* group). The

level of Bmp2 Exon3 expression in select cells in the dental pulp was not affected (yellow arrowhead) in Bmp2-cKO[od]. The odontoblasts in the Bmp2-cKO[od] were dysmorphic and failed to form a well-organized polarized structure, as noted by the red arrows in Figs. 1C and 1D. The thickness of the predentin at this early stage was also reduced (light red). The ameloblasts appeared morphologically normal, although there was a delay in the formation of pre-enamel, as noted by the darker red staining (Figs. 1C and 1D). By µCT of the 1st and 2nd molars of 4-month-old animals (Fig. 1E) and 1-month-old animals (Appendix Figs. 1A, 1B), volumetric measurements were carried out on the mineralized regions in enamel, crown dentin, crown pulp, root dentin, root pulp, and periodontium. We observed a 30 to 50% decrease in dentin volume of both crown and roots. By x-ray analysis, dentin thickness decreased 50% (Appendix Fig. 1C). This pronounced decreased dentin thickness was still observed in 8-month-old Bmp2-cKO[od] animals compared with controls (data not shown). When the sections from 4-month-old molars (Figs. 1F and 1G) were stained with Goldner (Intini *et al*., 2007), the dentin of the Bmp2-cKO[od] showed a patchy disorganized structure with disorganized dentinal tubules and reduced blood vessel size and number in the pulp. No significant change in the enamel or periodontal volume was noted, although there was a trend of increased periodontal volume in Bmp2-cKO[od], possibly due to the overall smaller teeth relative to growth of the jaw at 4 mos. These defects were permanent in the teeth as evaluated at 4 to 8 mos.

We first determined changes in the Bmp-dependent signaling pathway for possible mechanisms. There was a decrease of over 80% in the level of phospho-Smad1/5/8 immunoreactivity in odontoblasts of Bmp2-cKO[od] compared with control teeth at 5 post-natal days during active molar tooth growth and formation (n = 3 animals *per* group) (Figs. 2A and 2B). We noted a low level of Bmp signaling in the dental pulp in the control, and less in Bmp2-cKO[od]. We also found decreased phospho-Smad1/5/8 levels in the ameloblasts, suggesting an indirect role of odontogenesis in amelogenesis. However, we noted no significant permanent decrease in enamel volume at 1 or 4 mos (Fig. 1E; Appendix Fig. 1B).

A key transcription factor that regulates odontogenesis as well as osteoblastogenesis is the gene Osterix/Sp7 (Chen *et al*.,
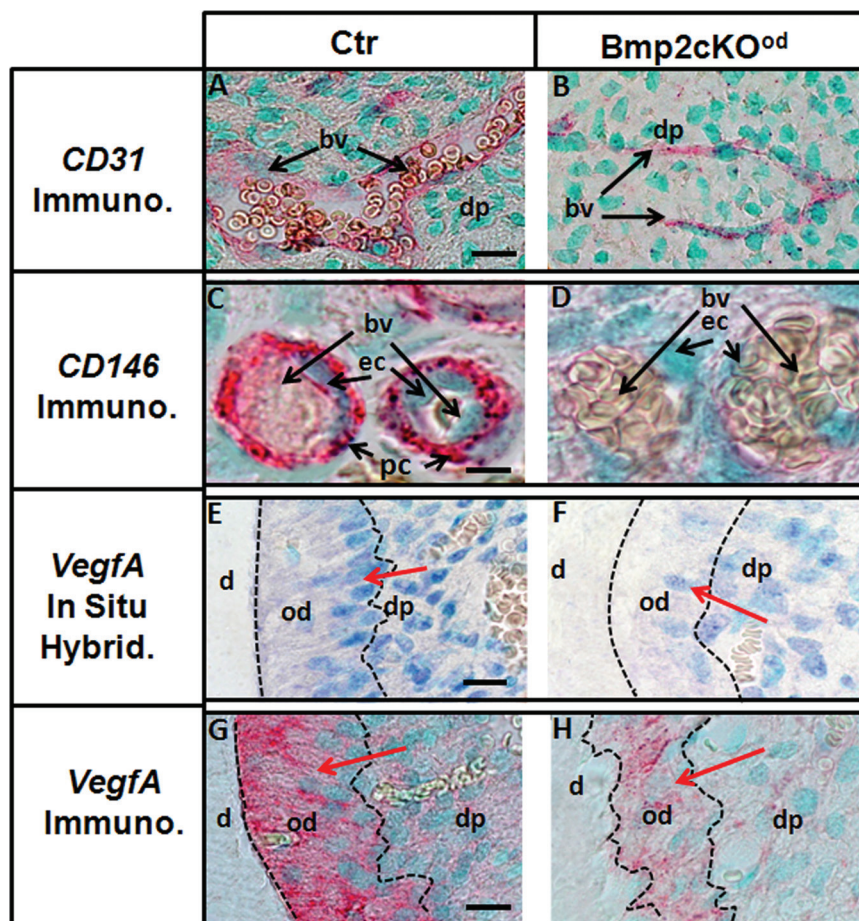

Figure 3. Blood vessels (CD31+) and associated CD146+ pericytes plus VegfA expression were reduced in the Bmp2-cKO[od] compared with control. (Panels **A, B**) The number of BV (CD31+ regions) in the Bmp2-cKO[od] dental pulp region was decreased 50% compared with Ctr in 5-day-old animals. Bar = 20 µm. (Panel **C**) Control, Ctr. (Panel **D**) Bmp2-cKO[od]. CD146+ cells/pericytes associated with BV. Total CD146+ immunoreactivity was reduced 90% in the Bmp2-cKO[od] mice in the dental pulp as shown for the 1st molars of 1-month-old animals (n = 3 *per* group). (See Appendix Figs. 3C and 3D for low magnification.) The numbers of CD146+ cells directly associated with a BV are also reduced by 70%, as shown by an extreme example in panels C and D. Bar = 10 µm. VegfA mRNA expression in incisors of 4-month-old control and Bmp2-cKO[od] is shown in Panels **E** and **F** (blue). VegfA was expressed in more odontoblasts and showed a stronger signal *per* odontoblast in control compared with Bmp2-cKO[od] animals (red arrows). The VegfA hybridization signal in the odontoblast layer, on average, was reduced over 60% in the Bmp2-cKO[od] compared with Ctr. Bar = 10 µm. VegfA immunocytochemistry (red) signal in incisors of 4-month-old animals was reduced 80% in Bmp2-cKO[od] compared with Ctr (Panels **G, H**, red arrows). See Appendix Figs. 3G and 3H for low magnifications, with reduced VegfA protein associated with blood vessels in the pulp and the odontoblast layer in the Bmp2-cKO[od] animals. Bar = 10 µm. Dental pulp (dp), blood vessels (bv), Pericyte (pc), endothelial cells (ec), odontoblasts (od), and dentin (d).

2009; Zhou *et al*., 2010). Osterix, a Zn2+ finger transcription factor, directly co-regulates many genes, including Col1a1, Dmp1 (Zhou *et al*., 2010), and Dspp. Osterix expression was reduced 60% in odontoblasts of 5-day molar odontoblasts (n = 3) (Appendix Figs. 2D, 2E, 2F). The major structural protein for dentin is type 1 collagen (Col1a1), which is decreased 70% during early tooth cytodifferentiation (data not shown). Even in the continuously erupting incisors (4 mos), we observed a 75% decrease in Col1a1 expression (Appendix Figs. 2A, 2B, 2C).
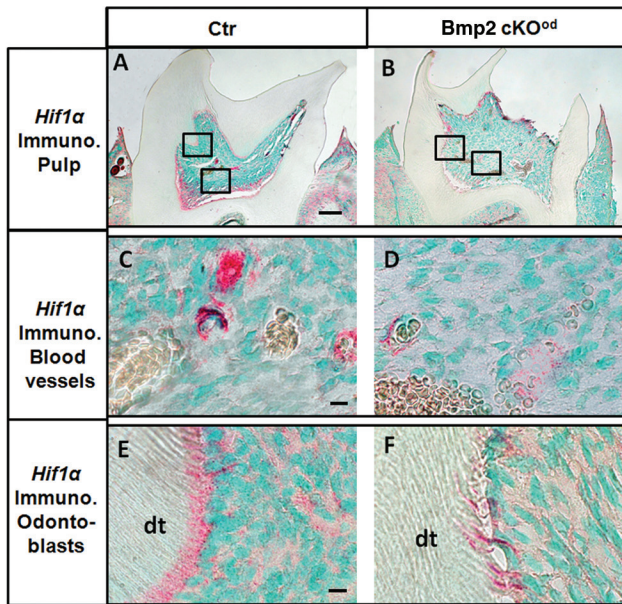
Figure 4. Hif1α immunocytochemistry of Ctr and Bmp2-cKO[od] animals, 1 mo old. (Panel **A**) Control, Ctr. (Panel **B**) Bmp2-cKO[ob]. Low magnification (bar = 100 μm) of 1st molars, showing overall Hif1α levels. (Panel **C**) Control, Ctr. (Panel **D**) Bmp2-cKO[ob]. High magnification (bar = 20 μm) of Hif1α levels in the pulp associated with the blood vessels. (Panel **E**) Control, Ctr. (Panel **F**) Bmp2-cKO[od]. High magnification (bar = 20 μm) showing Hif1α levels associated with the odontoblast layer. Note the disorganized odontoblasts in the Bmp2-cKO[od] and the permanent disorganized dentinal tubules (dt) in the Bmp2-cKO[ob].

Osterix was also reduced 60% in the 4-month-old continuously growing incisors (data not shown).

Dspp expression was reduced over 90% in the Bmp2-cKO[od] compared with control, 5-day 1st molars, indicating a failure in terminal differentiation (Figs. 2C, 2D) (n = 3). There was 60% reduced dynamic Dentin Formation Rate (DFR) as measured between 14 and 22 days, in Bmp2-cKO[od] mice, compared with control (Figs. 2E, 2F) (n = 2). A decrease in blood vessels in dental pulp of the Bmp2-cKO[od] animals was initially observed (Figs. 1F, 1G). The blood vessels in dental pulp provide at least one niche for dental pulp stem cells, and those in bone marrow do the same for skeletal stem cells (Gronthos *et al.*, 2002; Shi and Gronthos, 2003; Sacchetti *et al.*, 2007; Feng *et al.*, 2011). Osteoblast stem cells also move into developing bone along with invading blood vessels (Maes *et al.*, 2010a). CD146 expression is a marker for vascular-associated pericyte/stem cells, including bone marrow and muscle and neural tissue (Sacchetti *et al.*, 2007; Tormin *et al.*, 2011). CD146+ vascular-associated cells show all the required properties of a self-renewing stem cell population (Sacchetti *et al.*, 2007). CD146+ cells have also been proposed as dental pulp stem cells, or DPSC (Gronthos *et al.*, 2002; Shi and Gronthos, 2003). Using antibody to CD31, we observed a 50% reduction of blood vessels in the dental pulp of 5-day molars (Figs. 3A, 3B). Low magnification is shown in Appendix Figs. 3A and 3B. There was 90% reduction of total CD146+ cells in pulp in the Bmp2-cKO[od] compared with control and 70% actual reduction of CD146+ cells on blood

vessels. In many cases, in the Bmp2-cKO[od] blood vessels, we saw no positive CD146+ pericytes (Fig. 3D) compared with control (Fig. 3C) (n = 3 animals *per* group). Low magnification of these CD146+ results is shown in Appendix Figs. 3C and 3D.

VegfA (vascular endothelial cell growth factor A), a Bmp2-regulated protein, is a key factor in angiogenesis and blood vessel formation in bone marrow osteoblasts and dental pulp region (Deckers *et al.*, 2002; Gonçalves *et al.*, 2007; Zhang *et al.*, 2009; Maes *et al.*, 2010b). Bmp2 has been shown to promote proliferation and migration of endothelial cells through the VegfA/VegfR2 signaling pathway (Suzuki *et al.*, 2008). We observed a 65% reduction in VegfA mRNA expression signal (blue) in odontoblasts, as well as in a subset of dental pulp cells in the Bmp2-cKO[ob] (n = 3) (Figs. 3E, 3F). Low magnifications are given in Appendix Figs. 3E and 3F. By immunocytochemistry with a VegfA antibody (Figs. 3G and 3H; Appendix Figs. 3G and 3H, low magnification), we observed an 80% reduction of VegfA protein in the odontoblasts of the Bmp2-cKO[od] (n = 3).

Hif1α transcription factor expression is a sensitive read-out of hypoxic conditions, and the decreased blood vessels in dental pulp could potentially lead to hypoxia, which may affect terminal differentiation of the odontoblasts, as in bone (Araldi and Schipani, 2010) and in teeth (Amemiya *et al.*, 2003). VegfA is a direct transcriptional target for Hif1α that is activated by hypoxia. In the Bmp2-cKO[od] model, VegfA was actually decreased 60 to 70% in the odontoblasts, suggesting that hypoxia is not a major issue. We confirmed this hypothesis and, in fact, found Hif1α levels decreased in the pulp region associated with blood vessels and near the base of the pulp chamber in the Bmp2-cKO[od], with little or slightly decreased Hif1a levels in the odontoblast upper layer (Figs. 4A and 4B, overall low magnification; Figs. 4C and 4D, red immunostain in pulp and associated blood vessels; Figs. 4E and 4F, Hif1a immunostain associated with odontoblasts).

DISCUSSION

Deletion of the Bmp2 gene in early odontoblasts results in a permanent tooth phenotype in both the molars and incisors, with a decrease of dentin in the crown, and shows a more pronounced effect on root dentin. The quality of dentin is altered, with patchy unmineralized areas and dysmorphic dentinal tubules. There is a delay in amelogenesis in the absence of odontoblast Bmp2, but no overt change in the quantity of enamel. In the dental pulp, there are reduced blood vessels and associated pericytes. There appear to be no hypoxic conditions. The dentin and pulp phenotypes may be independent, but we hypothesize that the VegfA from terminal odontoblasts is required to link the terminal differentiation program to generation of new blood vessels and associated pericytes. These pericytes are at least one class of dental pulp stem cells, but are by no means the major niche in the context of incisor function (Feng *et al.*, 2011). The lack of blood vessels and associated pericytes most likely does not directly contribute to the major defects in dentin formation seen in the Bmp2-cKO[od]. There may be an overall link in a cycle of terminal differentiation and production of VegfA and other

unknown factors that play a role in blood vessel and pericyte formation. We summarize our results and model in Appendix Fig. 4, as 2 separate Bmp2-dependent pathways, linked by VegfA.

Why do Bmp2 and Bmp-4 differ in their individual roles in tooth cytodifferentiation? Previously, we reported that deletion of Bmp4 gene also leads to decreased terminal differentiation of odontoblasts (Gluhak-Heinrich *et al.*, 2010). However, the phenotype was milder in the Bmp4-cKO[od]. In Bmp2-cKO[od], we see no change in Bmp4 expression, suggesting that they are not linked and may signal through different receptor combinations (Cheifetz, 1999; Little and Mullins, 2009). The extent of interaction of Bmp4 and Bmp2 with extracellular heparin-sulfate proteoglycan and noggin-like inhibitor molecules may also play a role in the difference between how Bmp2 and Bmp4 function (Dutko and Mullins, 2011). Investigations with Bmp2 and Bmp4-cKO in bone and teeth are now being carried out to resolve some of these issues.

Thus, the primary mechanism for the decreased quantity and quality of dentin in the Bmp2-cKO[od] resides in the failure of functional odontoblasts to differentiate and to produce the necessary terminal products such as collagen type I and expression of Dspp and its products. At a morphological level, the odontoblasts in Bmp2-cKO[od] are not elongated and do not form well-polarized odontoblasts. Bmp2 in an autocrine fashion, produced by odontoblasts, directly signals to themselves and adjacent odontoblasts, drives differentiation, and activates the genetic program for proper dentin formation. A secondary consequence of this failure in function is the lack of the Bmp2-regulated gene product, VegfA. VegfA overexpression in bone marrow leads not only to new blood vessels and associated skeletal stem cells but also to massive new bone formation (Maes *et al.*, 2010b). We hypothesize that the lack of VegfA from odontoblasts leads to the loss of blood vessels in the pulp and associated pericytes. How much this vascular phenotype contributes to overall changes in quantity and quality of dentin is not known, but is worthy of further study.

ACKNOWLEDGMENTS

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