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Wnt5a regulates growth, patterning, and odontoblast differentiation of developing mouse tooth

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Abstract

Wnt/β-catenin signaling is essential for tooth development beyond the bud stage, but little is known about the role of non-canonical Wnt signaling in odontogenesis. Here we compared the expression of Wnt5a, a representative of noncanonical Wnts, with that of Ror2, the Wnt5a receptor for non-canonical signaling, in the developing tooth, and analyzed tooth phenotype in *Wnt5a* mutants. *Wnt5a* deficient mice exhibit retarded tooth development beginning from E16.5, leading to the formation of smaller and abnormally patterned teeth with a delayed odontoblast differentiation at birth. These defects are associated with upregulated *Axin2* and *Shh* expression in the dental epithelium and reduced levels of cell proliferation in the dental epithelium and mesenchyme. Retarded tooth development and defective odontoblast differentiation were also observed in *Ror2* mutant mice. Our results suggest that *Wnt5a* regulates growth, patterning, and odontoblast differentiation during odontogenesis, at least partially by modulating Wnt/β-catenin canonical signaling.

Keywords

Wnt5a; tooth development; patterning; growth

INTRODUCTION

Mammalian tooth development begins with determination of tooth forming sites and tooth types, followed by progression through distinct morphological stages, including the lamina, the bud, the cap, and the bell stages, to tooth root formation and tooth eruption. Reciprocal and sequential epithelial-mesenchymal interactions govern each step of tooth development, regulating growth, differentiation, and pattern formation (Zhang et al., 2005). Members of several growth factor families, including Bmp, Fgf, Hh, and Wnt, play pivotal in mediating such tissue interactions. During tooth development, these factors are often expressed in either the same tissue layer or in the adjacent one, and act synergistically or antagonistically to regulate gene expression. Different signaling pathways also often talk each other and form regulatory networks to control many aspects of tooth development.

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The Wnt signaling molecules have been implicated in the patterning, proliferation, and differentiation of a variety of organs and cell types during embryonic development (Cadigan and Nusse, 1997). Wnt proteins signal through the Frizzled trans-membrane receptors (Fz) and LRP5/6 co-receptors, activating either the β-catenin dependent canonical pathway or β-catenin independent noncanonical pathways (Veeman et al., 2003; van Amerongen and Nusse, 2009). The latter includes the planar cell polarity pathway and the Wnt/Ca2+ pathway. The "Wnt5a class" Wnt proteins, including Wnts -4, -5a, and -11, are classified as noncanonical Wnt family members and signals via noncanonical pathways. The noncanonical Wnt pathways have been known to inhibit the canonical Wnt/β-catenin signaling, possibly by promoting degradation of β-catenin in a Gsk3-independent manner (Topol et al., 2003).

Many lines of evidence have supported an essential role for the canonical Wnt signaling in tooth development (Liu and Millar, 2010). During determination of tooth forming site and tooth types at embryonic day 10.5 (E10.5), *Wnt7b* is expressed in the oral epithelium to interact with Shh signaling to set up the ectodermal boundaries between oral and dental ectoderm (Sarkar et al., 2000). From E11.5 on, several Wnt ligands, receptors, and effectors for the canonical Wnt signaling, including *Wnts -3, -4, -6, -7b, -10a, -10b*, and *Lef1*, are expressed in the developing mouse tooth germ (Kratochwil et al., 1996; Dassule and McMahon, 1998; Sarkar and Sharpe, 1999). Targeted inactivation of *Lef1* in mice leads to an arrest of tooth development at the bud stage, and overexpression of the canonical Wnt signaling inhibitor Dkk arrests tooth development at the lamina stage (Kratochwil et al., 1996; Andl et al., 2002). The absolute requirement of β-catenin in both the dental epithelial and mesenchymal compartments for early tooth development has been demonstrated recently by tissue specific inactivation of *Catnb*, the gene encoding β-catenin (Liu et al., 2008; Chen et al., 2009). In addition, mutations in *Wise* (*Ectodin*) that encodes an inhibitor of Wnt canonical signaling result in formation of supernumerary tooth in the diastemal region (Kassai et al., 2005; Ahn et al., 2010). Despite numerous studies on the Wnt/β-catenin signaling in tooth development, little is known regarding an involvement of noncanonical Wnt signaling in the regulation of odontogenesis.

Wnt5a is a representative of the noncanonical Wnts, having been shown to regulate convergent extension movement in vertebrates (Moon et al., 1993; Kilian et al., 2003), to inhibit secondary axis induction by *Wnt8* in *Xenopus* (Torres et al., 1996), and to block the canonical Wnt signaling in the developing limbs (Topol et al., 2003). Ror2, an orphan tyrosine kinase that serves as an alternative Wnt receptor, has been demonstrated to mediate Wnt5a-initiated noncanonical signaling and to be required for Wnt5a-mediated inhibition of the Wnt canonical signaling (Oishi et al., 2003; Mikels and Nusse, 2006). In the developing palate, Ror2-mediated Wnt5a noncanonical signaling regulates cell proliferation and directional cell movement (He et al., 2008). Additionally, the orhphan receptor Ryk was also shown to bind to Wnt5a as well as other Wnt ligands (Lu et al., 2004; Keeble et al., 2006). Interestingly, in the presence of Fz4 and Lrp5, Wnt5a can also activate Wnt/β-catenin signaling (Mikels and Nusse, 2006), suggesting a dual role for Wnt5a signaling in a receptor dependent manner.

Despite previous report on *Wnt5a* expression in the developing tooth (Sarkar and Sharpe, 1999), it has not yet been documented that if the absence of *Wnt5a* would cause defective tooth development. In the present study, we compared the expression of Wnt5a and its receptor Ror2 in the developing tooth and analyzed tooth phenotype in *Wnt5a* mutant mice. Consistent with a strong *Wnt5a* expression in the dental mesenchyme and differentiating odontoblasts, *Wnt5a* mutant teeth appear smaller and mis-patterned, and show a delayed odontoblast differentiation. Associated with the retarded tooth growth are reduced levels of cell proliferation in both dental epithelium and mesenchyme. Gene expression analyses

showed an upregulated expression of *Axin2* and *Shh* in the dental epithelium of *Wnt5a* mutants. Consistent with the expression of *Ror2* in the developing tooth and its role as Wnt5a receptor, retarded tooth development was also observed in *Ror2* mutants. Our results suggest that during tooth development, one of Wnt5a's roles is to modulate the Wnt/β-catenin canonical signaling, possibly mediated by Ror2.

RESULTS AND DISCUSSION

Expression of Wnt5a and its receptor Ror2 in the developing tooth

While *Wnt5a* expression in the early developing mouse teeth has been reported previously (Sarker and Sharpe, 1999), we re-examined *Wnt5a* expression, particularly at later stages that were not covered in the previous studies. Since Ror2 is known to mediate Wnt5a-initiated noncanonical signaling (Oishi et al., 2003; Mikels and Nusse, 2006), we also examined *Ror2* expression in parallel. Consistent with the previous report (Sarkar and Sharpe, 1999), *Wnt5a* expression was initially detected in the dental mesenchyme at E13.5 (data not shown), and remained in the dental papilla at E14.5 in both incisors and molars (Fig. 1A, 1D). At E16.5, *Wnt5a* transcripts were continuously present in the dental papilla of incisor and molar teeth (Fig. 1B, 1E). Strong expression was found in the papilla tissue adjacent to the forming secondary enamel knots in the molar germ (Fig.1B). At P0, *Wnt5a* expression was observed specifically in the differentiating odontoblasts in both molar and incisor (Fig. 1C, 1F), however, in the incisor, *Wnt5a* expression was also detected in the enamel epithelium including the cervical loop (Fig. 1F). While *Ror2* expression was reported previously in the molar at E16.5 (Schwabe et al., 2004), detailed expression patterns in the developing tooth have not yet been documented. We therefore examined *Ror2* expression in the developing molars at the bud, the cap, the bell, and the differentiating stages. Our in situ hybridization studies showed that at the E13.5 bud stage, *Ror2* is expressed in the dental epithelium and the condensed dental mesenchyme, with stronger expression in the tip of dental epithelium where the enamel knot will form (Fig. 2A). At the E14.5 cap stage, *Ror2* expression remains in both the epithelial and mesenchymal compartments, and again with a higher level of expression in the enamel knot (Fig. 2B). At E17.5 bell stage, *Ror2* transcripts were continuously detected in the dental epithelium and dental papilla (Fig. 2C). At P0, *Ror2* expression became downregulated in the developing tooth, but was still detectable in the inner enamel epithelium and the differentiating odontoblasts (Fig. 2D). The presence of *Ror2* expression in the developing tooth implicates a role of Wnt5a-mediated noncanonical signaling in tooth development.

Wnt5a mutant mice exhibit retarded tooth growth

Histological examination of developing tooth germs in *Wnt5a* mutants revealed normal tooth development until the E14.5 cap stage (data not shown). However, delayed development of tooth with obviously reduced size could be initially identified at E16.5, compared to the wild type control (Fig.3A, 3B). At P0, the mutant teeth became significantly smaller compared to the controls (Fig. 3C, 3D; Fig. 4A, 4B, 4E, and 4F). Decreased level of cell proliferation and induction of excessive cell death are two major cellular processes contributing to reduced size of a developing organ. Since the absence of *Wnt5a* alters the rates of cell proliferation but does not cause excessive cell death in the developing palate (He et al., 2008), we performed cell proliferation assays on $Wnt5a^{-/-}$ teeth by BrdU labeling. Our results demonstrated a significantly reduced level ($P < 0.01$) of cell proliferation in both the dental epithelium and mesenchyme at E16.5 compared to the littermate controls (Fig. 3E, 3F, 3G), consistent with a reduced size of *Wnt5a* mutant tooth at this stage. Gross examination of extracted *Wnt5a* mutant teeth at P0 revealed not only reduced size, but also deformed morphology (Fig. 4B, 4F). The mutant molars exhibited abnormally patterned cusps, including blunted cusps and lack of lingual 1 (L1) and distal (d) cusps (Fig. 3C), consistent

with the observations at histological level (Fig. 3C, 3D). Similarly, the incisors from mutants manifested an extremely reduced proximal-distal length and blunted end, compared to the controls (Fig. 4E, 4F). While BrdU labeling assay was not performed on incisors, given the similar *Wnt5a* expression pattern in both incisor and molar, we would assume that the reduced size of incisor could also be attributed to a decreased level of cell proliferation in the mutant incisor.

Since *Wnt5a*$^{-/-}$ mice develop a cleft palate phenotype and die a few hours after birth (He et al., 2008), we wondered if the reduced size and mis-patterned phenotype observed in *Wnt5*$^{-/-}$ teeth was simply a consequence of delayed development. In addition, *Wnt5a* mutant mice have truncated snout and mandible, which could potentially restrain tooth growth. To test these possibilities, we performed subrenal culture experiments by grafting incisor germs from E14.5 and molars from P0 of wild type controls and mutants, respectively. Similar to the defects identified at P0, grafted mutant teeth, after 4-week in subrenal culture, remained a smaller size and aberrantly patterned cusps, as compared to the wild type control (Fig. 4C, 4D, 4G, 4H). Thus, delayed development and hypoplastic mandible and maxilla do not represent the causatives of the reduced tooth size and dys-regulated tooth patterning in *Wnt5a* mutants. We conclude that *Wnt5a* regulates tooth growth and cusp patterning during odontogenesis.

Enhanced expression of Axin2 and Wnt/β-catenin signaling targeted genes in Wnt5a mutant tooth

Given the fact that *Ror2* expression overlaps with that of *Wnt5a* in the developing tooth and Ror2's function in mediating Wnt5a initiated noncanonical signaling to modulate the Wnt canonical signaling, we set to determine if the absence of *Wnt5a* would alter the activity of Wnt/β-catenin signaling in the tooth. It was shown previously that active Wnt/β-catenin canonical signaling is initially present in the dental epithelium before the bud stage, and then subsequently becomes restricted in the enamel knot which is known to serve as a signaling center to regulate tooth patterning (Liu et al., 2008). This Wnt/β-catenin signaling activity in the enamel knot and late in the dental epithelium was recently confirmed by an *Axin2-LacZ* reporter mouse line, although *Axin2* expression was also found in the dental mesenchyme and differentiating odontoblasts (Lohi et al., 2010). Since *Axin2* is a direct target and reliable indicator of Wnt/β-catenin signaling activity (Jho et al., 2002; Ontiveros et al., 2008; Wang et al., 2008), we chose to examine *Axin2* expression in the mutant tooth. We also examined the expression of several tooth developmental genes, particularly the tooth patterning genes *Shh* and *Fgf4* that are expressed in the enamel knot and are known to be regulated by the Wnt canonical signaling (Jernvall et al., 1994; Dassule et al., 2000; Gritli-Linde et al., 2002; Kratochwil et al., 2002; Liu et al., 2008). At E14.5 when the primary enamel knot forms in the wild type molar, we detected *Axin2* expression in the enamel knot (Fig. 5A), consistent with the observation in the previous report using the *TOPGAL* transgenic allele as the indicator of Wnt/β-catenin signaling and the *Axin2-LacZ* reporter line (Liu et al., 2008; Lohi et al., 2010). In *Wnt5a* mutant at the same stage, we observed an upregulated and expanded *Axin2* expression in the enamel knot area (Fig. 5B), indicating an enhanced Wnt/β-catenin signaling activity. The Wnt/β-catenin signaling acts as a positive upstream regulator of *Shh* and *Fgf4* expression in the enamel knot (Kratochwil et al., 2002; Liu et al., 2008). Coinciding with an increased Wnt/β-catenin signaling in the *Wnt5a* mutant enamel knot, we also found an upregulated level of *Shh* and *Fgf4* expression, as compared to their littermate controls (Fig. 5C–F).

Since *Wnt5a* expression is restricted in the dental papilla at this stage and the presence of *Ror2* in this tissue compartment, we also examined the expression of *Msx1*, which is expressed exclusively in the dental mesenchyme, and *Bmp4*, which is expressed in the dental mesenchyme and the enamel knot at this stage (E14.5) (Fig. 5G, 5I). Our results showed a

slightly reduced expression of these two genes in the dental papilla of the mutant (Fig. 5H, 5J). Interestingly, *Bmp4* expression in the enamel knot of mutants appeared to be up-regulated (Fig. 5J), suggesting differential gene expression regulation by *Wnt5a* in a tissue specific manner in the developing tooth. In fact, this differential regulation of *Bmp4* expression by *Wnt5a* has been observed in the palatal mesenchyme that has the same cranial neural crest origin as the dental mesenchyme (Chai et al., 2000; Ito et al., 2003; He et al., 2008). Slight downregulation of *Msx1* was also observed in the palatal mesenchyme of *Wnt5a* mutant (He et al., 2008). These results suggest a conserved role for *Wnt5a* in the regulation of *Msx1* and *Bmp4* expression in the developing tooth and palatal shelves.

Wnt5a$^{-/-}$ tooth exhibits a delayed odontoblast differentiation

During tooth development, odontoblast differentiation begins at the late bell stage, as assessed morphologically by polarized cell shape. In wild type control at P0, differentiated pre-odontoblasts were present at the tip of the cusp (Fig. 6A), and had begun to produce pre-dentin matrix molecules, including Dspp which could be strongly detected in the pre-odontoblasts but at a relatively low level in the ameloblasts as well (Fig. 6C). At the same time, the ameloblast differentiation marker *Amelogenin* is also strongly expressed in the polarized pre-ameloblasts (Fig. 6E). Since *Wnt5a* is exclusively expressed in the differentiating odontoblasts at this stage, we took a closer histological examination of the molars in *Wnt5a* mutants. In the mutants, while the odontoblasts indeed became elongated, they appeared to be relatively shorter in length but thicker in width, as compared to their wild type counterparts (Fig. 6B). Pre-dentin formation was not found in the mutant molar. However, the mutant ameloblasts appeared more or less comparable to the controls morphologically. Examination of differentiation markers revealed that in the mutant, while obvious *Dspp* expression could be observed in the pre-ameloblasts, the level of *Dspp* expression was extremely low in the odontoblasts (Fig. 6D), indicating a defective odontoblast differentiation. Consistent with the relatively normal morphology of the pre-ameloblasts, *Amelogenin* expression in the mutant was found at a level comparable to the controls (Fig. 6G, 6H). Histological examination of grafted teeth after 4- week subrenal culture, as shown in Fig. 2, revealed dentin formation in the mutants (Fig. 6H) comparable to the controls (Fig. 6G), indicating a delayed odontoblast differentiation in *Wnt5a* mutants.

We have demonstrated previously that Wnt5a functions through Ror2 to regulate cell proliferation in the developing palatal shelves (He et al., 2008). The reduced cell proliferation rate in the *Wnt5a* mutant tooth prompted us to examine tooth phenotype in *Ror2* mutant mice. Since it was reported previously that *Ror2* mutants exhibited normal size, shape, and number of molar at E16.5 (Schwabe et al., 2004), we examined *Ror2*$^{-/-}$ teeth at E17.5 and P0 (*Ror2* mutants die soon after birth due to a cleft palate defect). At E17.5, *Ror2*$^{-/-}$ molar appeared comparable to the littermate controls (data not shown). At P0, however, *Ror2*$^{-/-}$ molar exhibited retarded growth, as assessed by its smaller size, as compared to the wild type controls (Fig. 7A, 7B). Similar to the *Wnt5a*$^{-/-}$ molar, close examination revealed a lack of pre-dentin formation in *Ror2*$^{-/-}$ tooth at this stage, as shown in Fig. 7D. In line with this defect, the pre-odontoblasts, while polarized, appear relatively shorter as compared to the control (Fig. 7C, 7D). Interestingly, pre-ameloblasts in the mutant were also shorter than that in the control. Similarly, the mutant incisor also exhibited reduced length along the proximal-distal axis at P0, as compared to the control (Fig. 7E, 7F). These observations indicate retarded tooth growth and defective odontoblast and ameloblast development in *Ror2*$^{-/-}$ mice.

While *Wnt5a* expression is restricted in the dental mesenchyme and differentiating odontoblasts, its receptors are expressed in the dental epithelium and mesenchyme, suggesting an impact of *Wnt5a* on the development of both epithelial and mesenchymal compartments. Indeed, the absence of *Wnt5a* causes a significantly reduced level of cell

proliferation in the dental epithelium as well as the mesenchyme, which contributes to a retarded tooth growth. It has been demonstrated previously that Ror2 mediated Wnt5a signaling regulates cell proliferation in the developing palatal shelves (He et al., 2008). Given the fact that *Ror2* is expressed in both the dental epithelium and mesenchyme, and retarded tooth development is seen in *Ror2* mutants, it is conceivable that this Wnt5a/Ror2-rmediated cell proliferation regulatory pathway is conserved in the developing tooth, but at the late stage. This is because a retarded tooth development in *Ror2* mutants occurs later in development than that observed in *Wnt5a* mutant. Other receptors must play key role in mediating Wnt5a's signaling in the regulation of cell proliferation during early tooth development. While we do not know how *Wnt5a* regulates the expression of *Bmp4* and *Msx1* and what exact impact that the slightly down-regulated *Bmp4* and *Msx1* in the dental mesenchyme would have on tooth development in *Wnt5a* mutants, this slightly reduced *Msx1* expression may also contribute to the altered cell proliferation rate in *Wnt5a*$^{-/-}$ teeth, given the fact that the absence of *Msx1* causes significantly decreased level of cell proliferation in the palate and tooth (Zhang et al., 2002; Han et al., 2003). Nevertheless, it appears that the *Wnt5a-Bmp4-Msx1* regulatory pathway is also conserved in both developing palate and tooth (He et al., 2008; this study).

One function of Wnt5a-initiated noncanonical signaling is to inhibit the Wnt/β-catenin canonical signaling (Topol et al., 2003; Mikels and Nusse, 2006), which is known to be mediated by Ror2 (Oishi et al., 2003). Consistent with the expression of *Ror2* in the dental epithelium where active Wnt/β-catenin signaling is present, the absence of *Wnt5a* leads to an enhanced Wnt/β-catenin signaling activity in the dental epithelium, as evidenced by the upregulated *Axin2* expression. Along with the enhanced canonical Wnt signaling is the upregulation of *Shh* and *Fgf4* expression in the enamel knot. This is not a surprise because the canonical Wnt signaling is known to act upstream of *Shh* and *Fgf4* expression in the enamel knot (Kratochwil et al., 2002; Liu et al., 2008). Given the importance of Shh and Fgf4 in tooth patterning (Jernvall et al., 1994; Dassule et al., 2000; Gritli-Linde et al., 2002), the altered *Shh* and *Fgf4* expression in the enamel knot appears to be responsible for the patterning defects found in *Wnt5a* mutants.

The delayed odontoblast differentiation observed in *Wnt5a* mutant teeth could represent a cell autonomous effect. This is evidenced by the facts that *Wnt5a* is specifically expressed in the differentiating odontoblasts in mice and humans (Peng et al., 2010a; this study), and overexpression of *Wnt5a* was shown to promote differentiation of human dental papilla cells (Peng et al., 2010b). While the underlying mechanism warrants future investigation, the overlapped expression of *Ror2* with *Wnt5a* in the differentiating odontoblasts and similar defective odontoblast differentiation in both *Wnt5a* and *Ror2* mutants strongly support a role for Ror2 in mediating Wnt5a's role in the regulation of odontoblast differentiation. However, the possibility exist that altered expression of signaling molecules in the differentiating ameloblasts, such as an enhanced *Shh* expression (data not shown), in the absence of *Wnt5a* may exert regulatory effects on odontoblast differentiation. Despite a defective odontoblast differentiation, ameloblast differentiation appears normal in *Wnt5a* mutants. Thus Wnt5a acts on the enamel epithelium to regulate cell proliferation but does not have an impact on ameloblast differentiation.

In sum, our results presented here demonstrate a role for Wnt5a, a noncanonical Wnt signaling molecule, in the regulation of growth, patterning, and differentiation of tooth. Given its expression in the developing tooth and its property as the receptor for Wnt5s-initiated non-canonical signaling, Ror2 may participate in mediating Wnt5a's signaling in these processes by modulating, at least partially, the Wnt/β-catenin canonical signaling.

EXPERIMENTAL PROCEDURES

Animals

Wnt5a$^{+/-}$ mice (Yamaguchi et al., 1999) were obtained from the Jackson Laboratories, and *Ror2*$^{+/-}$ mice (Takeuchi et al., 2000) were provided by Dr. Yasuhiro Minami of Kobe University, Japan. PCR-based genotyping was performed to determine genotypes of mutant mice as described previously (Yamaguchi et al., 1999; Takeuchi et al., 2000). All animal studies were approved by the Tulane University Institutional Animal Care and Use Committee.

Histology, in situ hybridization, BrdU labeling assay, and subrenal culture

For histological and in situ hybridization analyses, embryos collected from timed pregnant females or tissue grafts were fixed in 4% paraformaldehyde (PFA) at 4°C for overnight, followed by dehydration, paraffin embedding and sectioning at 10-μm. Postnatal teeth (including the tooth grafts) were decalcified in 10% EDTA (pH 7.4) after fixation for 1 to 7 days depending on the ages. Sections were subjected to standard Hematoxylin/Eosin staining and non-radioactive in situ hybridization, as described previously (St. Amand et al, 2000). BrdU labeling assay was performed to determine cell proliferation rate, as described previously (Zhang et al., 2002). Timed pregnant mice were injected with BrdU at a dose of 1.5ml of labeling reagent/100 g body weight using the BrdU labeling and Detection kit II from Roche for 1 hour. Samples were fixed in Carnoy's fixative and paraffin sections were made at 5-μm. Three individual embryos of wild type and mutant were included, and three adjacent sections from each sample were counted for BrdU labeling. BrdU-positive cells were counted and were presented as percentage of labeled cells among total cells within a defined arbitrary area, and Student's *t*-test was applied to determine the significance of difference, as described previously. For subrenal culture, lower molars from P0 and lower incisor germs from E14.5 embryos were isolated respectively, and subjected for subrenal culture for 4-week as described previously (Zhang et al., 2003).

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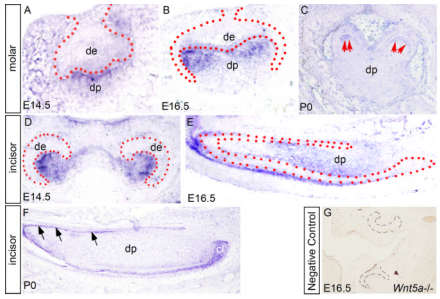

Figure 1.

Expression of *Wnt5a* in the developing tooth. (A, D) *Wnt5a* expression is detected by in situ hybridization mainly in the dental papilla of molar (A) and incisor (D) at E14.5. (B, E) Expression of *Wnt5a* is found in dental papilla of E16.5 molar (B) and incisor (E). Note in the molar (B) the expression is strongly localized in the areas immediately adjacent to the epithelial sites where the secondary enamel is forming. (C, F) At P0, *Wnt5a* expression is found restrictedly in the differentiating odontoblasts (arrows) of the molar (C) and incisor (F). *Wnt5a* expression is also detected in enamel epithelium, particularly in the cervical loop in the incisor at this stage (F). (G) Lack of *Wnt5a* expression in an E16.5 *Wnt5a* mutant molar, which serves as a negative control for in situ hybridization. All sections shown were made through coronal plane, except that in panels E and F were made through sagittal plane. Dot lines demarcate the boundary of dental epithelium. cl, cervical loop; De, dental epithelium; dp, dental papilla.

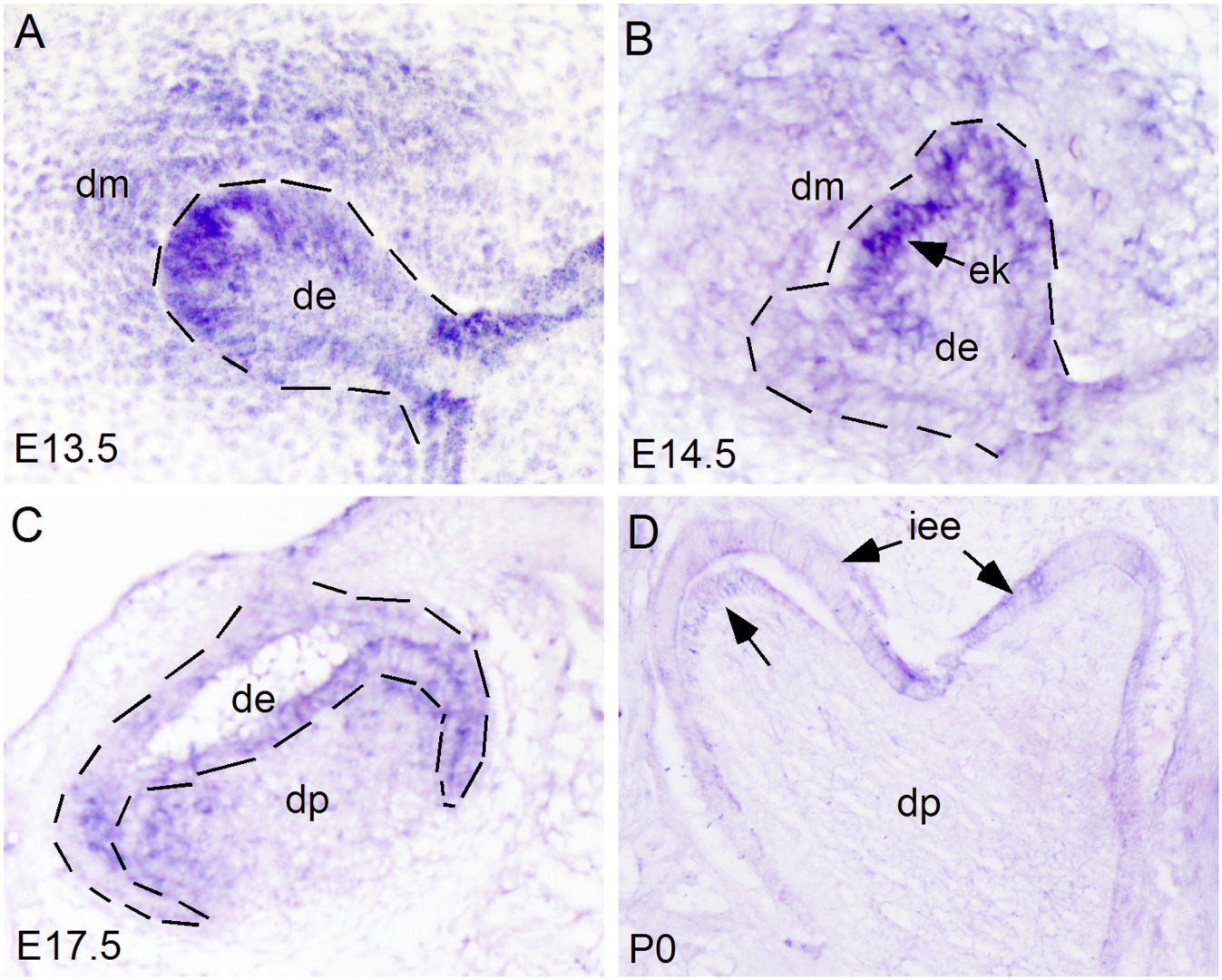

Figure 2.

Expression of *Ror2* in the developing molar. *Ror2* expression is observed in both the dental epithelium and dental mesenchyme at E13.5 (A), E14.5 (B), E17.5 (C), and P0 (D). Note strong *Ror2* expression in the site of future enamel knot at E13.5 (A) and the enamel knot at E14.5 (B). At P0 (D), *Ror2* expression becomes downregulated, but remains detectable in the inner enamel epithelium and the differentiating odontoblasts (arrow). Dot lines demarcate the dental epithelial boundary. de, dental epithelium; dm, dental mesenchyme; dp, dental papilla; iee, inner enamel epithelium.

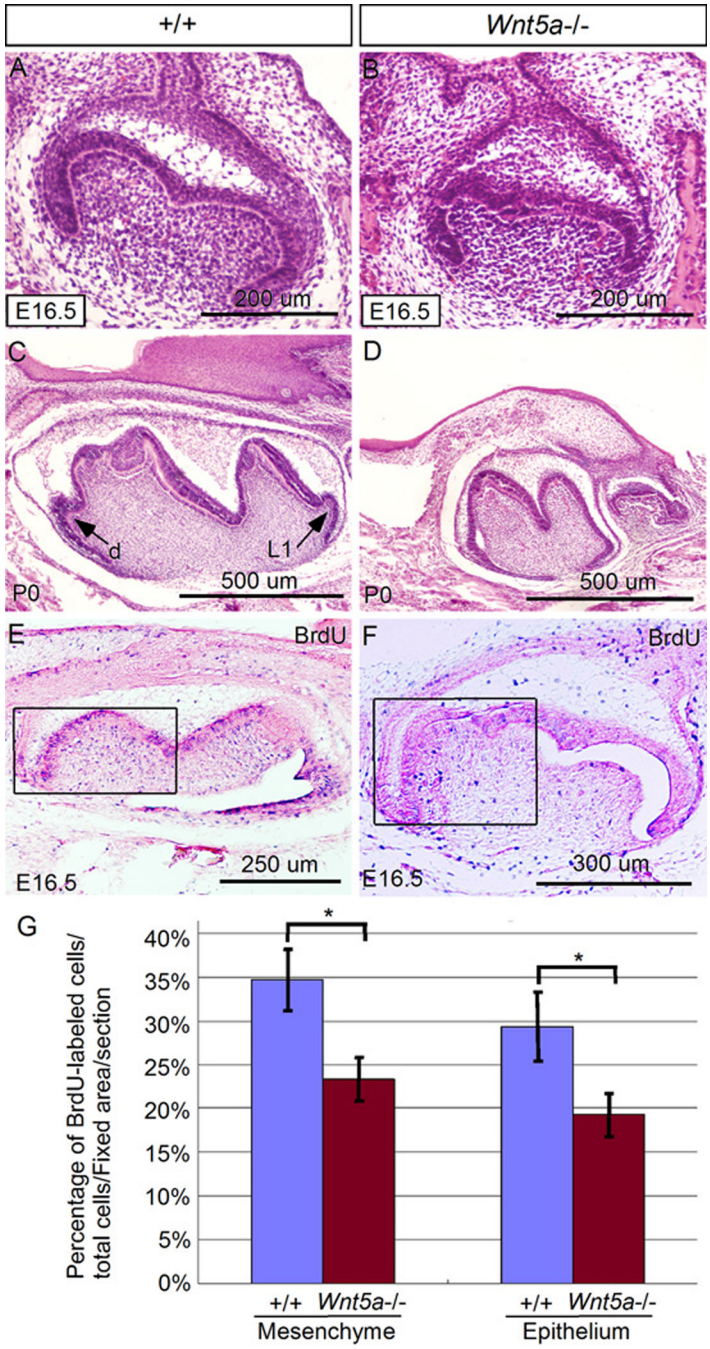

Figure 3.

Retarded tooth development and reduced cell proliferation rate in *Wnt5a* mutant. (A, B) Histological comparison of molars from the wild type control (A) and *Wnt5a* mutant (B) at E16.5 reveals a slightly smaller molar in the mutant. (C, D) At P0, the mutant molar (D) appears much smaller than its wild type counterpart (C). Note that the mutant molar has less and blunted cusps. The lingual 1 (L1) and distal (d) cusps are missing in the mutant molar. (E, F) BrdU labeling of wild type (E) and mutant molar (F) at E16.5. Boxes indicate the area where labeled cells and total cell number were counted and compared. (G) Comparison of percentage of BrdU-labeled cells in the fixed areas of molars from the wild type control and

Wnt5a mutant. Panels A and B are coronal sections, and panels C–F are sagittal sections. *: $P < 0.01$.

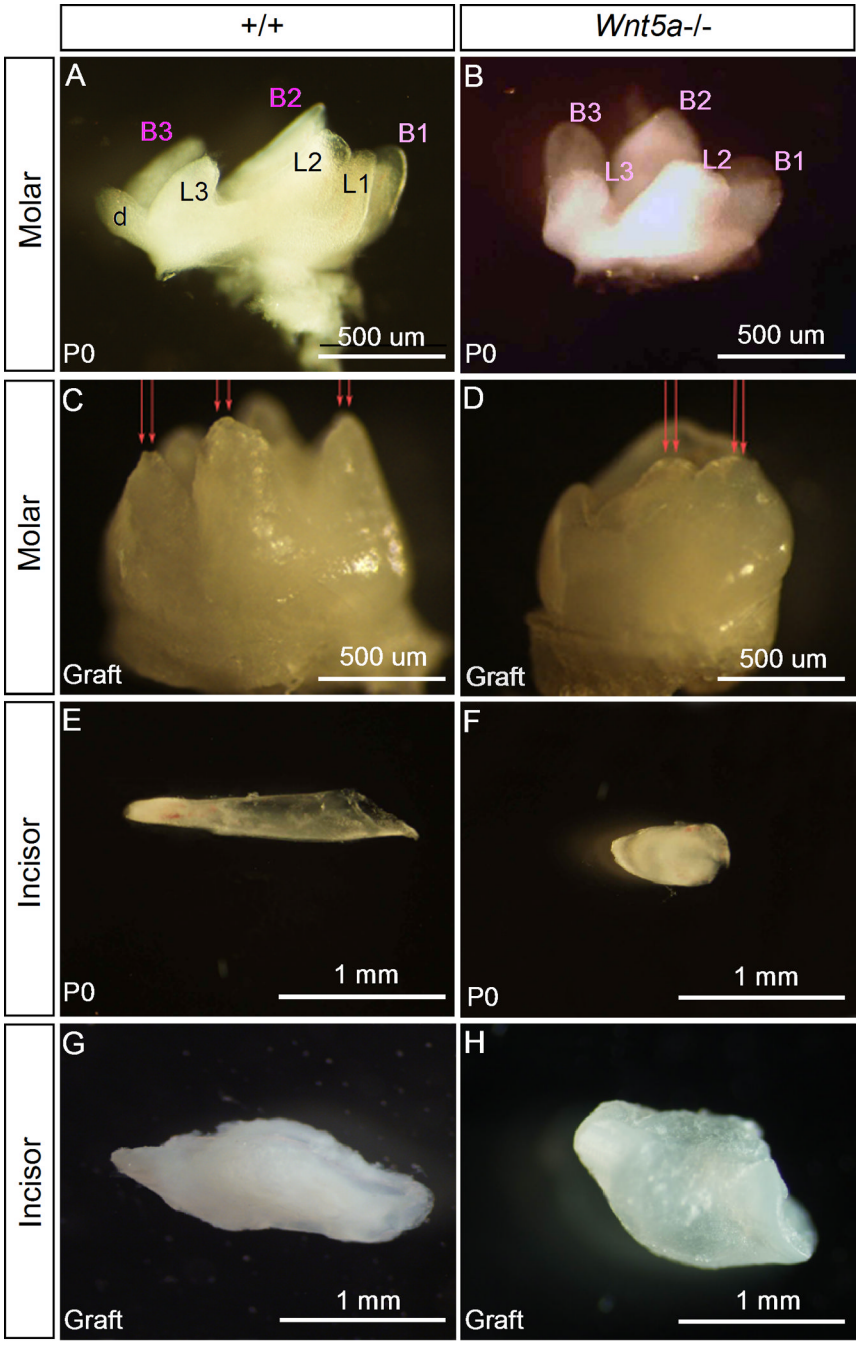

Figure 4.

Wnt5a is required for tooth development and patterning. (A, B) Whole mount view of the first molars from P0 wild type (A) and P0 *Wnt5a* mutant (B) reveals retarded tooth development and dis-regulated cusp patterning in the mutant. The mutant molar appears smaller and misses the lingual 1 (L1) and distal (d) cusps. All remaining cusps in the mutant are blunted. (C, D) After four weeks in subrenal culture, the mutant molar remains smaller and shows blunted cusps (arrows) (D), as compared to the wild type controls (C). (E–H) *Wnt5a* mutant incisor becomes largely shortened in proximal-distal length bluntly ended at P0 (F), and remains similarly morphologically after 4-week in subrenal culture (H), as

compared to the wild type controls (E, G). B1-3 represents buccal side cusps; and L1-3 indicate lingual site cusps.

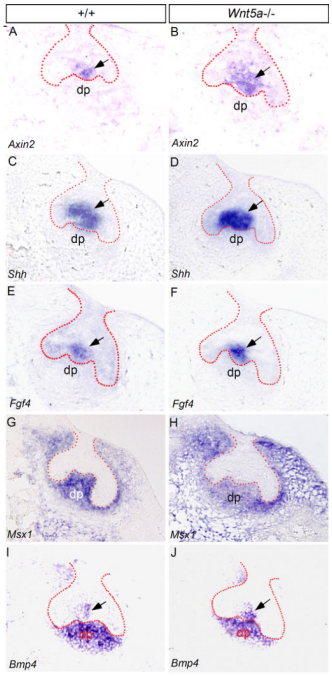

Figure 5.

Altered gene expression in *Wnt5a*$^{-/-}$ molar at E14.5. (A–F) In situ hybridization assays reveal up-regulated expression of *Axin2*, *Shh*, and *Fgf4* in the enamel knot of *Wnt5a*$^{-/-}$ molar (B, D, F), as compared to the wild type controls (A, C, E). (G–J) At the same stage, expression of *Msx1* and *Bmp4* is slightly reduced in the dental papilla of *Wnt5a*$^{-/-}$ molar (H, J), as compared to the controls (G, I). In contrast, *Bmp4* expression appears to be slightly up-regulated in the enamel knot of *Wnt5a* mutant (J). Dotted lines demarcate the boundary of dental epithelium. dp, dental papilla. Arrows point to the enamel knot.

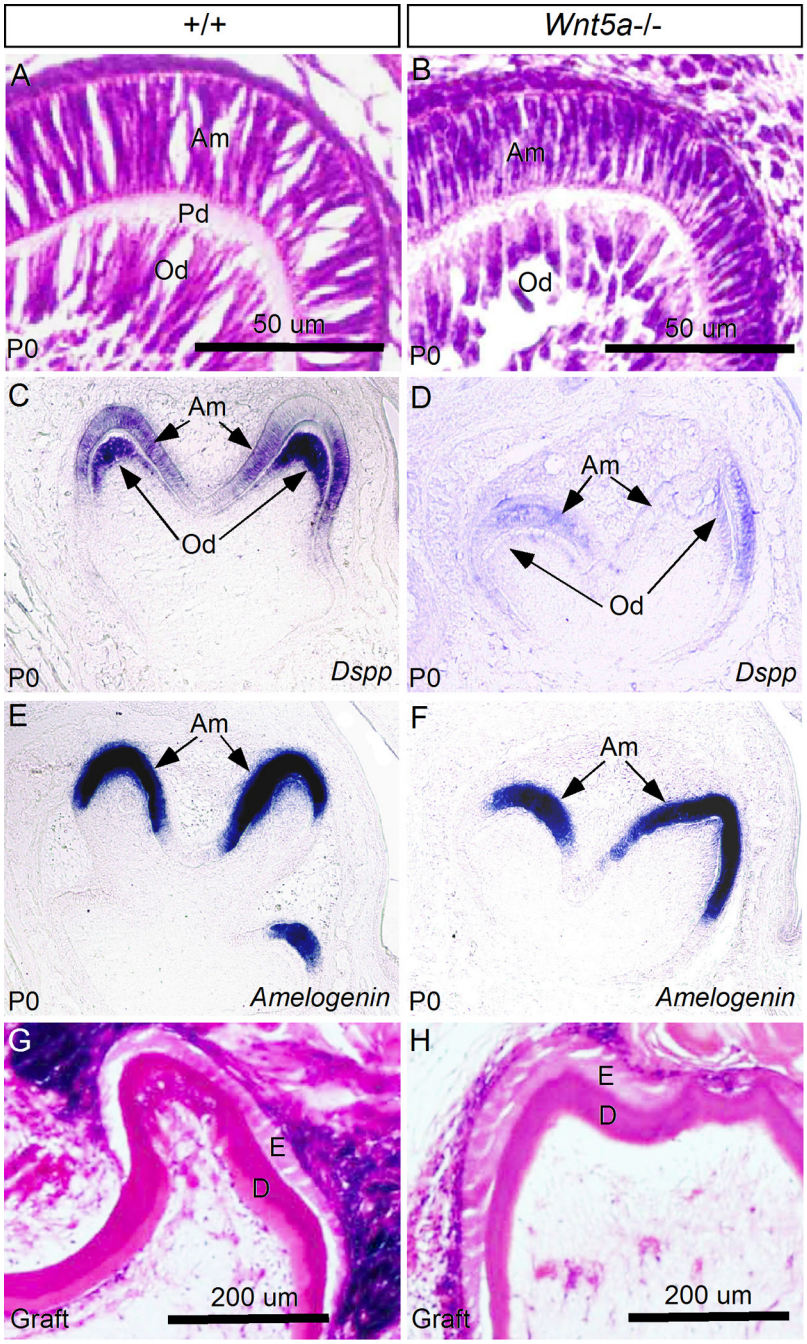

Figure 6.

Wnt5a$^{-/-}$ tooth displays delayed odontoblast differentiation. (A, B) Histological sections show abnormal morphology of differentiating odontoblasts and lack of pre-dentin formation in *Wnt5a*$^{-/-}$ molar at P0, as compared to the control (A). (C–F) In situ hybridization shows significantly down-regulated expression of *Dspp* in the differentiating odontoblasts but not the expression of *Amelogenin* in the ameloblasts in *Wnt5a*$^{-/-}$ molars (D, F), as compared to the wild type controls (C, E). (G, H) Histological sections through wild type (G) and *Wnt5a*$^{-/-}$ molar grafts (H) show deposition of dentin in both wild type control and mutant. D, dentin; E, enamel; Am, ameloblasts; Od, odontoblasts; Pd, pre-dentin.

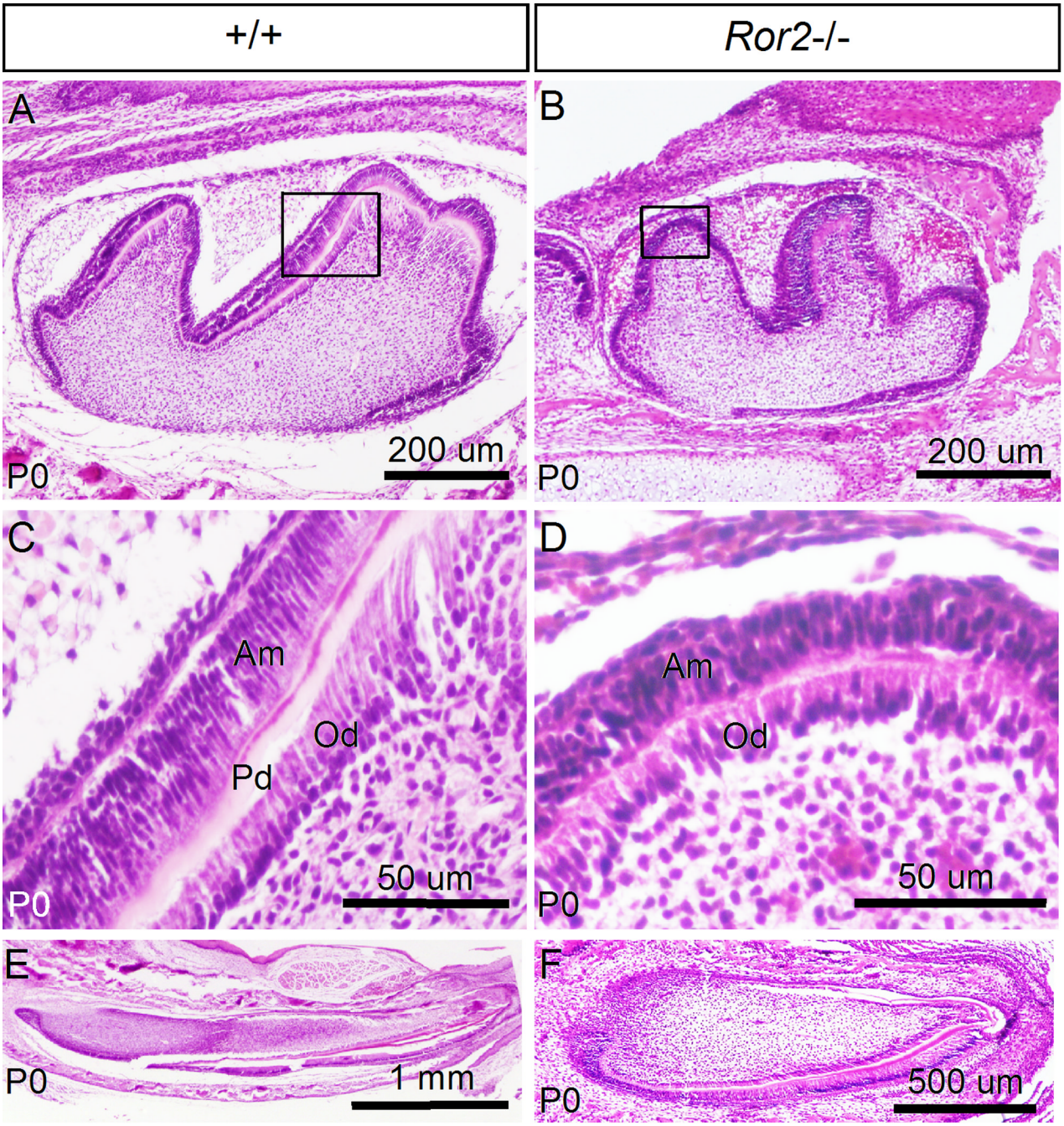

Figure 7.

Ror2$^{-/-}$ mice exhibits retarded development and defective differentiation of tooth. (A, B) Histological sections reveal retarded molar development in *Ror2* mutant at P0 (B), as compared to the control (A). (C) A higher magnification of wild type molar from the box in (A) shows formation of pre-dentin (Pd) and normal morphology of ameloblasts (Am) and odontoblasts (Od). (D) A higher magnification of *Ror2* mutant molar from the box in (B) shows absent pre-dentin formation and abnormal morphology of ameloblasts (Am) and odontoblasts (Od). (E, F) Sagittal sections reveal the normal size of a wild type incisor (E) and a shortened incisor in *Ror2* mutant (F) at P0.