Enamel Knots as Signaling Centers Linking Tooth Morphogenesis and Odontoblast Differentiation

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Abstract — Odontoblasts differentiate from the cells of the dental papilla, and it has been well-established that their differentiation in developing teeth is induced by the dental epithelium. In experimental studies, no other mesenchymal cells have been shown to have the capacity to differentiate into odontoblasts, indicating that the dental papilla cells have been committed to odontoblast cell lineage during earlier developmental stages. We propose that the advancing differentiation within the odontoblast cell lineage is regulated by sequential epithelial signals. The first epithelial signals from the early oral ectoderm induce the odontogenic potential in the cranial neural crest cells. The next step in the determination of the odontogenic cell lineage is the development of the dental papilla from odontogenic mesenchyme. The formation of the dental papilla starts at the onset of the transition from the bud to the cap stage of tooth morphogenesis, and this is regulated by epithelial signals from the primary enamel knot. The primary enamel knot is a signaling center which forms at the tip of the epithelial tooth bud. It becomes fully developed and morphologically discernible in the cap-stage dental epithelium and expresses at least ten different signaling molecules belonging to the BMP, FGF, Hh, and Wnt families. In molar teeth, secondary enamel knots appear in the enamel epithelium at the sites of the future cusps. They also express several signaling molecules, and their formation precedes the folding and growth of the epithelium. The differentiation of odontoblasts always starts from the tips of the cusps, and therefore, it is conceivable that some of the signals expressed in the enamel knots may act as inducers of odontoblast differentiation. The functions of the different signals in enamel knots are not precisely known. We have shown that FGFs stimulate the proliferation of mesenchymal as well as epithelial cells, and they may also regulate the growth of the cusps. We have proposed that the enamel knot signals also have important roles, together with mesenchymal signals, in regulating the patterning of the cusps and hence the shape of the tooth crown. We suggest that the enamel knots are central regulators of tooth development, since they link cell differentiation to morphogenesis.

Introduction

The terminal differentiation of odontoblasts is initiated during the bell stage of tooth morphogenesis at sites corresponding to the tips of the future cusps. Thereafter, differentiation proceeds as a gradient in the mesenchymal cells directly underlying the enamel epithelium. The odontoblasts derive from neural crest cells which migrate from the areas of midbrain and hindbrain to the frontonasal prominences and first branchial arch. These cells give rise to most connective tissues in the craniofacial region, including the bones of the calvarium, face, and jaws, as well as all components of the teeth, except the enamel, which is formed by epithelial ameloblasts. The epithelium covering the facial processes has a central role in the regulation of the fate of the neural crest cells after they have completed migration to the facial prominences. During these early stages, epithelial signals from oral ectoderm induce odontogenic potential in the mesenchymal cells and initiate tooth formation (Mina and Kollar, 1987; Lumsden, 1988; Sharpe, 2001). Our review paper deals with subsequent epithelial signals which regulate the determination and differentiation of the odontoblast cell lineage.

The odontogenic mesenchymal cells condense around the budding epithelium, and during the transition from bud to cap stage, mesenchymal cells nearest the tip of the epithelial bud give rise to the dental papilla. The dental papilla cells are the progenitors of odontoblasts, whereas the more peripheral mesenchyme forms the dental follicle, giving rise to periodontal tissues. The dental papilla subsequently grows rapidly in concert with the growth and folding of the enamel epithelium. Finally, those papilla cells which directly underlie the enamel epithelium differentiate into odontoblasts, whereas the rest of the dental papilla cells form the dental pulp (Thesleff and Nieminen, 2000).

Both the formation of the dental papilla and the terminal differentiation of the odontoblasts are regulated by epithelial-mesenchymal interactions. Curiously, both processes are also tightly linked with epithelial morphogenesis. The dental papilla forms during the transition from bud to cap stage at the site where the first convex epithelial folding occurs, and the terminal differentiation of odontoblasts is initiated where the epithelium folds into local convexities at the sites of the future cusps. In our laboratory, signaling centers were discovered at the sites of epithelial convexities, and we have studied their roles in the regulation of tooth morphogenesis and molar cusp patterns (Jernvall *et al.*, 1994, 2000). In the following, we shall discuss the associations of these signaling centers, called the enamel knots, with odontoblast determination and differentiation and the possible roles of the enamel knot signals in these processes.

Primary Enamel Knot and the Induction of the Dental Papilla

Although the enamel knot had already been discovered, more than 100 years ago, as a morphological structure in a cap-stage tooth, its role as a signaling center was realized only during the 1990s (Jernvall and Thesleff, 2000). The enamel knot cells express at least ten different signaling molecules, including Shh (sonic hedgehog) and several members of the FGF (fibroblast growth factor), BMP (bone morphogenetic protein), and Wnt families (Fig. 1; Vaahtokari *et al.*, 1996; Keränen *et al.*, 1998; Jernvall and Thesleff, 2000).

The function of the enamel knot is tightly linked with epithelial-mesenchymal interactions. It is formed by epithelial cells at the tip of the tooth bud and has differentiated fully by the cap stage. Its formation is regulated by signals from the mesenchyme, in particular BMP4. This was first demonstrated by *in vitro* bead experiments (Jernvall *et al.*,

Key Words

Enamel knot, signal molecules, epithelial-mesenchymal interactions, odontogenesis.

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1998) and, more recently, in elegant studies on *Msx1* mutant mice, in which enamel knots fail to form. The *Msx1* mutant dental mesenchyme does not express *Bmp4*, and tooth development is arrested at the bud stage. Interestingly, their tooth development could be completely rescued by BMP4 protein (Bei *et al.*, 2000).

Curiously, the enamel knot cells themselves do not divide, but apparently they stimulate the proliferation of nearby epithelial cells which form the cervical loops, as well as the mesenchymal cells forming the dental papilla. We have shown that FGFs expressed in the enamel knot can stimulate cell division in the enamel epithelium and the dental papilla (Jernvall *et al.*, 1994; Kettunen *et al.*, 1998). However, the enamel knot cells themselves do not express FGF receptors and thus are unable to respond to the mitogenic stimuli of FGFs (Kettunen *et al.*, 1998). Therefore, we have suggested that the lack of proliferation in the enamel knot, together with stimulated proliferation around it, regulates the epithelial folding and the transition of the bud to the cap stage (Jernvall *et al.*, 1994; Jernvall and Thesleff, 2000).

The dental papilla cells are first seen as a local morphological change within the larger mesenchymal condensate at the tip of the bud, next to the developing primary enamel knot. As the growth of the epithelial cervical loops continues, the dental papilla becomes encompassed by epithelium (Figs. 3A, 3B, 3D). So far, a few molecular markers have been identified which correlate with dental papilla development (http://bite-it.helsinki.fi). These include *Cbfa1 (Run x 2)*, a transcription factor which is essential for osteoblast differentiation and function. *Cbfa1* expression is regulated by the dental epithelium and by FGFs, presumably originating from the enamel knot (D'Souza *et al.*, 1999). *Cbfa1* is necessary for normal tooth morphogenesis, since in the *Cbfa1* mutant mice, only a rudimentary dental papilla forms, and the hypoplastic tooth germs are arrested at an aberrant cap stage. Interestingly, FGFs from the enamel knot also regulate the expression of *Fgf-3*, which is another marker of the dental papilla (Bei and Maas, 1998; Kettunen *et al.*, 2000). Tooth development is also arrested at the onset of the cap stage in the *Lef1* mutant mice, in which no dental papilla forms. In this mouse, the defect was localized in the dental epithelium, and it appears that some signal(s) in the enamel knot are regulated by the transcription factor Lef1 (Kratochwil *et al.*, 1996; Kratochwil, personal communication). More than ten signal molecules have so far been localized in the enamel knot (see http://bite-it.helsinki.fi), and it is probable that different signals have different functions. Recent evidence indicates that Shh is not required for the formation of the dental papilla, although its expression in the enamel knot is necessary for normal morphogenesis and cusp development in the tooth (Dassule *et al*, 2000; see below). So far, only FGFs have been linked with the formation of the dental papilla, and they may have a role in the determination of the dental mesenchymal cell lineage.

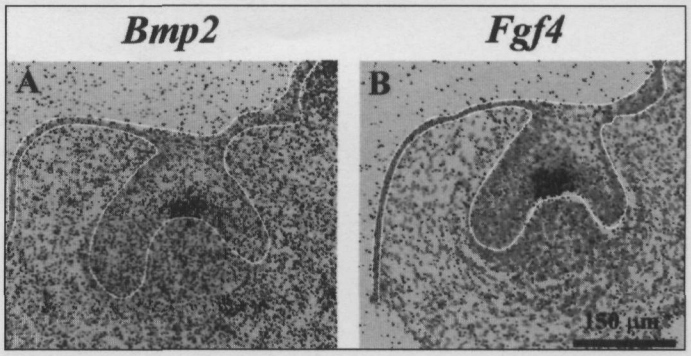

Fig. 1 — The primary enamel knots express several signaling molecules like *Bmp-2* and *Fgf-4*. *In situ* hybridization analysis of sections through cap-stage tooth germs.

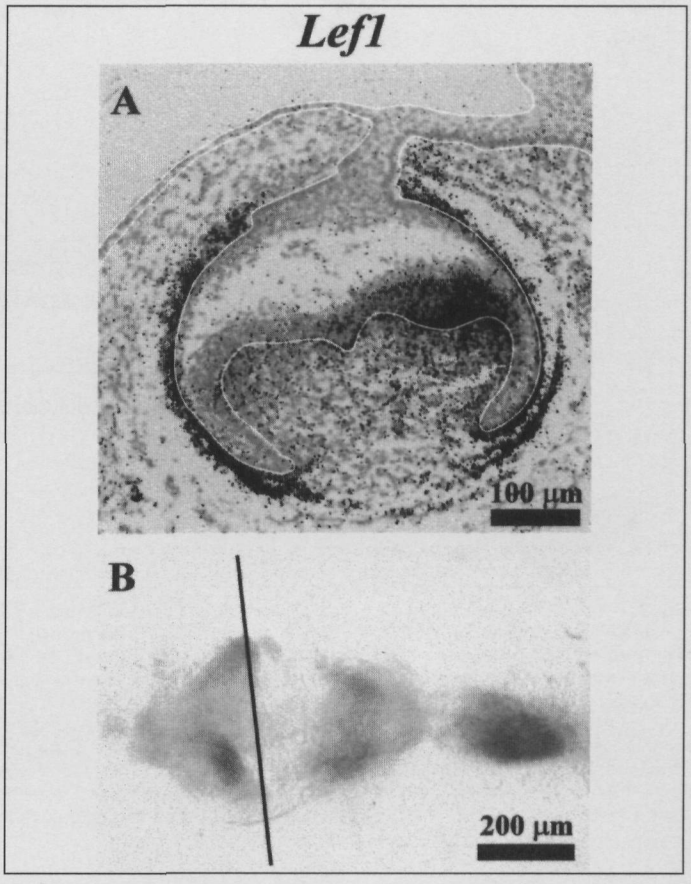

Fig. 2 — The secondary enamel knots express many signaling molecules in the FGF, BMP, hedgehog, and Wnt families. In addition, some transcription factors, including Lef1, are co-localized with the signals. (A) Tissue section of a bell-stage tooth germ showing Lef1 expression in secondary enamel knot. (B) Whole-mount *in situ* hybridization of the tooth germ demonstrates that the Lef1-expressing enamel knots correspond to forming cusps. This occlusal view shows four secondary enamel knots in the first molar and the primary enamel knot in the second molar (right). The line shows the location of the section in (A).

Secondary Enamel Knots and the Induction of Terminal Odontoblast Differentiation

In molar teeth, the primary enamel knot is removed by apoptosis for the most part, except for the region which gives rise to the first cusp (Jernvall *et al.*, 2000). Subsequently, new enamel knots appear exactly at locations corresponding to other cusps. These enamel knots were named secondary enamel knots, and they also express several signaling molecules. These include *Fgf-4,9*, *Shh*, *Wnt-10a,b*, and *Bmp-4,7* (Keränen *et al.*, 1998; Kettunen and Thesleff, 1998). As in the primary enamel knots, *p21*, the cyclin-dependent kinase inhibitor, as well as the transcription factors *Msx2* and *Lef1* are also expressed (Keränen *et al.*, 1998; Fig. 2). The secondary enamel knots correlate with cusp patterns as shown by comparison of the enamel knots and tooth crowns in mice and voles, two rodents with markedly different molar cusp patterns (Keränen *et al.*, 1998; Jernvall *et al.*, 2000). Quantitative comparisons of gene expression patterns and morphologies of the developing cusps by the GIS-mapping techniques (Geographic Information Systems) indicated that the signals in the enamel knots start to be expressed before any morphological change is observed in the epithelium (Jernvall *et al.*, 2000). Furthermore, the species-specific cusp positions are determined very early by spatial shifts in expression patterns of primary enamel knot signals (Jernvall *et al.*, 2000). That the secondary enamel knots are directly related to the development of cusps was shown by the analysis of the tooth

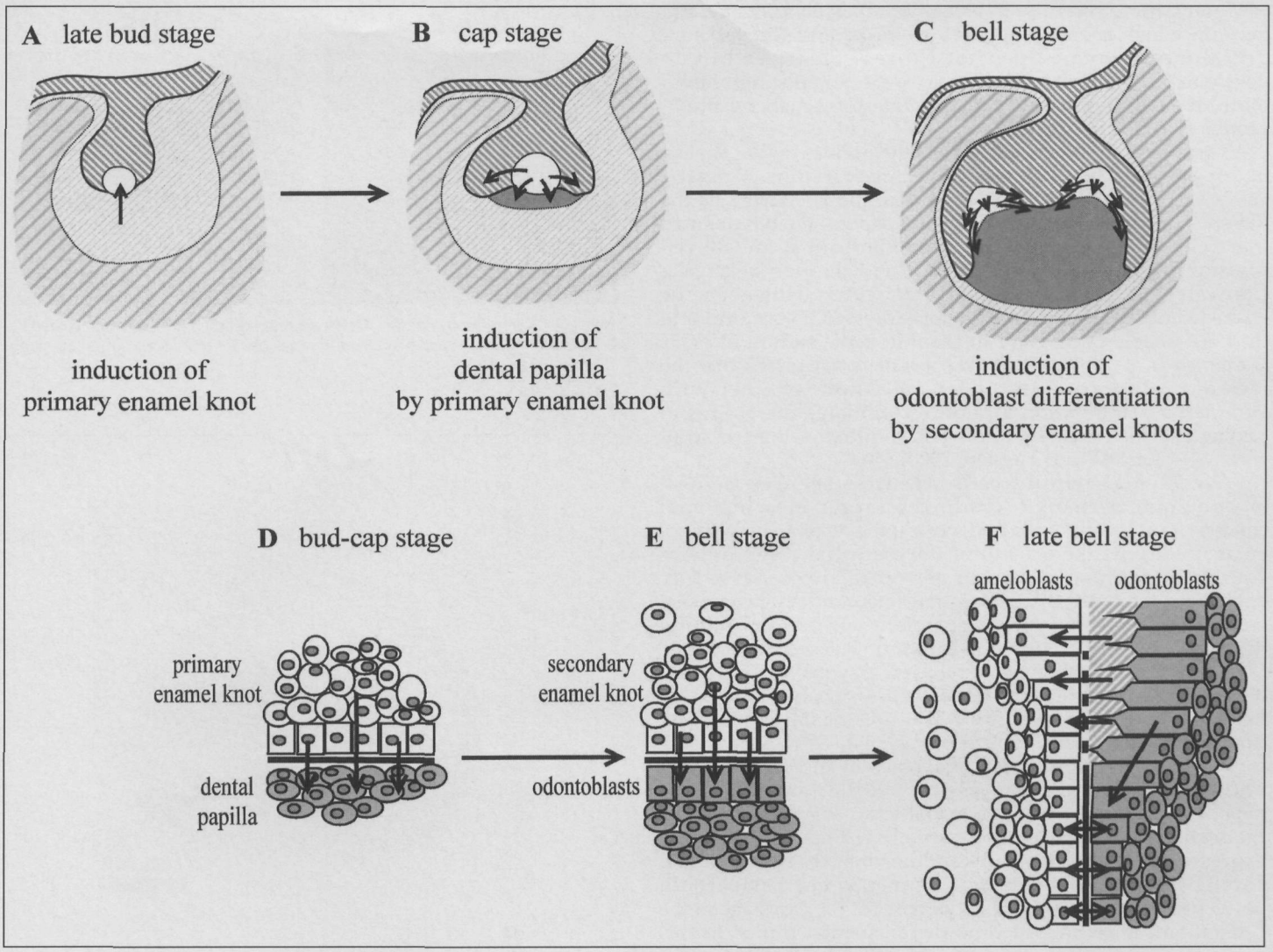

Fig. 3 — Schematic presentation of the association of enamel knot signaling with morphogenesis and odontoblast differentiation. (A) During the bud stage, the condensed odontogenic mesenchyme induces the formation of the primary enamel knot at the tip of the epithelium. (B) During the cap stage, the enamel knot expresses signaling molecules, which regulate the formation of the dental papilla and growth of the cervical loops of the epithelium. (C) During the bell stage, signals from the secondary enamel knots regulate the formation of cusps and may induce the initiation of terminal differentiation of odontoblasts. Differentiation proceeds toward the intercuspal areas and cervical loops. (D) Closer view of B shows the induction of dental papilla cells in the dental mesenchyme underlying the primary enamel knot. (E) Closer view of the cusp tip at the time of secondary enamel knot formation (stage slightly preceding that in C) shows the induction of odontoblast differentiation in the dental papilla cells underlying the secondary enamel knot. (F) Closer view of the odontoblast differentiation proceeding at the slope of the cusp (region of the vertical arrow in the left cusp in C). (After initiation of odontoblast differentiation at the cusp tip, the differentiation signals may come from the epithelium in which the expression of several enamel knot signals are spreading [arrows from left to right] and/or the signals may be relayed by differentiating odontoblasts [vertical arrow]. The odontoblasts secrete dentin and induce the terminal differentiation of ameloblasts [arrows from right to left].)

phenotype of *Tabby* mouse mutants, which lack the function of a tumor necrosis factor, ectodysplasin (Pispa *et al.*, 1999). The receptor of ectodysplasin, edar is expressed in the primary enamel knots (Laurikkala *et al.*, 2001), which are hypoplastic in the *Tabby* mutant teeth. Interestingly, most secondary enamel knots in *Tabby* mutant first molars are fused, resulting in correspondingly fused and fewer cusps as compared with wild-type mice (Pispa *et al.*, 1999).

The molecular pre-patterns, like those manifested by the enamel knots, are realized by the growth and differentiation processes which they control. The cusp patterns predicted by the secondary enamel knots are fixed by the terminally differentiated odontoblasts when they begin to deposit the mineralized dentin matrix. The terminal differentiation of odontoblasts always starts from the tips of the cusps, and proceeds in a cervical or intercuspal direction (for review, see Butler, 1956). Numerous studies during the last 50 years indicate that this differentiation is induced by the epithelium (Thesleff and Hurmerinta, 1981; Ruch *et al.*, 1995). Hence, the first odontoblasts differentiate from those dental papilla cells which directly underlie the secondary enamel knots (Figs. 3C, 3E). The timing of odontoblast differentiation is in line with a signaling function of the secondary knots, since the first alignment of the papilla cells under the epithelium is seen shortly after the folding of the epithelium has started. Many signals expressed in the secondary enamel knots have been previously associated with odontoblast differentiation based on expression patterns. Several members of the BMP and FGF families as well as Shh are expressed in the enamel epithelium (Åberg *et al.*, 1997; Keränen *et al.*, 1998; Kettunen and Thesleff, 1998).

In most cases, it is not known whether the signal proteins expressed in the epithelium actually cross the basement membrane and affect mesenchymal cells. However, the transfer of Shh protein from dental epithelium to mesenchyme was recently demonstrated by immunohistochemistry, and together with the observed induction of the expression of *Ptc*, a target of Shh signaling in dental mesenchyme, this indicates that, of the enamel knot signals, at least Shh affects mesenchymal cells (Gritli-Linde *et al.*, 2001). The requirement of Shh for tooth development was recently analyzed in mouse mutants in which

Shh activity had been removed by a conditional allele (Dassule *et al.*, 2000). The size and shape of teeth were affected, and the polarity and organization of the odontoblast as well as the ameloblast layer were disrupted. However, odontoblasts differentiated and formed dentin matrix. Hence, Shh, which is expressed in the dental epithelium first during initiation of budding, then in the primary enamel knot and subsequently in the secondary enamal knots and inner enamel epithelium, appears not to be necessary for the determination of the cell fate or for the differentiation of odontoblasts.

Interestingly, although the enamel knot signals are first restricted to the enamel knots, the expression of most of them spreads rapidly to the surrounding enamel epithelium (so far *Fgf-4* is the only signal strictly restricted to the primary and secondary enamel knots) (Jernvall *et al.*, 1994; Keränen *et al.*, 1998). Most enamel knot signals thus appear to be correlated with the progress of odontoblast differentiation. The advancing odontoblast differentiation could therefore be regulated by these or other epithelial signals, or by a lateral relay mechanism between the odontoblasts (Fig. 3F). However, in both cases, signals from the secondary enamel knots seem to determine the location and time of the onset of terminal odontoblast differentiation.

Concluding Remarks

At the cellular level, the differentiation of odontoblasts from the neural crest cells is a long process involving several intermediate steps. This is comparable with the process of osteoblast differentiation, which starts from stem cells differentiating into progenitor cells, pre-osteoblasts, and finally into terminally differentiated functional osteoblasts. Based on the intimate associations between tooth morphogenesis and odontoblast differentiation, as well as the fact that epithelial-mesenchymal interactions regulate various steps of morphogenesis, we propose that the key steps in the advancing differentiation within the odontoblast cell lineage depend on epithelial signals. First, early signals from oral ectoderm induce the odontogenic identity of the neural crest cells. Second, signals from the primary enamel knot at the late bud stage of tooth development induce the dental papilla cells, and finally, signals from the secondary enamel knots at the bell stage induce the terminal differentiation of odontoblasts at the cusp tips.

The morphogenesis of teeth is regulated by interactions between the epithelial and mesenchymal tissue components, and, because the differentiation of odontoblasts is intimately linked with tooth morphogenesis in space and time, it is conceivable that the same interactions regulate both processes. The enamel knots seem to offer a suitable mechanism for linking morphogenesis and cell differentiation. The exact roles of the different signal molecules expressed by the enamel knots have not yet been clarified, but we propose that they both pattern the folding morphogenesis of the epithelium from bud to bell stage and control its final realization by inducing odontoblast differentiation and thereby the formation of the dental hard tissues. The primary and secondary enamel knots express mostly the same signals, but there are some differences. For example, the strong *Bmp2* expression in the primary enamel knot is not seen in the secondary enamel knots during the early bell stage (Åberg *et al.*, 1997; Keränen *et al.*, 1998). This suggests functional stage-specific differences between the primary and secondary enamel knots, and these differences may also be involved in the step-wise differentiation of the odontoblasts. Likewise, the incisors have a primary enamel knot expressing the same signals as the molar primary enamel knots (our unpublished observations), and thus the regulation of dental papilla and cap formation seem to be similar in the different tooth families. Incisors do not have separate secondary enamel knots, but whether the expression profile of signals is changed in the incisor enamel knot in association of odontoblast differentiation, as in molars, is not known.

BMPs and FGFs, in particular, have been implicated as signals from the early ectoderm, inducing the odontogenic identity of the neural crest cells (Sharpe, 2001). Signals in these two families are also expressed in the enamel knots and are good candidates for signals acting at later stages of odontoblast determination. As noted above, FGFs from the primary enamel knot may be involved in the differentiation of dental papilla cells, since they stimulate the expression of *Cbfa1* and *Fgf-3*, two markers of dental papilla cells. FGFs and BMPs also regulate the terminal differentiation of odontoblasts in experimental studies (Bègue-Kirn *et al.*, 1992; Martin *et al.*, 1998; Lesot *et al.*, 2001; Tziafas *et al.*, 2001). It is noteworthy that FGFs and, in particular, BMPs also act as differentiation signals during several steps of osteoblast differentiation (Wozney, 1992; Erlebacher *et al.*, 1995; Rice *et al.*, 2000). Indeed, the extracellular mineralizing matrices of dentin and bone share many similarities, and it is likely that the regulation of differentiation of osteoblasts and odontoblasts may involve the same signaling molecules. However, these signals induce odontoblast differentiation only in odontogenic and not in osteogenic neural-crest-derived mesenchyme, and therefore the early steps in the determination of the odontoblast lineage during the initiation and morphogenesis of the individual tooth germs appear to be important in the regulation of their cell fate (Mina and Kollar, 1987; Lumsden, 1988). Because the enamel knot patterns of the individual teeth are also determined at these early stages (Mina and Kollar, 1987; Tucker *et al.*, 1998), the morphogenesis and cell differentiation in dental tissues may be more intimately linked than previously appreciated.

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