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# The life history of an embryonic signaling center: BMP-4 induces *p21* and is associated with apoptosis in the mouse tooth enamel knot

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## SUMMARY

**The enamel knot, a transient epithelial structure, appears at the onset of mammalian tooth shape development. Until now, the morphological, cellular and molecular events leading to the formation and disappearance of the enamel knot have not been described. Here we report that the cessation of cell proliferation in the enamel knot in mouse molar teeth is linked with the expression of the cyclin-dependent kinase inhibitor *p21*. We show that *p21* expression is induced by bone morphogenetic protein 4 (BMP-4) in isolated dental epithelia. As *Bmp-4* is expressed only in the underlying dental mesenchyme at the onset of the enamel knot formation, these results support the role of the cyclin-dependent kinase inhibitors as inducible cell differentiation factors in epithelial-mesenchymal interactions. Furthermore, we show that the expression of *p21* in the enamel knot is followed by *Bmp-4* expression, and subsequently by apoptosis of the differentiated enamel knot cells. Three-dimensional reconstructions of serial sections after in situ hybridization and Tunel-staining indicated an exact codistribution of *Bmp-4* transcripts and apoptotic cells. Apoptosis was stimulated by BMP-4 in isolated dental epithelia, but only in one third of the explants. We conclude that *Bmp-4* may be involved both in the induction of the epithelial enamel knot, as a mesenchymal inducer of epithelial cyclin-dependent kinase inhibitors, and later in the termination of the enamel knot signaling functions by participating in the regulation of programmed cell death. These results show that the life history of the enamel knot is intimately linked to the initiation of tooth shape development and support the role of the enamel knot as an embryonic signaling center.**

Key words: Epithelial-mesenchymal interaction, Odontogenesis, *p21*, *Bmp*, Bone morphogenetic protein, Mouse, Apoptosis

## INTRODUCTION

The morphogenesis of organs requires exact control of proliferation and differentiation of cells in time and space. This intricate control of patterns and shapes during organogenesis is well manifested in the evolutionary diversity of mammalian molar tooth shapes (Jernvall, 1995; Jernvall et al., 1996). Molar tooth shapes are created by different combinations of cusps, which are produced by unequal growth of the enamel epithelium accompanied by growth of the whole tooth germ. We have recently suggested that a transient epithelial structure, the enamel knot has a central function in the control of growth and patterning of the tooth cusps (Jernvall et al., 1994; Jernvall, 1995; Vaahtokari et al., 1996a).

The enamel knot appears during early tooth morphogenesis in the center of the tooth bud epithelium above the forming dental papilla. The enamel knot cells express several signaling molecules including Sonic hedgehog (Shh), Bone morphogenetic proteins – BMP-2, BMP-4 and BMP-7, as well as Fibroblast growth factor-4 (Fgf-4) (Vaahtokari et al., 1996a). As the same signals are expressed by well-studied vertebrate signaling centers, such as the notochord, the apical ectodermal ridge and the zone of polarizing activity, we have put forward a hypothesis that the enamel knot represents a signaling center for tooth morphogenesis (Vaahtokari et al., 1996a). However, the combination of expressed signaling molecules appear unique to the enamel knot. For example, the apical ectodermal ridge and the zone of polarizing activity in the developing limb each express a subset of the signaling molecules expressed in the enamel knot. In addition to the expression of the signal molecules, the cells of the enamel knot cease to proliferate (Jernvall et al., 1994). The cessation of cell proliferation is followed by the upregulation of *Fgf-4* expression in the enamel knot. In contrast, FGF-4 protein promotes cell proliferation in dental epithelia and mesenchymes in vitro (Jernvall et al., 1994). Therefore, the enamel knot appears to fulfill the minimum requirement for the control of tooth cusp development: it may cause the unequal growth of the enamel epithelium by concurrently remaining non-proliferative and by stimulating growth around it (Jernvall et al., 1994; Jernvall 1995; Vaahtokari et al., 1996a). Similar non-proliferative secondary enamel knots expressing *Fgf-4* are present at the sites of additional cusps in multicusped teeth (Jernvall et al., 1994; Jernvall 1995). However, the first (primary) enamel knot is present in all tooth types at the beginning of tooth shape development and may play a role in the determination of

secondary enamel knots by regulating the growth of the tooth crown base from which individual cusps develop.

Extensive programmed cell death (apoptosis) was recently reported in the enamel knot (Lesot et al., 1996; Vaahtokari et al., 1996b). Apoptosis has been traditionally identified as an important mechanism of embryonic development, classic examples being interdigital cell death (e.g., van der Hoeven et al., 1994) and death of the palatal midline epithelium (e.g., Mori et al., 1994). Apoptosis is also localized in the apical ectodermal ridge, suggesting that programmed cell death may be a general mechanism for silencing embryonic signaling centers (Vaahtokari et al., 1996b). However, molecular events leading to the formation and disappearance of embryonic signaling centers have not been previously described.

In this paper, we have further investigated the cellular and molecular basis of the formation and disappearance of the enamel knot. The molecular and morphological changes in developing teeth are very fast and the temporal and spatial order of events are not easily depicted from serial sections (Lesot et al., 1996). Therefore, we have reconstructed the order of events by using three-dimensional reconstructions of the tooth germs and the patterns of different cellular activities, allowing the identification of morphogenetically interesting aspects of molecular change.

We have monitored cell proliferation, programmed cell death and gene expression of *Bmp-4*, *Msx-2* and cyclin-dependent kinase inhibitor (CKI) *p21* (*p21*$^{Cip1/WAF1}$) in the mouse first lower molar. We were interested in the presence of *p21* activity in the enamel knot because it is known to inhibit cell proliferation at $G_1$/S transition (Steinman et al., 1994; Parker et al., 1995; Gorospe et al., 1996; Harper and Elledge, 1996). Moreover, previously *p21* has been shown to be involved in the terminal differentiation of muscle cells and also to associate with the cessation of cell proliferation in the apical ectodermal ridge of the limb bud (Steinman et al., 1994; Parker et al., 1995; Harper and Elledge, 1996). Similar mechanisms may be used in the developmental control of cell cycle in other animals (De Nooij et al., 1996). While *p21* can be considered a candidate molecule for the enamel knot cell differentiation, *Bmp-4* and *Msx-2* have been reported to be involved in apoptosis in rhombomeres and limbs (Graham et al., 1994; Yokouchi et al., 1996; Zou and Niswander, 1996; Macias et al., 1997) and may also be involved in the apoptosis of enamel knot cells.

*Bmp-4* also participates in epitheliomesenchymal induction of tooth development (Vainio et al., 1993). As tooth development is regulated by reciprocal epithelial-mesenchymal interactions (Thesleff et al., 1995; Thesleff and Nieminen, 1996; Maas and Bei, 1997), it is conceivable that the formation and disappearance of the enamel knot is controlled by these inductive tissue interactions. The enamel knot appears at the late bud stage prior to the formation of the dental papilla and tissue recombination experiments have shown that, during this stage, the potential to induce tooth formation resides in the dental mesenchyme (Kollar and Baird, 1969; Mina and Kollar, 1987; Lumsden, 1988). Hence, the enamel knot is likely to be induced by mesenchymal signals. To supplement the first part of this study, we have experimentally investigated the possible roles of mesenchymal signals in the regulation of the enamel knot. We carried out a set of in vitro experiments in which we studied the effects of the putative mesenchymal signal BMP-4 on the expression of several genes in isolated dental epithelia, including the putative differentiation factor *p21*.

## MATERIALS AND METHODS

### Teeth

The first lower molars were dissected from E13-E15 (vaginal plug = day 0) mouse embryos (CBA × NMRI) and fixed in 4% paraformaldehyde for in situ hybridization and TUNEL assay and in 70% ethanol for cell proliferation assay. The tissues were embedded in paraffin and serially sectioned. Because the stage of tooth development can vary considerably between individuals, the comparisons of teeth illustrated below are not necessarily from similar aged embryos. Instead, the comparisons were made between similar staged teeth.

### Cell proliferation and apoptosis assays

Cell proliferation was localized by mapping the distribution of S-phase cells. 1.5 ml/100 g 5-bromo-2′-deoxyuridine (BrdU, Boehringer Mannheim) was administered to etherized mice, which were killed after 2 hours and incorporated BrdU was localized in sections by immunohistological detection (Jernvall et al., 1994).

Apoptotic cells were localized by detecting DNA fragmentation. A digoxigenin-based modification of terminal deoxynucleotidyl transferase-mediated labeling (TUNEL) was used for histological sections (Vaahtokari et al., 1996b).

### In situ hybridizations

The RNA probes used were synthesized from a murine 285 bp *Bmp-4 Pst*I-*Eco*RI cDNA fragment inserted into the pGEM3 vector (Promega), from a murine 740 bp *p21 Eco*RI-*Eco*RI cDNA fragment inserted into the pBluescript SK(+/–) vector (Stratagene), from a murine 850 bp *Msx-2 Hin*dIII-*Bgl*II cDNA fragment inserted into the pSP72 vector (Promega), from a rat 2.6 kb *Shh* cDNA fragment inserted into the Bluescript SK+ vector (Stratagene) and from a 620 bp *Fgf-4* cDNA fragment inserted into the Bluescript vector (Stratagene). In situ hybridization for serial paraffin sections of dissected tooth germs and tissue explants (*Fgf-4*, *Shh* and *Bmp-4*) was performed as described by Vainio et al. (1993).

The tissues for whole-mount in situ hybridization were first fixed for 5 minutes in 100% methanol and then as described in Henrique et al. (1995). The in situ hybridization was done mainly as described by Henrique et al. (1995), but the tissues were treated with proteinase K (10 μg/ml in 37˚C for 5 minutes), refixed for 20 minutes in 4% PFA/0,2% GA, the hybridization buffer contained 0.1% Tween20, 1% blocking powder (Boehringer-Mannheim), 100 μg/ml yeast tRNA, 50 μg/ml heparin and both the prehybridization and hybridization were done at +54˚C. The probe concentrations were about 1 μg/ml. The post-hybridization washes were done as previously described (Henrique et al., 1995). The concentration of anti-digoxigenin/alkaline phosphatase in blocking solution was 1/2000. The antibody blocking was done overnight in 4˚C. After extensive washes (during the last three washes before the color reaction and during the color reaction, the tissues were also treated with 20 mM Levamisole), the color reactions in all explants in the same series were stopped at the same time.

### Three-dimensional reconstructions

Three-dimensional computer reconstructions were done of serial paraffin sections. Each section was digitized as a bright-field image using Macintosh PPC computers with Cohu 4912-5000 CCD (Cohu, CA, USA) camera and Scion LG-3 Frame Grabber card (Scion, MR, USA). Digitizing and reconstructions were done using the public domain NIH Image 1.61 program (US National Institutes of Health,

available from the Internet by anonymous FTP from zippy.nimh.nih.gov). Projections of BrdU-and Tunel-labelled cells, as well as in situ silver grains were made of aligned and inverted bright-field image stacks. The basement membrane was drawn directly onto the digitized images and rendered in Extreme 3D (Macromedia). Elevation and depth maps were prepared from rectified datasets.

### In vitro experiments

First mandibular molar tooth germs were dissected from day 13 (gene induction and apoptosis experiments) and day 14 (apoptosis experiments) mouse embryos (CBA × NMRI) and epithelium and mesenchyme were separated (Vainio et al., 1993). In order to improve the survival of isolated epithelia, a drop of Matrigel (Collaborative Biochemical Products, Bedford, MA, USA) was put on filters and a drop of Matrigel was also pipetted on top of the tissue. Affi-Gel blue agarose beads (BioRad) were incubated in 100 ng/μl recombinant BMP-4 and BMP-2 (Genetics Institute, MA, USA) or PBS for 30 minutes at 37˚C and placed in contact with the tissues. The explants were cultured in a Trowell-type organ culture in Minimum Essential Medium supplemented with 10% fetal calf serum, fixed after 24 hours and processed for whole-mount in situ hybridization (*p21, Msx-2, Shh*) or serial section in situ hybridization (*Shh, Fgf-4, Bmp-4*). To detect the effect of FGF-4 on apoptosis, epithelia with FGF-4 beads (25 ng/μl recombinant FGF-4 [Human sequence, British Biotechnology Products, UK] incubated with acrylic beads containing 1000 μg/ml heparin [Sigma]) were incubated with 75 ng/μl recombinant BMP-4 in the medium. Immunofluorescence detection (ApopTag, Oncor, MD, USA) was used for detection of apoptotic cells.

## RESULTS

### *p21* and initiation of the enamel knot

The first sign of the enamel knot is seen in the mesial (anterior) part of the epithelium in the bud-stage molar tooth germ. This was readily visible as an area of epithelium lacking cells that incorporate BrdU (Fig. 1) and transcripts of *p21* were present in the enamel knot area (Fig. 1). However, a closer examination of serial sections indicated that the upregulation of *p21* slightly precedes the cessation of cell proliferation. We have earlier shown that *Bmp-2* and *Bmp-7* as well as *Shh* are expressed in the enamel knot area at the bud stage (Vaahtokari et al., 1996a) but *p21* transcripts appear to correspond to the cessation of cell proliferation more closely. At this stage, *Bmp-4* transcripts are present only in the mesenchyme in the bucco-ventral side of the tooth bud (Fig. 1). *Msx-2* transcripts are present in the epithelium adjacent to the *Bmp-4* transcripts in the mesenchyme (Fig. 1).

### Apoptosis and *Bmp-4* expression begin in the fully formed enamel knot

At the cap stage, some 18 hours after its initiation, the enamel knot has enlarged and now extends to the distal (posterior) part of the tooth germ. The whole tooth germ has grown wider and the dental epithelium has begun to grow around the dental mesenchyme (dental papilla) thus manifesting the onset of crown (and cusp) formation. At this stage, the areas of *p21* expression and non-proliferating cells are at their maximum (Figs 1, 3). The domain of *p21* expression slightly exceeds that of BrdU-negative cells. Three-dimensional reconstructions reveal that *Bmp-4* transcripts, which were not present in the dental epithelium during the initiation of the enamel knot, have appeared exclusively in the distal half of the enamel knot (Figs 1, 3) and apoptotic cells are also present. The domain of *Bmp-4* expression closely matches the distribution of apoptotic cells which are also restricted to the distal half of the enamel knot (Figs 2, 3). In the mesenchyme, the *Bmp-4* transcripts continue to be present along the whole tooth germ (Fig. 1). The 3-D analysis of shape development reveals that epithelial cells are proliferating in areas of epithelium downgrowth (Figs 3, 4).

### The enamel knot disappears from back to front and forms the crown base

Some 8-12 hours after the previous stage (14.5 day embryo) the enamel knot has diminished to half its length (Fig. 3). The distal part of the knot has disappeared and no *p21, Bmp-4* or *Msx-2* transcripts are present in this area (Fig. 3). Furthermore, cell proliferation has resumed in the inner enamel epithelium in the distal part of the tooth germ (Fig. 3). This indicates that the distal half of the enamel knot, which is the last part to form, is the first half to disappear. The remaining half (mesial) of the enamel knot continues to express *p21* and *Msx-2* as well as *Fgf-4*, *Shh*, *Bmp-2* and *Bmp-7* (Vaahtokari et al., 1996a; unpublished observations). *Bmp-4* transcripts and apoptotic cells, which were first detected in the now missing distal half of the enamel knot, are now abundant in the distal part of the remaining enamel knot (Figs 2, 3). Thus, the termination of the enamel knot gradually proceeds in mesial direction and, by day 15 (cap/bell stage), it has disappeared almost completely (not shown). The 3-D analysis of shape development shows that the only portion of the epithelial-mesenchymal interface that grows up (towards the oral cavity) is the area of disappearing enamel knot (Fig. 4). Thus, the disappearance of the enamel knot establishes the tooth crown base and apoptosis can be presumed to be an integral part of this morphogenetic event.

### Induction of *p21* and *Msx-2* expression by BMP-4 in dental epithelium

The beads releasing BMP-4 and placed in contact with isolated dental epithelia stimulated the expression of *p21* in the epithelia (Fig. 5A). BMP-4-releasing beads are known to induce *Msx-2* in the dental mesenchyme (Vainio et al., 1993) and this we show to be the case in the E13 dental epithelia also (Fig. 5B). BMP-2-releasing beads had similar effects as BMP-4 beads (Fig. 5C,D). Compared to the induction of genes in the mesenchyme, the induced *p21* and *Msx-2* expression domains had a different appearance; only a portion of the epithelia expressed *p21* and *Msx-2* with usually no apparent gradient away from the bead. Furthermore, the domains of gene-expressing cells were sharply bordered by non-expressing cells (Fig. 5A-D) giving the appearance of epithelial ‘compartments’. In some cases, the positive areas did not continue into regions between the beads (Fig. 5B). This may indicate that the gene expression patterns in the isolated dental epithelium can mimic the in-vivo-like patterns (i.e., the enamel knot) and suggests that only a portion of the epithelium may respond to BMP signals or that the signals cause a generic organization of the epithelium. The localized induction of *p21* and *Msx-2* by BMP-4 resembled the effect of dental mesenchyme on epithelium, the effect of mesenchyme being, however, more limited (Fig. 5E,F). It is noteworthy that the mesenchyme is conceivably a source of several growth factors and thus the isolated epithelia may not

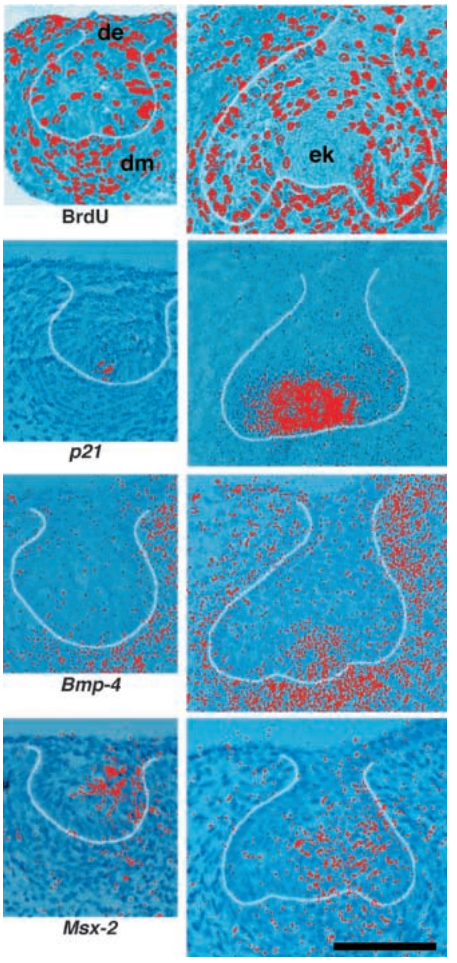


**Fig. 1.** Cell proliferation as detected by BrdU labeling and in situ hybridization analysis of the expression of *p21*, *Bmp-4* and *Msx-2* in the enamel knot. Left: initiation of the enamel knot (bud stage E13). Right: fully formed enamel knot (cap stage E14). Lack of cell proliferation is seen in the enamel knot, although intense cell proliferation is detected in other areas of the developing tooth. The expression domains of *p21* cover the enamel knot. *Bmp-4* is detected in the dental mesenchyme at bud stage and it appears in the distal part of the enamel knot at cap stage. *Msx-2* distribution extends beyond the enamel knot in the dental epithelium, facing the expression domain of BMP-4 in the mesenchyme. de, dental epithelium; dm, dental mesenchyme; ek, enamel knot. Scale bar indicates 100 μm. Basement membrane highlighted, buccal is toward the right.

respond the same as epithelia with mesenchyme. Almost every BMP-bead induced expression of *p21* (12 out of 13) or *Msx-2* (9 out of 10). Control beads soaked in PBS ($n$=12) had no effect on *p21* and *Msx-2* expression (Fig. 5G,H), and explants hybridized with the sense probes were negative in all cases (not shown). BMP-4 beads did not induce *Fgf-4*, *Bmp-4* or *Shh* expression in the epithelia (the two former detected using paraffin sections with radioactive in situ and the latter detected using both whole mount and paraffin sections, not shown).

### Effects of BMP-4 and FGF-4 on apoptosis in dental epithelium

In approximately one third of explants (10 out of 32), apoptosis

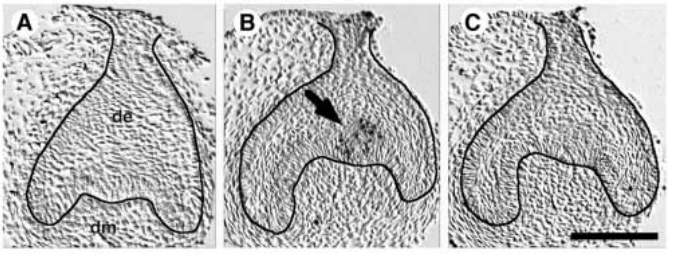


**Fig. 2.** Three sections of a late cap stage mouse molar (E14.5-15) showing apoptotic and non-apoptotic areas. The enamel knot has not yet disappeared in the most mesial end of the tooth germ (A) and no apoptosis is detected. Abundant apoptosis is visible in the enamel knot in the mid-portion of the tooth germ (arrow in B) and in the distal end of the tooth germ, no apoptosis and no enamel knot is detected (C). de, dental epithelium; dm, dental mesenchyme. Scale bar indicates 100 μm. Basement membrane is marked with a black line, buccal is toward the right.

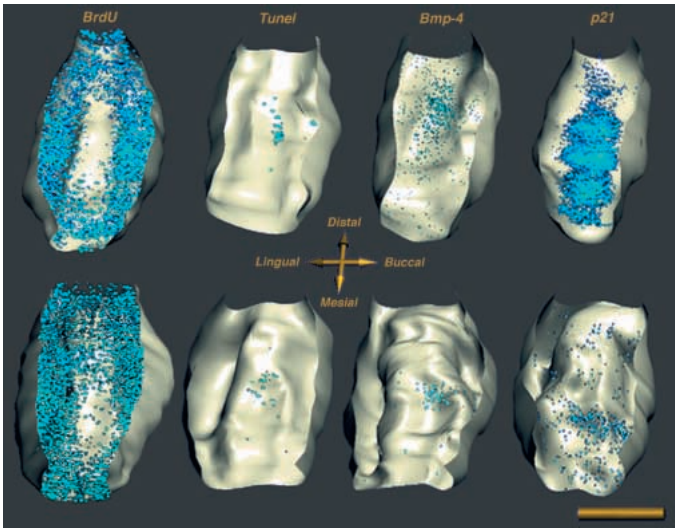


**Fig. 3.** Three-dimensional computer reconstructions of cap (top, E14) and late cap (bottom, E14.5-E15) stage mouse first lower molars showing the distribution of cell proliferation (BrdU), apoptosis (Tunel) and expression domains of *Bmp-4* and *p21*. The epithelial-mesenchymal interface (gray) is shown from above (the oral side). The mesial (anterior) ends are toward the observer; teeth are rotated 60˚ (counterclockwise) about the x-axis and 10˚ (clockwise) about the y-axis. The cap stage enamel knot is about 230 μm long and practically no incorporation of BrdU is detected in the knot cells. Note that the *p21* expression domain covers the whole extent of the enamel knot while *Bmp-4* expression and apoptosis are only detected in the distal portion of the knot. In the late cap stage (bottom), only the mesial portion of the enamel knot remains and *Bmp-4* transcripts and apoptosis are seen in its distal part. After the enamel knot disappears, the mesenchyme grows into the area previously occupied by the knot and forms the crown base. *Bmp-4* transcripts are present in the dental papilla mesenchyme and in the surrounding mesenchyme along the whole tooth germ in its buccal side (not shown). Scale bar indicates 200 μm.

was detected in a 5-cell-thick zone around the BMP-4-releasing beads and placed in contact with isolated dental epithelia (Fig. 6A). The remaining explants cultured with BMP-4 beads (Fig. 6B) showed only marginal or no increase

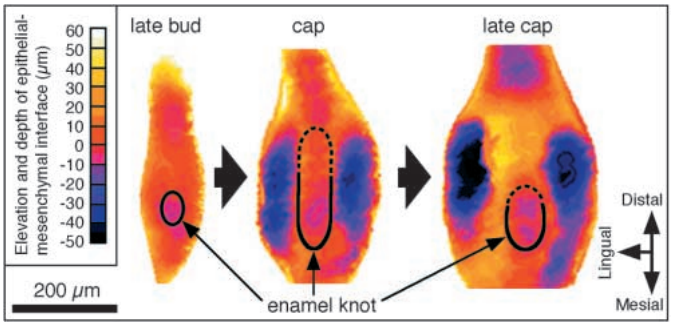


**Fig. 4.** Analysis of shape change in relation to the enamel knot in mouse first lower molar. Epithelial-mesenchymal interface is shown at a perpendicular angle from above, and the relative height differences are from the epithelial-mesenchymal interface underneath the mesial end of the enamel knot. Darker colors (depth) mark the downgrowth, lighter colors (elevation) mark the upgrowth of the epithelial-mesenchymal interface. At the late bud stage, the epithelial-mesenchymal interface is relatively flat and the tooth bud is widest in the mesial-end where the enamel knot is already present. By the cap stage, the epithelium has surrounded the dental papilla as a result of downgrowth (up to 40 μm) of both buccal and lingual protrusions (darker colors). The distal area of the enamel knot appear to be relatively stationary, even though some of the cells in the distal part of the knot (marked with hatched line) express *Bmp-4* and some are apoptotic. At the late cap stage, the distal part of the enamel knot has disappeared and the underlying papilla mesenchyme has grown up to replace the vacated space. This has resulted in the elevation of the epithelial-mesenchymal interface (up to 40 μm) and formation of the tooth crown base that the cusps will develop from. The enamel knot is still present in the mesial end of the tooth germ but in its distal end there is a thin, mesially moving, region of apoptosis and *Bmp-4* expression. The epithelial protrusions continue their downgrowth.

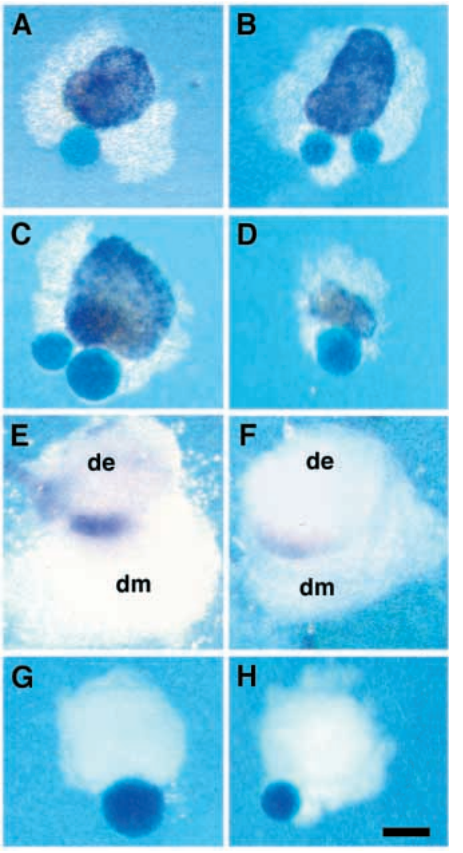


**Fig. 5.** Stimulation of *p21* (left) and *Msx-2* (right) expression in isolated dental epithelia by beads releasing BMP-4 and BMP-2 protein. The beads releasing BMP-4 stimulated a localized area of the epithelium to express *p21* (A) and *Msx-2* (B). The induced expression domains (purple-brown) were sharply delineated with no marked gradients. One expression domain was observed per explant, usually in the center region surrounded by non-expressing (white) epithelial tissue. BMP-2 beads (C,D) had a similar effect as BMP-4 beads. Dental mesenchyme stimulated small expression domains of both *p21* and *Msx-2* in the epithelia (E,F). Control beads (PBS) had no effect on *p21* or *Msx-2* expression in the epithelia (G,H). de, dental epithelium; dm, dental mesenchyme. Scale bar indicates 100 μm.

in apoptosis compared to tissues cultured with control beads. The control beads (10 explants) had only sporadic apoptotic cells around them (Fig. 6C). The explants cultured with BMP-4-releasing beads were smaller (mean difference 14%) than the control explants, indicating that BMP-4 affected the growth and survival of the epithelia. FGF-4 has been previously shown to stimulate cell proliferation in isolated dental epithelia (Jernvall et al., 1994) and the beads releasing FGF-4 also prevented epithelial apoptosis in the presence of BMP-4 in the media (Fig. 6D). The mesenchyme cultured in contact with the epithelium (Fig. 6F) prevented apoptosis in the vicinity of the mesenchyme, suggesting the presence of survival factors (like FGFs) in the mesenchyme.

## DISCUSSION

### Association of *p21* and *Bmp-4* with differentiation of the enamel knot

Two lines of evidence suggest that the cyclin-dependent kinase inhibitor (CKI) *p21* is involved in the differentiation of the epithelial enamel knot. First, the *p21* expression pattern in teeth closely correlates with the withdrawal of the enamel knot cells from the cell cycle. In fact, our comparisons of the 3-D reconstructions of serial sections indicated that the upregulation of *p21* expression slightly preceded the absence of BrdU incorporation. Hence, p21, which inhibits progression through the $G_1$ phase of the cell cycle (Harper et al., 1993; Elledge and Harper, 1994), is conceivably part of the molecular mechanisms leading to the cessation of cell division in the enamel knot

Second, *p21* expression in the epithelium was induced by the putative mesenchymal signal BMP-4. Because *p21* expression has been associated with early cell differentiation in several cell lineages (Elledge and Harper, 1994; Harper and Elledge, 1996), and because it also appeared to be a very early marker of enamel knot differentiation, it could be hypothesized that *p21* is regulated by differentiation signals. Earlier data from tissue recombination studies indicate that, at the time when the enamel knot is induced (the bud stage), the potential to induce tooth development resides in the mesenchyme (Kollar and Baird, 1969; Mina and Kollar, 1987; Lumsden, 1988; Thesleff et al., 1995). The most obvious putative signal that is expressed in the dental mesenchyme at the bud stage is

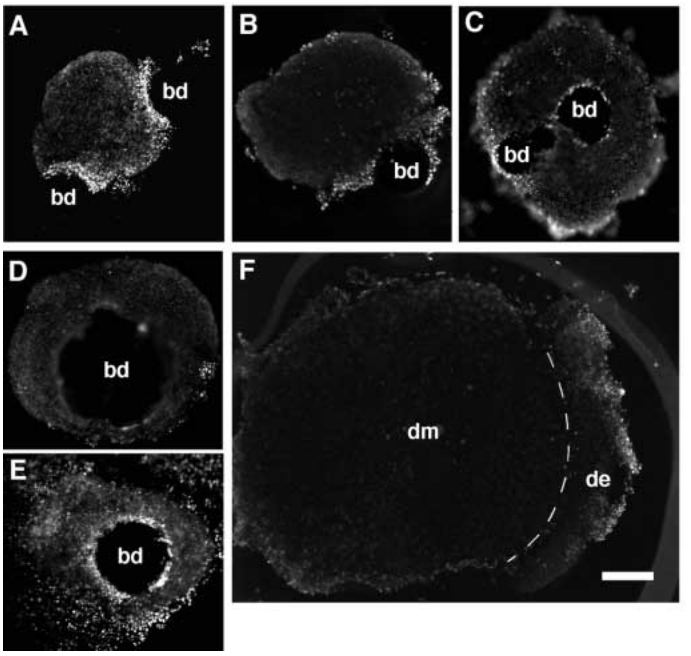


**Fig. 6.** Tunel analysis of the effect of beads releasing BMP-4 and FGF-4 on apoptosis. BMP-4 increased apoptosis in one third of the isolated epithelia (A). The remaining explants had only sporadic apoptosis (B) similar to that observed in the tissues cultured with control beads (C). FGF-4 prevented apoptosis (in the presence of BMP-4 in the media) and induced growth of the tissue resulting in the thickening of epithelium around the beads (D) while control beads did not (E). Epithelium recombined with mesenchyme lacked apoptosis in the area next to the mesenchyme, whereas apoptosis was detected in the peripheral epithelium (F). This indicates that, in addition to BMP-4, the mesenchyme is a source of factors improving the survival and growth of the epithelium (which BMP-4-releasing beads are not). de, dental epithelium; dm, dental mesenchyme; bd, bead. Scale bar indicates 100 μm.

BMP-4. Our findings indicate that BMP-4 is a potent inducer of *p21* in the enamel knot. Also BMP-2, which is expressed in the budding tooth epithelium, induced *p21* expression in the isolated epithelia. Therefore, it is possible that *Bmp-2*, or the combined effect of both *Bmp-2* and *Bmp-4* are needed for the induction of the enamel knot. However, different BMPs commonly have similar effects in experimental assays and the extent to which different BMPs substitute for each other in vivo is currently not known (see Hogan, 1996; Macias et al., 1997; Schultheiss et al., 1997). In addition, as BMP-4-releasing beads did not induce the expression of other early markers of the enamel knot, *Shh* and *Fgf-4*, in isolated dental epithelia, BMPs may not be sufficient signals for enamel knot induction in vivo. In any case, the very localized (almost enamel-knot-like) expression domain of *p21* in the isolated dental epithelia (Fig. 5) suggests a complex (e.g., differential epithelial competence or generic effects sensu Newman and Comper [1990]) induction event caused by BMP signals. As the centralized expression of *p21* was independent of absolute tissue size, a self organization in the epithelia is possible.

To our knowledge, this is the first demonstration that BMPs regulate the expression of *p21*. It is possible that this is a common mechanism in the mediation of the effects of BMPs on cell differentiation. BMPs regulate the differentiation of osteoblasts (Wozney et al., 1988) and odontoblasts (Bégue-Kirn et al., 1992) and have also been associated with ameloblast differentiation (Vainio et al., 1993). We have also detected *p21* expression in differentiating ameloblasts and odontoblasts (unpublished observations).

Previously, *p21* has been shown to be induced by transforming growth factor-β (TGF-β) (Datto et al., 1995). Interestingly, TGF-β has also been shown to change the proportions of CKI-cyclin-dependent kinase complexes by inducing particular CKIs (*p15* over *p27*; Reynisdottir et al., 1995). Since *Tgf-β1* is expressed in the bud stage tooth epithelium (Vaahtokari et al., 1991), it is possible that TGF-β1 is also involved in the modulation of CKIs in developing teeth.

*p21* is apparently not absolutely required for the formation of the enamel knot because mutant mice lacking the *p21* gene develop quite normally and no tooth abnormalities were reported (Deng et al., 1995). A likely explanation for this is that *p21* is functionally redundant with other inhibitors of cell proliferation. Indeed, several other CKIs are known (such as *p15*, *p27*, *p57*, for ref. see Harper and Elledge, 1996) which may be part of a developmental 'insurance policy' against loss of normal cell cycle control. Presently it is not known which of the CKIs are expressed in the enamel knot.

BMP beads also induced the expression of *Msx-2* in the epithelium. In vivo, the expression patterns of *Bmp-4* and *Msx-2* were strikingly correlated. *Bmp-4* was expressed in the mesenchyme underlying the buccal aspect of the budding dental epithelium, which expressed *Msx-2*. We have earlier shown that BMP-4 stimulates the expression of *Msx-2* in dental mesenchyme (Vainio et al., 1993), and the present results indicate that the same regulatory pathway may function from the mesenchyme to the epithelium.

Another BMP-4 target gene in the epithelium may be the transcription factor *Lef-1*, the expression of which was recently shown to be induced by BMP-4 (Kratochwil et al., 1996). Mutant mice lacking functional *Lef-1* have their molar tooth development arrested at bud stage (Van Genderen et al., 1994). Kratochwil et al. (1996) have shown that, for tooth morphogenesis to take place, *Lef-1* expression is required in the epithelium prior to the stage of the formation of the enamel knot. Moreover, the downstream effects of *Lef-1* are transferred from the epithelium to the mesenchyme. It is not currently known whether *Lef-1* acts upstream or downstream from *p21*, but it is possible that *Lef-1* is associated with the mesenchymal induction of the enamel knot (Kratochwil et al., 1996).

### Association of *p21* and *Bmp-4* with apoptosis of the enamel knot

The differentiated and functional state of the enamel knot cells lasts only about 24 hours. This functionally active phase of the enamel knot cells is reflected by the expression of several morphogenetic signals (Vaahtokari et al., 1996a), whereas the final fate of the enamel knot cells is apoptosis (Fig. 2; Vaahtokari et al., 1996b). This parallels the life history of another epithelial signaling center, the apical ectodermal ridge (AER), in the limbs. The AER controls limb outgrowth by stimulating cell proliferation (via FGF-4) in the underlying mesenchyme and it also participates in limb patterning via interactions with ZPA. This interaction includes complex regulatory loops between *Shh*, *Fgf-4* and *Hox*-genes

(Niswander et al. 1994; Tickle, 1995). Like the enamel knot, the cells of the AER do not proliferate, they express *p21* and *Msx-2* (Parker et al., 1995) and they also undergo apoptosis (Vaahtokari et al., 1996b). Moreover, *Bmp-4* is expressed in the AER (Roberts and Tabin, 1994; Tickle and Eichele, 1994). Therefore, *p21*, *Msx-2* and *Bmp-4* may be components of a common molecular machinery regulating apoptosis leading to the silencing of embryonic signaling centers.

The expression of *p21* continued in the enamel knot until the cells of the enamel knot disappeared by programmed cell death. Hence, an obvious question is the exact role of *p21* (and CKIs in general) in the enamel knot apoptosis. It has been well established that *p21* participates in the apoptotic pathway following damage by UV irradiation, and that it then acts downstream of the tumor suppressor gene *p53* (Liu and Pelling, 1995). However, *p21* is also expressed independently of *p53*, in particular in association with cell differentiation (Steinman et al., 1994; Parker et al., 1995; Harper and Elledge, 1996). For example, the activation of *p21* manifests the initiation of muscle cell differentiation (Parker et al., 1995). Therefore, *p21* does not necessarily participate the enamel knot apoptosis. Another possibility is that *p21* (and other CKIs) has a role in both enamel knot formation and apoptosis. Canman et al. (1995) have shown that activation of *p53/p21* after irradiation can have different outcomes depending on growth factors; irradiation of a murine hematopoietic cell line in the presence of growth factor *interleukin-3* (*IL-3*) induces $G_1$ arrest, while irradiation in the absence of *IL-3* results in apoptosis. This suggests that growth factors may modulate the function of CKIs. Indeed, FGF-4 suppresses apoptosis both in the dental epithelium (Fig. 6D) and mesenchyme (Vaahtokari et al., 1996b). Therefore it is possible that one function of FGF-4 in the enamel knot is to prevent its premature apoptosis. Thus the effects of *p21* in the enamel knot can depend on the molecular context which itself depends on the stage of development.

The pattern of expression of *Bmp-4* in the enamel knot differed clearly from those of *Bmp*-2, *Bmp-7, Shh*, *p21* and *Fgf-4. Bmp-4* transcripts appeared in the enamel knot later than the other genes, in the cap stage and then only in the distal part of the enamel knot. The expression of *Bmp-4* is strikingly associated with apoptosis in the enamel knot cells. The 3-D reconstructions (Figs 3, 4) depict how the enamel knot disappears soon after the upregulation of *Bmp-4*. Interestingly, *Bmp-4* was first associated with apoptosis in the neural crest cells of odd-numbered rhombomeres, where it was shown to induce apoptosis and to upregulate *Msx-2* expression (Graham et al., 1994). *Msx-2* transcripts are also present in the enamel knot (MacKenzie et al., 1992; Fig. 1). However, the expression pattern of *Msx-2* is more widespread in the dental epithelium than that of *Bmp-4* which is restricted to the distal part of the enamel knot. This supports the suggestion that BMP-4 may be the actual mediator triggering apoptosis (Graham et al., 1994). Recently, *Bmp-4* was also shown to be required for the apoptosis of interdigital tissue in the developing chick limbs (Yokouchi et al., 1996; Zou and Niswander, 1996) and has been also proposed to participate in apoptosis in mammary glands (Phippard et al., 1996). Hence, BMP-4 may be an autocrine regulator of embryonic programmed cell death in various tissues and *Msx-2* appears to be part of the apoptotic pathway. However, in our bead experiments, the beads releasing BMP-4 stimulated apoptosis in a third of the explants, which may indicate the need for additional factors (like upregulation of receptors and *Bmp-4* itself) in the initiation of the enamel knot cell apoptosis in vivo. As the apoptosis was induced only next to the beads, it may depend on concentration of BMP-4 (differing from that needed for the induction of *p21* and *Msx-2*).

We do not currently have an explanation for the biological role of the shorter life span of the distal part of the enamel knot. However, the same pattern holds for all mammalian lower molar enamel knots studied so far (Jernvall, 1995) and we hypothesize that the distal part of the enamel knot is an evolutionary novelty. Therian mammals evolved the distal part of their lower molars (talonid) as a gradual distal protrusion of the mesial part (trigonid) (e.g., Butler, 1990) and this more recent evolutionary origin might still be visible during the initiation of the enamel knot (Fig. 4). Also, the timing of formation and disappearance of the distal enamel knot may be a mechanism for modulating cusp patterns in molar teeth (Jernvall, 1995).

### BMP-4 signal is used repeatedly during tooth morphogenesis

*Bmp-2, Bmp-4* and *Bmp-7* have been associated with epithelial-mesenchymal interactions in several organs, including the teeth (Lyons et al., 1991; Vainio et al., 1993; Dudley et al., 1995; Liem et al., 1995; Thesleff and Nieminen, 1996; Bellusci et al., 1996; Hogan, 1996; Åberg et al., 1997). In teeth, *Bmp-4* is involved in the inductive interactions already prior to the formation of the enamel knot (Vainio et al., 1993). *Bmp-4* is first expressed in the dental epithelium during initiation of tooth development and the expression transfers to the dental mesenchyme during budding, at the time of the shift in the tooth inductive potential from epithelium to mesenchyme (Vainio et al., 1993). BMP-4 and BMP-2 recombinant proteins induce the expression of transcription factors *Msx-1* and *Msx-2* in the dental mesenchyme (Vainio et al., 1993). Mice lacking a functional *Msx-1* gene have their tooth development arrested at the bud stage (Satokata and Maas, 1994) and, in these mice, *Bmp-4* expression in the dental mesenchyme is also deficient (Chen et al., 1996; Maas and Bei, 1997). Thus, the arrest of tooth development at bud stage in the *Msx-1* null mutant mice may result from failure of the induction of the enamel knot by mesenchymal BMP-4. This is suggested by the rescue experiment in which the *Msx-1* null mutant tooth buds reach the cap stage when cultured in the presence of BMP-4 (Chen et al., 1996). Interestingly, *Msx-1* expression is unaffected in the *Lef-1* mutants, and thus *Lef-1* either acts downstream to *Msx-1* or in another pathway (Kratochwil et al., 1996; Chen et al., 1996).

In Fig. 7, we present a (simplified) model for the enamel knot life history illustrating the repeated use of the BMP-4 signal. We suggest that the inductive effects of dental epithelium on mesenchyme are first partly mediated by BMP-4-inducing expression of *Msx-1*, which subsequently is needed for the expression of *Bmp-4* in the mesenchyme, which in turn induces the formation of the enamel knot back in dental epithelium (perhaps via *p21*, other CKIs, *Lef-1* ; Fig. 7). Hence, BMP-4 is intimately associated with the shifts in inductive potential between epithelium and mesenchyme tissues. The final stage in the life history of the enamel knot is the expression of *Bmp-4* in the enamel knot cells themselves and their subsequent apoptosis.

Even though *Bmp-4* expression may play multiple roles during

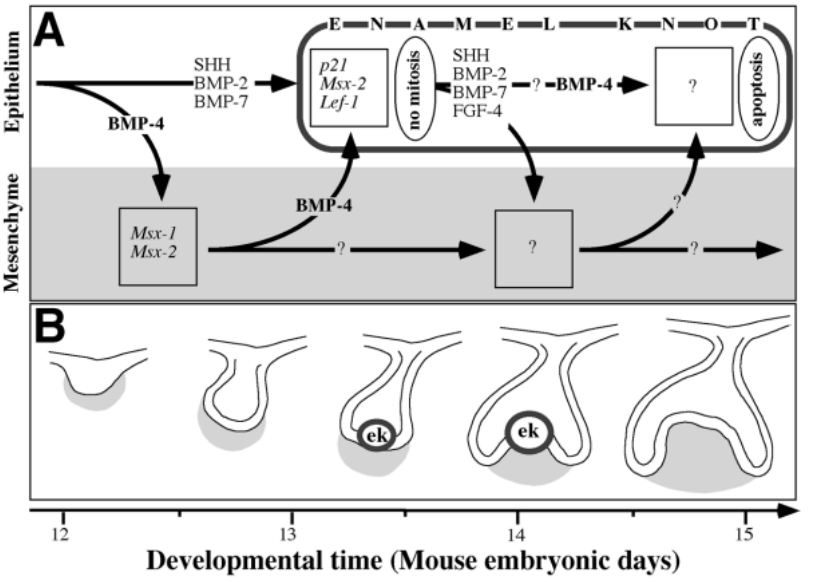


**Fig. 7.** Schematic model of the enamel knot life history. The molecular cascades are shown above (A) and corresponding morphological stages below (B). In A, curved arrows crossing tissues represent paracrine signals and horizontal arrows indicate autocrine signals. Note how BMP-4 (in bold) may have a role in both paracrine and autocrine signaling. The transcription factors known to be important for particular developmental stages are indicated in the squares. During initiation of tooth development, the epithelium exerts inductive influences on mesenchyme (first BMP-4 signal). During the bud stage (E13), mesenchymal-derived signals presumably induce the formation of the enamel knot (second BMP-4 signal) and the knot cells start to express *p21* and cease to proliferate. The signals expressed by the enamel knot cells may act both on mesenchyme and as planar signals within the epithelium (arrows). During the late cap stage, the enamel knot disappears by apoptosis (third BMP-4 signal). In reality, each signal-transcription factor cascade probably contains several nested molecular cascades. Illustration scheme adapted from Davidson (1993). Boxes represent intracellular signals, ovals cellular responses and arrows intercellular signals.

tooth morphogenesis, it may not be capable of inducing all the necessary signals in the epithelium, such as *Fgf-4*, *Shh* and *Bmp-4* (in fact *Bmp-4* expression appears so late in the enamel knot that it is unlikely to be induced by itself in vivo). Therefore, additional factors appear to be needed also for the enamel knot apoptosis. In teeth, the regulatory feedback loops between the different signals are likely to be complex (Fig. 7) because a greater number of expressed signaling molecules has been documented in the enamel knot than in other signaling centers (e.g., Vaahtokari et al., 1996a). While these molecular cascades are difficult to investigate experimentally, the precise association of *Bmp-4* transcripts to distinct morphological changes support multiple roles for *Bmp-4* in tooth crown development.

Taken together, BMP-4 signaling may be involved in at least three different regulatory changes during early tooth morphogenesis: the induction of the mesenchyme, the induction of the enamel knot and the apoptosis of the enamel knot. The repeated BMP-4 signals are apparently part of cascades of signaling events where cells respond by expressing new sets of transcription factors (e.g., *Msx*s and *Lef-1*), receptors and signals. Whatever the exact downstream target genes of BMP-4, it is remarkable that, while the biochemical functions of BMP-4 may be the same, the biological responses of the cells appear to be different, depending on the prevailing developmental context.

## CONCLUSIONS

Our study provides the first analysis of the life history of an embryonic signaling center by integrating morphological and molecular data. We show that early tooth shape development is a spatiotemporally dynamic process. To this end, these results show that signaling molecule BMP-4 appears to have several different developmental regulatory functions in the life history of the enamel knot. BMP-4 may function as a paracrine differentiation signal between the mesenchymal and epithelial tissue layers, inducing the formation of the enamel knot via upregulation of *p21* (and perhaps other CKIs, *Msx-2* and *Lef-1*). We hypothesize that *p21* is part of the mechanisms that allow cells of the enamel knot not to proliferate while expressing growth stimulatory *Fgf-4*. During more advanced development, in a different context, BMP-4 can participate in the regulation of apoptosis of the same cells perhaps by an autocrine mechanism involving the homeobox-containing transcription factor *Msx-2*. Data from other developmental systems suggest that these molecular pathways are widely used for developmental regulation, in particular during cell interactions controlling morphogenesis and cell differentiation, including apoptosis.

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