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Soile V.E. Keränen · Thomas Åberg · Päivi Kettunen
Irma Thesleff · Jukka Jernvall

Association of developmental regulatory genes with the development of different molar tooth shapes in two species of rodents

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Abstract While the evolutionary history of mammalian tooth shapes is well documented in the fossil record, the developmental basis of their tooth shape evolution is unknown. We investigated the expression patterns of eight developmental regulatory genes in two species of rodents with different molar morphologies (mouse, *Mus musculus* and sibling vole, *Microtus rossiaemeridionalis*). The genes *Bmp-2*, *Bmp-4*, *Fgf-4* and *Shh* encode signal molecules, *Lef-1*, *Msx-1* and *Msx-2*, are transcription factors and *p21*$^{CIP1/WAF1}$ participates in the regulation of cell cycle. These genes are all known to be associated with developmental regulation in mouse molars. In this paper we show that the antisense mRNA probes made from mouse cDNA cross-hybridized with vole tissue. The comparisons of gene expression patterns and morphologies suggest that similar molecular cascades are used in the early budding of tooth germs, in the initiation of tooth crown base formation, and in the initiation of each cusp's development. Furthermore, the co-localization of several genes indicate that epithelial signalling centres function at the three stages of morphogenesis. The earliest signalling centre in the early budding epithelium has not been reported before, but the latter signalling centres, the primary and the secondary enamel knots, have been studied in mouse. The appearance of species-specific tooth shapes was manifested by the regulatory molecules expressed in the secondary enamel knots at the areas of future cusp tips, whilst the mesenchymal gene expression patterns had a buccal bias without similar species-specific associations.

Key words Tooth morphogenesis · Evolution · Mouse · *Microtus rossiaemeridionalis* · Enamel knot

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S.V.E. Keränen (✉) · T. Åberg · P. Kettunen · I. Thesleff
J. Jernvall
Developmental Biology Program, Institute of Biotechnology,
Viikki Biocentre, P.O. Box 56, University of Helsinki,
FIN-00014, Finland

Introduction

The evolution of mammalian molar morphology is well documented in the fossil record, but while the evolution of molar shape has been shown to be influenced by environment (e.g. Hunter and Jernvall 1995; Jernvall et al. 1996), the developmental basis of evolutionary change is largely unknown (Jernvall 1995; Butler 1995; Thesleff and Nieminen 1996). The genetic basis of molar development has been primarily studied in mouse (*Mus musculus*) and these studies have elucidated some of the molecular mechanisms linked to shape development (Vaahtokari et al. 1996a; Thesleff and Nieminen 1996; Thesleff and Sharpe 1997; Maas and Bei 1997). While mouse has been a useful model organism in genetic research, studies comparing development of different molar morphologies, and thus different taxa, should be used to identify genetic pathways linked to the evolution of species-specific tooth shapes.

In this study, in order to explore the developmental mechanisms of tooth shape evolution, we compared the gene activities during shape development in the first lower molar of the house mouse with the first lower molar of the sibling vole (Arvicolidae, *Microtus rossiaemeridionalis*, formerly known as *M. epiroticus*). Based on immunological evidence, the mouse and vole lineages have been suggested to have diverged in the Miocene, about 20–25 million years ago (Nikoletopoulos et al. 1992), but the morphological and ecological radiation of voles began in the Pliocene (Guthrie 1965).

Both species have roughly the same body size, about as long a gestation time and equal numbers of teeth; three molars and one incisor in each jaw quadrant. However, these two species have very different molar shapes (Fig. 1). The first lower molar of mouse is low crowned and consists of two bucco-lingual pairs of cusps joined by transverse lophs and two fused anterior (mesial) cusps (the anteroconid; Fig. 1). A small, distal cusp (the hypoconulid) is usually present. In contrast, the first lower molar of vole is high crowned and ever growing (hypselodont). Individual vole cusps are prismatic in shape but

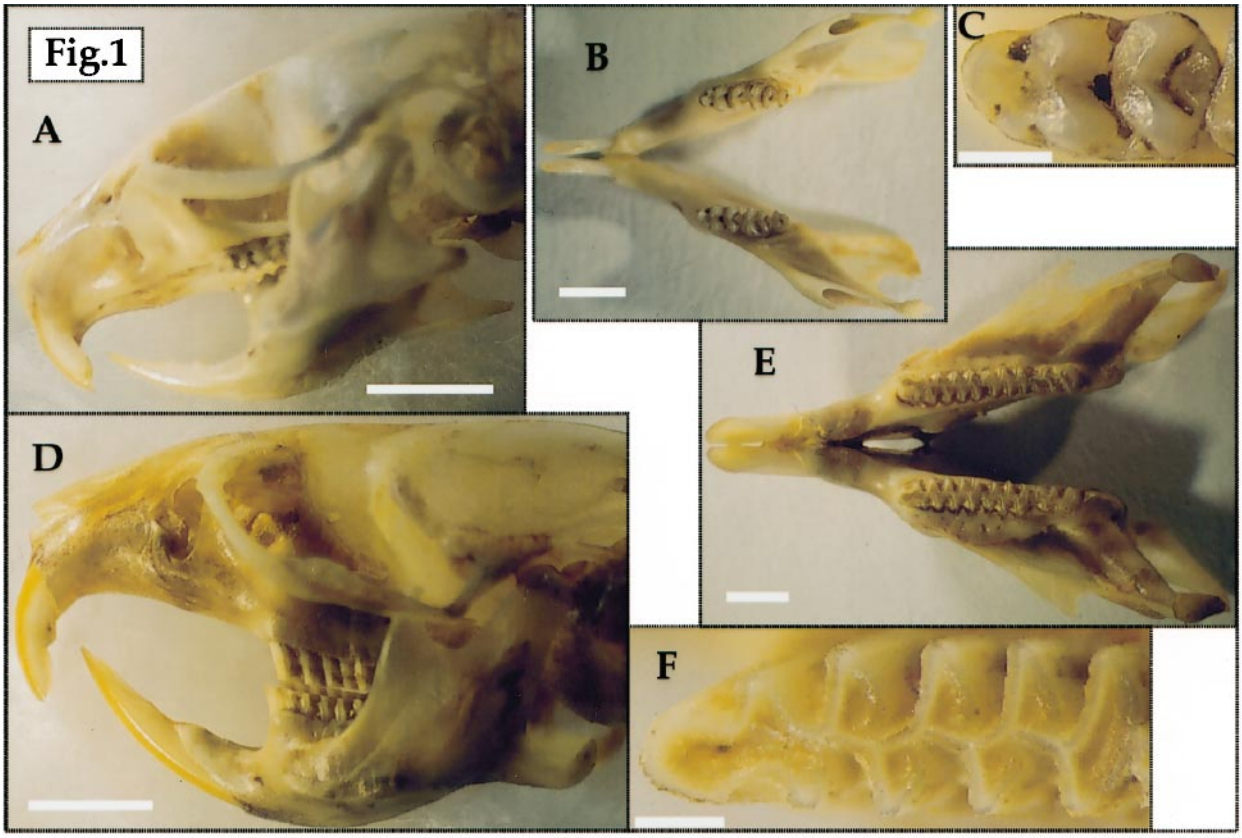
Fig.1
A
B
C
D
E
F

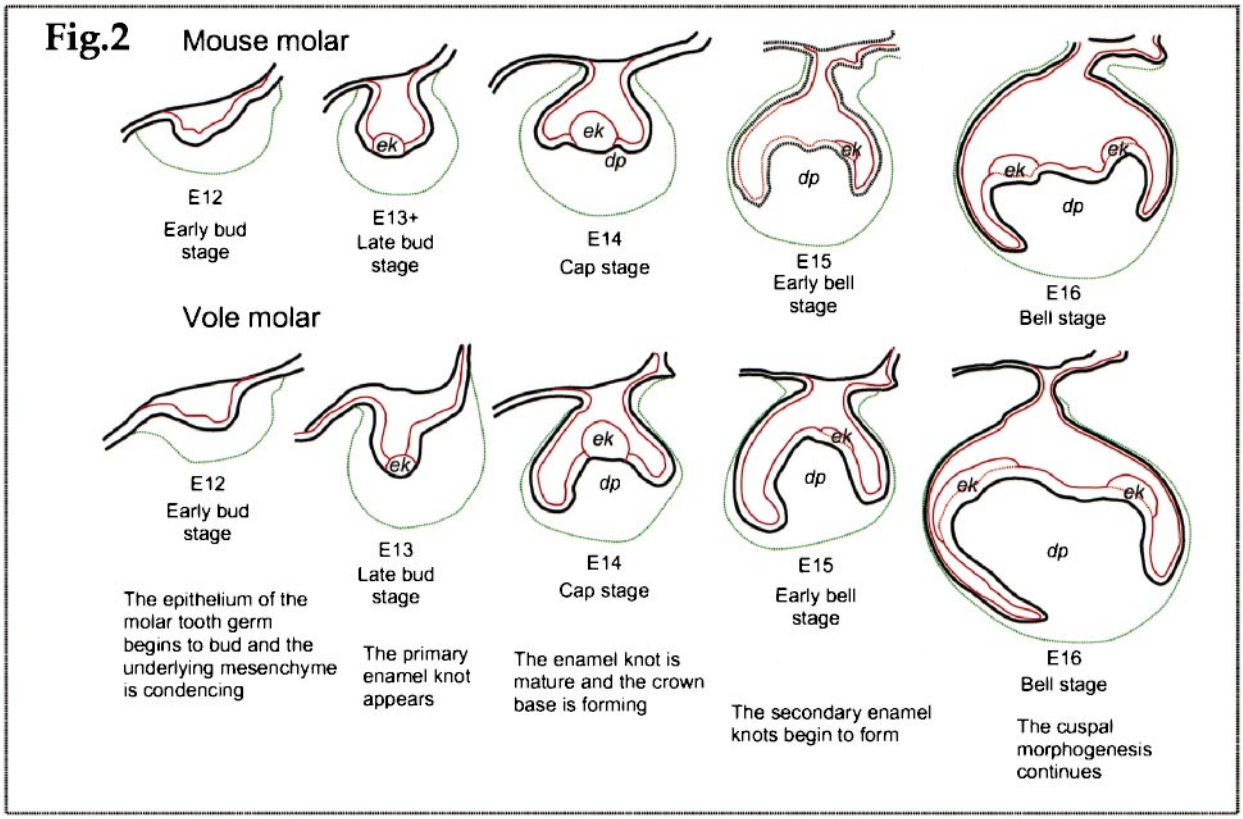
Fig.2
Mouse molar
E12
Early bud stage
E13+
Late bud stage
E14
Cap stage
E15
Early bell stage
E16
Bell stage
ek
dp
Vole molar
E12
Early bud stage
E13
Late bud stage
E14
Cap stage
E15
Early bell stage
E16
Bell stage
The epithelium of the molar tooth germ begins to bud and the underlying mesenchyme is condencing
The primary enamel knot appears
The enamel knot is mature and the crown base is forming
The secondary enamel knots begin to form
The cuspal morphogenesis continues

all the cusps are joined together along the central ridge. Four buccal and five (wider) lingual prisms protrude laterally from a central ridge forming a zigzag pattern when viewed occlusally (Fig. 1). The anteroconid is also present.

Based on previous morphological and genetical studies on mouse lower first molar development we selected for this study eight developmental regulatory genes; *Bmp-2, Bmp-4, Fgf-4, Lef-1, Msx-1, Msx-2, p21$^{CIP1/WAF1}$* and *Shh* (MacKenzie et al. 1992; Vainio et al. 1993; Jernvall et al. 1994; Satokata and Maas 1994; Kratochwil et al. 1996; Vaahtokari et al. 1996a; Jernvall et al. 1998). All these genes are involved in signalling and/or differentiation, either within the cell, like the transcription factors *Lef-1, Msx-1* and *Msx-2* and the cell cycle control gene *p21,* or between the cells, like the signalling molecules *Bmp-2, Bmp-4, Fgf-4* and *Shh.* Besides tooth development, the genes are used in a variety of organ systems and also in different developmental stages of the same organ within the same species (Niswander and Martin 1992; van Genderen et al. 1994; Bitgood and McMahon 1995; Davidson 1995; Parker et al. 1995).

Most of the genes in this study have been shown to have relatives even in distantly related animals like *Caenorhabditis elegans* (Coulier et al. 1997) and *Drosophila* (Padgett et al. 1993; Fietz et al. 1994; Davidson 1995; Sutherland et al. 1996), and each of the studied mouse genes has a human (sequence) homologue (Padgett et al. 1993; Oosterwegel et al. 1993; Davidson 1995; Harper and Elledge 1996; Roessler et al. 1996; Coulier et al. 1997). Therefore, it was reasonable to hypothesize that the sibling vole, a muroid rodent like the mouse, had functioning homologues of these genes.

In mouse teeth, all of the studied genes, except *Msx-1*, are expressed in the primary enamel knot, a region of non-dividing epithelial cells (Vaahtokari et al. 1996a; Kratochwil et al. 1996; Jernvall et al. 1998). The primary enamel knot may represent an organizing centre that regulates development of tooth shape by inducing the formation of secondary enamel knots at the future tips of each cusp (Jernvall 1995; Thesleff and Jernvall 1997). The enamel knots have, however, been documented using molecular markers only in the mouse. The secondary enamel knots are associated with mouse cusp development, and therefore we were particularly interested in the presence and exact location of the secondary enamel knots in the vole molars. On the other hand, from E12 onwards the dental mesenchyme has been shown to control tooth identity (Kollar and Mina 1991). Thus the mesenchymally expressed genes may also be important for patterning in the formation of tooth shape.

Materials and methods

The mouse (*Mus musculus*) teeth and jaws were obtained from crosses between inbred CBA male and outbred NMRI female mice, and plug day was taken as embryonic day E0. The sibling vole (*Microtus rossiaemeridionalis* or *M. epiroticus*) tissues were obtained from a colony kept at the Department of Animal Physiology (University of Helsinki), and the animals were allowed to get used to each other in separate cages for a day, then mated over night and the following day was counted as E0.

The tissues were fixed overnight in 4% paraformaldehyde (PFA) and taken through the ethanol series via xylene into paraffin and serially sectioned at 7 or 10 µm for histology and radioactive in situ hybridization. The in situ hybridization was done as described by Vaahtokari et al. (1996a).

The probes used were murine *Bmp-2* (240-bp cDNA fragment; Vainio et al. 1993), murine *Bmp-4* (285-bp cDNA fragment; Vainio et al. 1993), murine *Fgf-4* (620-bp cDNA fragment; Jernvall et al. 1994), murine *Lef-1* (660-bp 3'-truncation of the GL1 clone described in Travis et al. 1991 at NdeI site), murine *Msx-1* (600-bp cDNA fragment; Vainio et al. 1993), murine *Msx-2* (800-bp cDNA fragment; Vainio et al. 1993), murine *p21$^{CIP1/WAF1}$* (740-bp fragment; Jernvall et al. 1998) and rat *Shh* (2.6-kb cDNA fragment; Vaahtokari et al. 1996a).

The bright field and dark field images of each section were digitized using a Macintosh PPC computer with Cohu 4912-5000 CCD (Cohu, Calif.) camera and Scion LG-3 Frame Grabber card (Scion, MD.). Digitizing was 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). For Figs. 3 and 4 the grains from dark field pictures were selected, coloured black and added to the bright field pictures in Photoshop 4. The expression patterns for both species are also available in http://honeybee.helsinki.fi/toothexp, which is our database of gene expression patterns in teeth.

Results

Morphogenesis of the species-specific molar shapes

The molars in mouse and vole go through the same general developmental stages, starting with the thickening of the dental lamina, followed by epithelial budding and morphogenesis into cap and bell stages (Fig. 2). The gross histology of the tissues seems similar between the species (see also Butler 1956). For example, the primary and secondary enamel knots in both species showed a packing of epithelial cells, although the vole secondary enamel knots were shallower and less clearly delineated than in mouse. Despite these similarities, tooth morphogenesis in the two species proceeds into increasingly different directions, and at the bell stage, the species-specif-

Fig. 1 **A** Skull of a mouse viewed from left, **B** lower jaw of a mouse from above and **C** the left first lower molar of a mouse. The tooth is smaller than in vole and the seven cusps point upwards and to the mesial. Distal is to the *right* and mesial to the *left*. **D** Skull of a sibling vole viewed from left, **E** lower jaw of a sibling vole viewed from above and **F** left first lower molar of a sibling vole from above. The cusps are connected by a zigzag shaped ridge in the middle from above. Distal is to the *right* and mesial to the *left*. The photographs of the two species have been taken with same magnification. The *scale bar* indicates in **A** and **D** 4 mm, in **B** and **E** 2 mm, in **C** and **F** 0.5 mm

Fig. 2 The anatomy of mouse and vole first lower molar development as drawn from frontal sections of E12 to E16 embryos (where formation of plug is taken as embryonic day E0). Dental epithelium in *red* and dental mesenchyme in *green*. Oral surface of the epithelium and the basal lamina are in *black*. Buccal is to the *right* and lingual to the *left (dp* dental papilla, *ek* enamel knot)

ic crown shapes become apparent (Fig. 2). Hence, the final molar shapes in voles do not develop from mouse-like intermediate forms or vice versa.

Subtle morphological differences are already evident at E12. The presumptive dental epithelium is thicker buccally in voles than in mice, and at E13 the vole buds are longer (vole 48 µm, mouse 38 µm) and thinner than mouse buds, though the depth is about the same. The transition to cap stage is faster in voles than in mice; by E14, the lateral protrusions of the vole molar have grown down at least four times the distance of mouse molar protrusions (Fig. 2). Therefore, the dental papilla (mesenchyme surrounded by epithelium) of the vole is larger than mouse papilla and this difference is retained during later development. However, the relative difference between mouse and vole papilla heights slightly decreases at later developmental stages so that while in E15 the difference in height is around 55% (170/110 µm), the difference is only around 20% in E16 (200/170 µm) and E17 (390/320 µm).

The morphologies at E16 and E17 correspond to the early bell stage of tooth formation and the mouse- and vole-specific cusp patterns appear at this stage. In vole molars, the growth in height appears to slow down during cusp pattern formation, whereas the molar length continues to increase faster than in mouse molars. In E15 vole, the molar is 41% longer than mouse molars (550/390 µm), but the difference is 77% by E17 (1060/600 µm), similar to the difference of fully formed molars (79%, 2.5/1.4 mm). Vole molars have twice as many cusps (prisms) along the longitudinal axis as mouse molars, and the rapid longitudinal growth during E16–E17 seems to reflect the formation of this longer cusp pattern. Indeed, the final vertical growth of the vole molars into hypselodont type begins after the cusp pattern is completed (E17 onwards). The differences in molar widths are relatively small (in fully formed tooth 25%, vole 1.0 mm, mouse 0.8 mm).

All the genes studied were detected by in situ-hybridization in mouse and vole teeth using the same cDNA probes

Though all the studied genes seem to function in many other developmental systems, they were chosen for their known or supposed function in mouse tooth development. *Bmp-2*, *Bmp-4*, *Fgf-4* and *Shh* encode secreted signal molecules, and thus they can also affect other cells, whilst *Lef-1*, *Msx-1*, *Msx-2* and *p21* encode intracellular molecules which are involved in mediating the effects of extracellular signals.

All the used probes have been earlier shown to detect mouse transcripts (see Materials and methods). The mouse probes (*Bmp-2,-4*, *Msx-1,-2*, *Fgf-4*, and *p21*) and the rat probe (*Shh*) detected transcripts in vole tissues (Figs. 3 and 4). As the expression patterns were limited to similar portions of the tooth germ in mouse and vole, we assume that the probes detected the same gene transcripts. The sense controls did not show any hybridization in either species (data not shown).

Gene expression patterns during early tooth morphogenesis

Whilst the earliest bud stages (E12) were morphologically quite nondescript, several gene transcripts were detected in the tooth germs of both species at this stage. Strong expression of *Bmp-2*, *Lef-1* and *Shh*, and weaker of *Bmp-4*, *Msx-2* and *p21* were detected in the presumptive dental epithelium (Table 1, Figs. 3, 4). The only clear difference between the species was the weak *Fgf-4* expression in the lingual epithelium in vole, but not in mouse (Fig. 3, Table 1).

Interestingly, *Bmp-2*, *-4*, *Lef-1*, *Shh* and *p21* transcripts were co-localized in a restricted area at the lingual side of the forming buds and their expression appeared to correspond to a slight lingual swelling (Figs. 2–4). The cell population expressing these genes probably represents an early epithelial signalling centre.

By the middle bud stage (E13), the expression of these genes was downregulated in both species (Figs. 3, 4). Thus, the early epithelial signalling centre seems to be separate from the primary enamel knot which appears by E14.

Msx-1 transcripts were detected only in the mesenchyme whilst *Lef-1*, *Bmp-4* and *Msx-2* transcripts were expressed in both epithelium and mesenchyme. The latter two show a clear buccal bias in E12 and E13 mesenchyme. *Msx-2* transcripts also had a buccal bias in the epithelium.

Gene expression during the cap stage

The transition to cap stage begins at the late bud stage and is marked by the formation of the primary enamel knot at the tip of the epithelium (Jernvall et al. 1994; Fig. 2). The lateral protrusions, later forming the cervical loops, begin to form beside the enamel knot. By E14 in both species, the primary enamel knot expresses every gene except *Msx-1*, which is strictly mesenchymal in developing molars throughout tooth development. In general, the gene expression domains of the studied genes were more compartmentalized at this stage (i.e. restricted to the primary enamel knot) than in other developmental stages (Table 1).

We have earlier shown in the mouse primary enamel knot that *Bmp-4* expression is upregulated first in its distal part (Vaahtokari et al. 1996a; Jernvall et al. 1998) and we found this to be the case in the vole primary enamel knot as well (not shown). Hence, the primary enamel knot in both species is at least partially removed via apoptosis before the cusp pattern becomes apparent.

The buccal bias of *Bmp-4* transcripts in the mesenchyme and *Msx-2* transcripts in the epithelium continued in both species but by E14, *Bmp-4* and *Msx-2* expressions were also strong in the dental papilla (Figs. 3, 4).

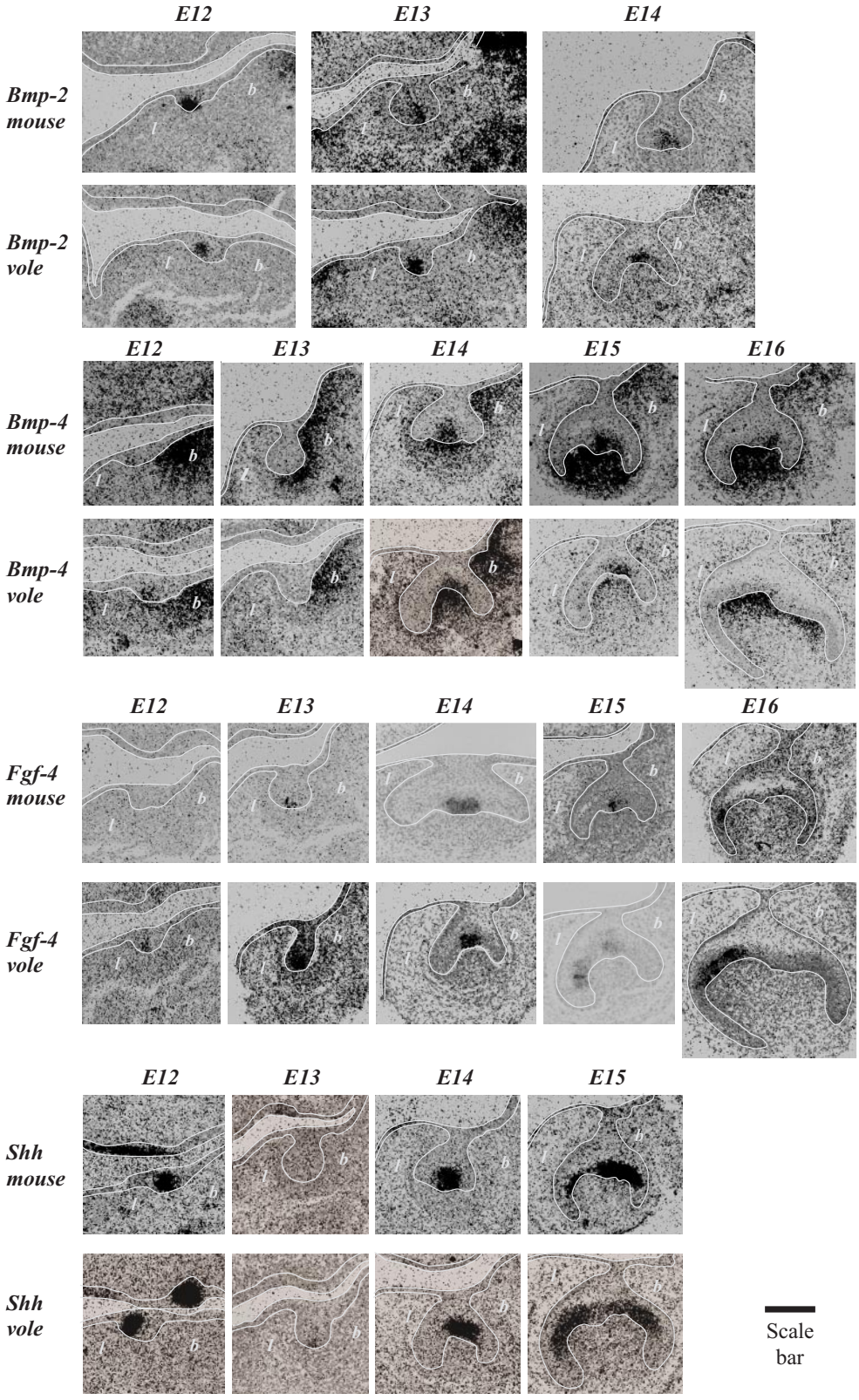

Fig. 3 In situ hybridization analysis of the expression patterns of the signalling molecules *Bmp-2*, *Bmp-4*, *Fgf-4* and *Shh*. The mouse *Fgf-4* E14 and vole *Fgf-4* E15 are bright field images. Other pictures have been treated as described in Materials and methods. To make the morphology clearer, *grey lines* were drawn over the basal lamina and the oral surface of the epithelium to enhance the tissue borders (*b* buccal, *l* lingual, *scale bar* indicates 150 µm)

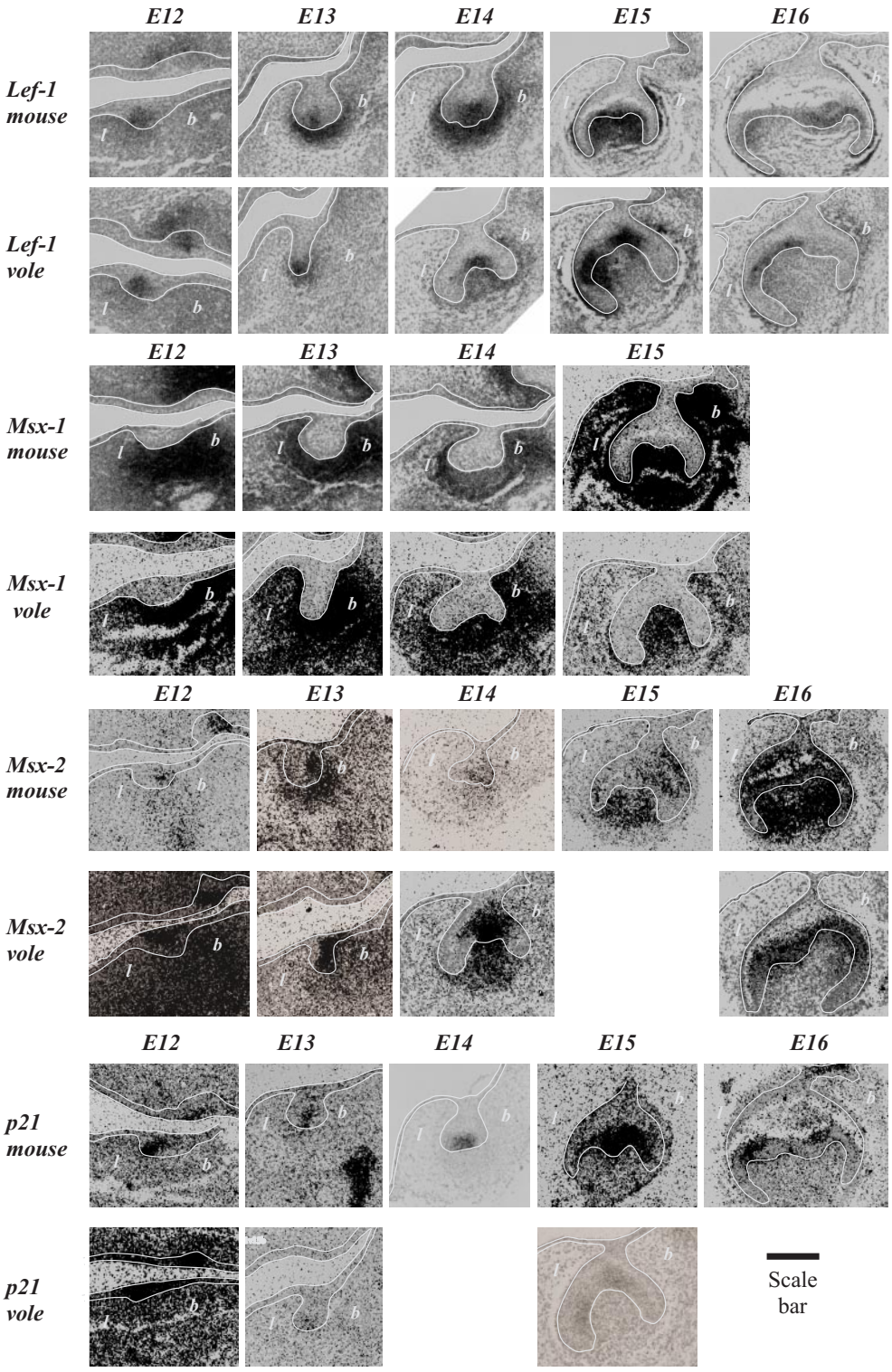

Fig. 4 In situ-hybridization analysis of the expression patterns of the transcription factors *Lef-1*, *Msx-1* and *Msx-2*, and the cell cycle controller $p21^{CIP1/WAF1}$. The genes are arranged into horizontal blocks by age with oldest stages to the right. *Lef-1*, mouse *Msx-1* E12, E13 and E14, mouse *p21* E13 and vole *p21* E15 are bright field images. The rest of the pictures have been treated as described in Materials and methods (*b* buccal, *l* lingual, *scale bar* indicates 150 µm)

Gene expression patterns during the development of cusp patterns

In the mouse molar, the first cusps can be morphologically detected first at E16 when the tooth germ has reached early bell stage. The development of the cusps in mouse molars is characterized with the formation of secondary enamel knots, which are seen as clusters of dental epithelial cells at the tips of the forming cusps (Jernvall et al. 1994; Jernvall 1995). In voles, the secondary enamel knots were shallower and less clearly delineated than in mouse. The vole secondary enamel knots were associated with the developing prisms at E16 (Fig. 2). In both species the secondary enamel knots begin to appear at

Table 1 Correlations between gene expression and specific morphological features within epithelial and mesenchymal tissues of mouse and vole molars. The morphological features are: at E12, the anterior lingual epithelial swelling or the mesenchyme under it; at E13, the tip of the bud or the mesenchyme around it; at E14, the primary enamel knot or the underlying crown base mesenchyme; at E15 and E16, epithelium at the cusp tips (secondary enamel knots) and underlying cuspal mesenchymes. Note that the correlation values illustrate the degree of spatial correlations and hence do not indicate intensity of gene expression in either species. ++: expression correlates strictly with the morphological feature; +: gene expression overlaps the morphological feature, but the expression pattern is either wider or narrower; –: no detectable correlation to morphology; *0*: no expression in the tissue; *NA*: not analysed. Buccal lingual asymmetry is shown as *bb* for strong and *b* for weak buccal bias. The values were visually estimated from serial sections

E12	*Bmp-2*	*Bmp-4*	*Fgf-4*	*Shh*	*Lef-1*	*Msx-1*	*Msx-2*	*p21*
Mouse epithelium	+ (+)	++	0	++	+ (+)	0	b	+ (+)
mesenchyme	0	bb	0	0	+	– (b)	b	0
Vole epithelium	+ (+)	+ (+)	++	++	+ (+)	0	(+) (b)	+
mesenchyme	0	b	0	0	+	– (b)	b	0
E13	*Bmp-2*	*Bmp-4*	*Fgf-4*	*Shh*	*Lef-1*	*Msx-1*	*Msx-2*	*p21*
Mouse epithelium	+ (+)	0	+	0 (+)	++	0	(+) bb	+
mesenchyme	0	b (b)	0	0	+ b	– (b)	+ b	0 (+)
Vole epithelium	+	0	+	+	+ (+) (b)	0	+ bb	+
mesenchyme	0	bb	0	0	+ b	– b	+ b	0
E14	*Bmp-2*	*Bmp-4*	*Fgf-4*	*Shh*	*Lef-1*	*Msx-1*	*Msx-2*	*p21*
Mouse epithelium	+ (+)	+ b	++	+ (+)	+	0	+ bb	+ (+)
mesenchyme	0	(+) b	0	0	b	– (b)	b (b)	(+)
Vole epithelium	+ (+)	+	+ (+)	+ (+)	+	0	(+) b	NA
mesenchyme	0	+ b (b)	0	0	b	b	b	NA
E15	*Bmp-2*	*Bmp-4*	*Fgf-4*	*Shh*	*Lef-1*	*Msx-1*	*Msx-2*	*p21*
Mouse epithelium	+ (+)	+	+ (+)	+	+	0	– (b)	(+)
mesenchyme	0	(+) b	0	0	(+) (b)	– (b)	–	0
Vole epithelium	+ (+)	+	+ (+)	+	+	0	NA	+
mesenchyme	0	+ b (b)	0	0	+ b	– b	NA	0
E16	*Bmp-2*	*Bmp-4*	*Fgf-4*	*Shh*	*Lef-1*	*Msx-1*	*Msx-2*	*p21*
Mouse epithelium	NA	+	++	(+)	+	0	–	(+)
mesenchyme	NA	(+) (b)	0	0	+ (b)	(+)	–	0
Vole epithelium	NA	+	+ (+)	(+)	+	NA	–	NA
mesenchyme	NA	+	0	0	+ b	NA	+	NA

E15, before the cusps were morphologically distinguishable (Figs. 3, 4).

We have reported earlier that *Fgf-4* is expressed in the secondary enamel knots in the mouse molars (Jernvall et al. 1994). Of all the genes studied here, *Fgf-4* showed the closest correlation with the secondary enamel knots both in mouse and vole (Table 1). *Fgf-4* expression was restricted to sites of cusp tips in mouse molars and the tips of the developing prisms and central ridge in voles (Fig. 3).

Lef-1 and *Shh* genes were expressed more widely in the inner enamel epithelium and by E17 they had no clear correlation to cusp pattern left (Figs. 3, 4), but the intensity of *Bmp-2* expression declined rapidly (Table 1; see also Åberg et al. 1997). The epithelial expressions of *Msx-2* and *p21* showed a weak association with secondary enamel knots and *Bmp-4* was present in some of them at E16. It is possible that the sporadically detected *Bmp-4* transcripts mark the apoptosis of the secondary enamel knots (Vaahtokari et al. 1996b). Thus, both the mouse and vole secondary enamel knots express almost the same set of molecules as the primary enamel knot, *Bmp-4, Fgf-4, Lef-1, Msx-2, p21* and *Shh* (*Bmp-2* was downregulated), which all correlated similarly with morphology in both mouse and vole (Figs. 3, 4; Table 1).

In adult mouse molars the cusps are arranged in bucco-lingual pairs, whereas in voles the prisms are connected diagonally to each other (Fig. 1). In mouse molars, *Fgf-4* transcripts have two separate expression domains corresponding to the lingual and buccal cusps. In contrast, the expression domains of *Fgf-4* in the vole molar are diagonally arranged, like the developing prisms, and by E17 *Fgf-4* has a long continuous undulating expression area (not shown).

In the mesenchyme, *Msx-1* and *Lef-1* transcripts were detected in overlapping but wider areas than *Bmp-4* and *Msx-2* transcripts and also later, at E15 and E16 (Figs. 3, 4). During later bell stage, the dental papilla express transcription factor *Lef-1*, *Msx-1* and *Msx-2* with decreasing intensity, though still higher than in oral mesenchyme surrounding the tooth germ (Table 1, Fig. 4).

Discussion

In this study we compared the molecular basis of disparate tooth shapes in two species of muroid rodents to the expression patterns of eight developmental regulatory genes. We chose to study mouse and sibling vole because, though their molar morphologies are very dissimilar, they are relatively close phylogenetically and thus their gene sequences should be quite similar. Indeed, all of the mouse probes and a rat *Shh* probe also detected vole mRNA. Additionally, mouse probes for *Barx-1*, *Fgf-8*, *Otlx-2* and *Pax-9* cross-hybridize specifically with vole mRNA (data not shown). Genes of the same gene families (paralogous genes, e.g. Bmp-2 and -4) appeared to be detected correctly. In general, as gene duplications in an evolutionary lineage have usually preceded taxonomic divergence, paralogous genes belonging to the same gene family are more different within species than same genes are among species (Fietz et al. 1994; Coulier et al. 1997). Compared to the ancient gene duplication events (e.g. in Fgf-family; Coulier et al. 1997), mouse and vole lineages are phylogenetically very recent (Nikoletopoulos et al. 1992). Therefore, it is unlikely that the mouse probes used recognized some unknown paralogous genes in vole.

Gene expression patterns and tooth shape development

The studied genes have been shown to participate in morphogenesis by affecting processes like cell proliferation (e.g. *Bmp-2* and *Fgf-4*; Niswander and Martin 1993), cell differentiation (e.g. *Bmp-2, p21* and *Shh*; Parker et al. 1995; Lough et al. 1996; Ericson et al. 1996), and apoptosis (e.g. *Bmp-4* and *Msx-2*; Graham et al. 1994). In addition they appear to be associated with cell adhesion (e.g. *Lef-1*; Huber et al. 1996). The biological roles of the studied genes are not exclusive to one organ and, in addition, there can be functional redundancy between the co-expressed genes. For example, BMP-2 and BMP-4 have similar inductive effects on dental epithelium and mesenchyme in vitro (Vainio et al. 1993; Jernvall et al. 1998). On the other hand, co-expression of several developmental regulatory genes within an embryonic region can be thought to be indicative of molecular modules that function in controlling organ development. Several known embryonic regions (signalling centres) co-express a set of the genes studied here. *Shh* is expressed in the developing limb in the zone of polarizing activity (ZPA) which regulates anteroposterior patterning (Riddle et al. 1993). *Fgf-4* is expressed in the limb apical ectodermal ridge (AER) which controls proximodistal growth in the limb and interacts with the ZPA (Niswander and Martin 1992; Riddle et al. 1993). Both ZPA and AER express *Bmp*s (Tickle 1995). *Shh* is also expressed in the notochord, which regulates patterning of the neural tube and somites and the ectoderm overlying the neural tube expresses *Bmp*s (Echelard et al. 1993; Fan and Tessier-Lavigne 1994; Liem et al. 1995).

In this study we found in both species three different stages when several genes were co-expressed in the dental epithelium or mesenchyme. Initially the nested expression patterns were detected prior to the visible species-specific morphologies. At the early bud stage (E12) *Bmp-2, -4, Shh, Lef-1*, and *p21* were co-expressed in the lingual portion of the emerging epithelial bud (Figs. 3, 4; Table 1). *Bmp-4* was also expressed in the buccal portion in the mesenchyme (Fig. 3; Table 1), like *Msx-2*, which was also expressed in the buccal epithelium (Fig. 4; see also Turecková et al. 1995).

The presence of an apparent signalling centre in the epithelium and the alternating patterns of epithelial and mesenchymal gene expression domains appear to mark the induction of dental mesenchyme by epithelium at E12 (e.g. Maas and Bei 1997). At this time, the inductive potential is known to transfer from dental epithelium to mesenchyme (Kollar and Mina 1991). Based on the similar patterns of gene expression in mouse and vole molar teeth, it is possible that rather than determining species-specific morphologies, this early developmental stage is associated more with the final establishment of tooth location by epithelial-mesenchymal interactions. The presence of *Fgf-4* in the early epithelial signalling centre at E12 in vole but not in mouse was the only clear species-specific difference we detected. Whether it has a role in the morphogenesis of the early molars of the two species remains to be studied.

After E12, the expressions of *Bmp-2*, *-4*, *Shh*, and *p21* decline in both mouse and vole epithelium (Figs. 3, 4). Crown base begins to form after a putative signalling centre, the primary enamel knot (Thesleff and Jernvall 1997), has appeared at the tip of the epithelial bud (late E13). In both species, the primary enamel knot expressed several genes: *Bmp-2*, *-4*, *Fgf-4*, *Shh*, *Lef-1*, *Msx-2*, and *p21* (see also Vaahtokari et al. 1996a; Åberg et al. 1997; Jernvall et al. 1998; Kettunen and Thesleff 1998). Whilst the gene expressions in the primary enamel knot seem quite alike between the species (Figs. 3, 4), the cap stage morphologies are different. Two ridges, the enamel grooves, delineate the enamel knot from the rest of the inner enamel epithelium and are distinct in mouse whereas less well developed in the vole molar. The general morphology of the E14 vole crown base is higher but rounder than the shallow but more angular mouse molar crown base (Fig. 2).

The disappearance of the primary enamel knot begins in both species at E14 when some of the cells in the distal part of the knot begin to express *Bmp-4* and some go into apoptosis (Fig. 3; Jernvall et al. 1998). The removal of the primary enamel knot is immediately followed by the formation of the secondary enamel knots. Expression domains of *Shh, Lef-1, Msx-2* and *p21* covered more of the occlusal part of the inner enamel epithelium whereas the *Fgf-4* expression domains were more restricted to the secondary enamel knots and show the clearest association with the species-specific cusp patterns (Figs. 3, 4). Thus, *Fgf-4* appears to be a good genetic marker for species-specific cusp pattern formation in mammals.

It is noteworthy that the development of the cusp patterns is accompanied by rapid growth of the tooth germs. The only areas that do not proliferate during early cusp pattern formation (E15–16) are the secondary enamel knots (Jernvall et al. 1994) and thus the folding of the epithelial sheet can be partly regulated by the secondary knots causing differences in the rates of cell proliferation.

In the mesenchyme, expression patterns appeared to have a buccal bias (*Bmp-4, Lef-1* and *Msx-2*; Fig. 4). The maximum buccal bias of the studied genes was detected at E13–14 and the bias disappeared at E15 (Table 1). Thus, the buccal and lingual portions of the mesenchyme appear to differ in gene activity at the time when the primary enamel knot is present. As tooth development is controlled by epithelial-mesenchymal interactions (Thesleff and Nieminen 1996; Maas and Bei 1997), it can be postulated that the asymmetry of the mesenchymal gene expression domains may be associated with the buccolingual differences in tooth shape.

The transition from the bud to the cap stage which begins with the appearance of the primary enamel knot, is characterised with the start of epithelial folding and the formation of the dental papilla. This process appears to be regulated by complex gene networks, and mice lacking a functional *Msx-1, Lef-1* or *Pax-9* gene all have their tooth development arrested at the bud stage (Satokata and Maas 1994; van Genderen et al. 1994; Peters et al. 1998). Interestingly, for tooth morphogenesis to take place, *Lef-1* expression is required in the epithelium prior to the formation of the primary enamel knot (Kratochwil et al. 1996). Also, tooth buds of *Msx-1* null mutant mice reach the cap stage when cultured in the presence of BMP-4, which is only expressed in the mesenchyme at the bud stage (Chen et al. 1996). We have recently shown that BMP-4 is indeed a potent inducer of *p21,* which is one of the earliest molecular markers of the primary enamel knot (Jernvall et al. 1998). In addition to showing that the formation of the primary enamel knot requires epithelial-mesenchymal interactions, these experiments suggest that the formation of the primary enamel knot may be a general “check point” of tooth morphogenesis. Based on our results, we suggest that this critical stage may also mark the activation of molecular cascades determining species-specific cusp patterns.

Evolution and development of vole and mouse molar shapes

Though mouse and vole molars are morphologically very different, we found that the relationships of the gene expression patterns compared to the morphologies are similar. Also, the formation of each cusp was found to associate with a secondary enamel knot expressing the same set of genes. Therefore, at least based on the genes studied in this paper, the general cusp patterns in both mouse and vole molars result from repetition of the same molecular cascade, i.e. the cusps are serially homologous, and thus new genes are not necessarily required for the evolution of new cusps.

The vole lineage (*Arvicolidae*) has had species turnover rates of up to some hundreds of thousands of years as measured by changes in prism shapes and tooth size measurements (Brunet-Lecomte and Chaline 1991), i.e. the changes in molar morphogenesis seem to arise easily. The evolution of new cusps would be relatively simple, if only slight changes in the mechanisms defining the pattern would be needed. Thus the evolution of additional cusps could result from slight temporal variations in the regulation of gene expression producing changes in growth rate and timing of secondary enamel knot formation. The evolution of additional prisms (cusps) in voles (Guthrie 1965) could be an example of this kind of developmental control.

Though the exact functions of genes in morphogenesis still remain unknown, some genes, like *Fgf-4*, have closer spatial correlation to morphology than others, and it is possible that such genes are more relevant to the patterning and evolution of the species-specific shapes than other genes, such as *Msx-1*, which are otherwise essential for the normal tooth development.

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