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Gene expression patterns associated with suppression of odontogenesis in mouse and vole diastema regions

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Abstract Rodents have a toothless diastema region between the incisor and molar teeth which may contain rudimentary tooth germs. We found in upper diastema region of the mouse (*Mus musculus*) three small tooth germs which developed into early bud stage before their apoptotic removal, while the sibling vole (*Microtus rossiaemeridionalis*) had only a single but larger tooth germ in this region, and this developed into late bud stage before regressing apoptotically. To analyze the genetic mechanisms of the developmental arrest of the rudimentary tooth germs we compared the expression patterns of several developmental regulatory genes (*Bmp2, Bmp4, Fgf4, Fgf8, Lef1, Msx1, Msx2, p21, Pitx2, Pax9* and *Shh*) between molars and diastema buds of mice and voles. In diastema tooth buds the expression of all the genes *differed* from that of molars. The gene expression patterns suggest that the odontogenic program consists of *partially independent signaling cascades* which define the exact location of the tooth germ, initiate epithelial budding, and transfer the odontogenic potential from the epithelium to the underlying mesenchyma. Although the diastema regions of the two species differed, in both species the earliest difference that we found was weaker expression of mesenchymal *Pax9* in the diastema region than in molar and incisor regions at the dental lamina stage. However, based on earlier tissue recombination experiments it is conceivable that the developmental arrest is determined by the early oral epithelium.

Key words Developmental genes · Mouse · *Microtus rossiaemeridionalis* · Rudimentary · Tooth

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Introduction

Mouse (*Mus musculus*) and sibling vole (*Microtus rossiaemeridionalis*) are muroid rodents which, according to immunological data, diverged from a common ancestor about 20 million years ago (Nikoletopoulos et al. 1992; Robison et al. 1997). Both species have one incisor and three molars in each jaw quadrant, and between the incisor and the first molar is a toothless gap, a diastema. The diastema has existed in rodents since at least the Eocene epoch, or for over 50 million years (Luckett 1985; Meng et al. 1994), but many rodents, including the mouse, have rudimentary tooth germs in the diastema region (Luckett 1985; Peterková et al. 1995). Rudimentary diastema tooth germs are believed to be remnants of the primitive Eutherian dental formula of three incisors, a canine, four premolars, and three molars in each jaw quadrant (Luckett 1985; Peterková et al. 1995), and studies on the genetic mechanisms of their developmental arrest may hence shed light on the mechanisms of the evolution of rodent dentition. Furthermore, the developmental arrest of diastema teeth represents a natural "experiment" which can be compared to the loss of teeth in gene knockout experiments.

All teeth, regardless of shape or identity, pass through the same developmental stages, and consist of the same tissues (Butler 1995; Stock et al. 1997). The oral epithelium invaginates into underlying mesenchyma and forms the tooth bud. The mesenchyma condenses, and an enamel knot is induced into the tip of the bud at late bud stage. The cervical loops are formed lateral to the enamel knot, and these grow to surround the tooth crown base during the cap stage. Crown morphogenesis and cytodifferentiation occur during the bell stage. After the tooth crown is mineralized, it begins to erupt. In rudimentary tooth germs the development may be arrested at any of these stages, and the tooth germ may then regress (Luckett 1985; Moss-Salentijn 1978). Because the basic development is similar in all teeth, they are considered to be serially homologous (Stock et al. 1997). Hence the genetic mechanisms involved in basic morphogenesis and

cytodifferentiation should be conserved between teeth of different identity, as they are between different species, for example, mouse and vole (Keränen et al. 1998).

Tooth development is regulated by a complex series of epithelial mesenchymal interactions. The odontogenic potential, which originally resides in the oral epithelium, is transferred into the underlying neural crest derived mesenchyma, and mesenchymal signals are necessary for further epithelial morphogenesis and cytodifferentiation. Extensive studies on mouse odontogenesis have revealed several genes which regulate the tooth morphogenesis and differentiation. To analyze the genetic basis of the rudimentary diastema tooth germ development we compared the expression patterns of genes for several secreted signaling molecules (*Bmp2, Bmp4, Fgf4, Fgf8* and *Shh*) and their intracellular targets (*Lef1, Msx1, Msx2, Pitx2, Pax9* and *p21*) between the diastema tooth germs and molars. It has been shown that these genes function in four major, interacting signaling pathways, namely bone morphogenetic protein BMP), fibroblast growth factor (FGF), Sonic Hedgehog (Shh), and Wingless (Wnt) signaling pathways (for reviews, see e.g., Maas and Bei 1997; Stock et al. 1997; Thesleff and Pispa 1998; Thesleff and Sharpe 1997).

All of these genes are known or suggested to have important functions in regulation of tooth development (Hardcastle et al. 1998; Maas and Bei 1997; Neubüser et al. 1997; Thesleff and Jernvall 1997). For example, *Bmp4* and *Fgf8* are expressed in the early dental epithelium, and BMP4 induces the mesenchymal expression of *Msx1, Msx2,* and *Lef1*, while FGF8 induces mesenchymal *Pax9* and *Msx1* (Jernvall et al. 1998; Kettunen et al. 1998; Neubüser et al. 1997; Vainio et al. 1993). *Msx1* and *Pax9* regulate mesenchymal *Bmp4*, which acts on the epithelium, regulating, for example, *p21* expression, and is essential for the transition from bud to cap stage (Chen et al. 1996; Jernvall et al. 1998; Peters et al. 1998). Epithelial Lef1 is also essential for transition from bud to cap stage (Kratochwil et al. 1996), and misexpressed *Lef1* can induce ectopic tooth germs (Zhou et al. 1995). *Lef1* is induced in the epithelium by BMP2 and BMP4 (our unpublished results), but Lef1 can also mediate Wnt-signaling (Kengaku et al. 1998). In mice lacking the function of Lef1, Msx1 or Pax9 tooth development is arrested at late bud stage; while when both *Msx1* and *Msx2* genes are nonfunctional, odontogenesis is arrested already at dental lamina stage (Maas and Bei 1997). On the other hand, Shh can induce ectopic epithelial invaginations in oral epithelium (Hardcastle et al. 1998), and it can also induce *Pitx2* expression in the chicken node (St Amand et al. 1998). Haploinsufficiency of the transcription factor Pitx2/RIEG causes missing teeth in humans (Semina et al. 1996). *Shh* is coexpressed in molars with *Bmp2, Fgf4, Lef1,* and *p21*. The coexpression of these genes is typical to primary and secondary enamel knots, which apparently are epithelial signaling centers at cap and early bell stage (Jernvall et al. 1998). The expression of these genes in an anterior lingual swelling in early bud stage molars suggests that this swelling is an early epithelial signaling center, analogous to the enamel knots and necessary for normal morphogenesis (Keränen et al. 1998). Hence, the comparison of spatial and temporal expression patterns of the studied genes between diastema buds and molars may pinpoint the parts of the signaling networks, which are lacking in rudimentary tooth germs.

We found that although the final dental formulas of mouse and sibling vole are the same, mouse upper diastema region contains three small rudimentary tooth germs which develop into early bud stage before their apoptotic removal, while the single larger vole diastema tooth germ develops into late bud stage before its degeneration. The initiation of tooth buds in the diastema region indicates that the odontogenic program is partially activated. We found significant differences in the downregulation or upregulation of various developmental regulatory genes in mouse and vole diastema buds as compared to the molars. However, the changes were not similar in different genes, which suggests that the basic odontogenic program consists of partially separate subprograms involving semi-independent signaling cascades, some of which appeared to be primarily affected. Because none of the developmental genes is likely to have been lost, the evolution of the stage of developmental arrest and the numbers of the rudimentary tooth germs probably results from spatiotemporal changes in the early expression patterns of some but not all epithelial genes. Our results support the model by which tooth development requires a strong induction of *Pax9* expression by FGF8, but they also indicate that the lack of strong *Pax9* expression is not the only factor causing the evolution of tooth loss in murine diastema region.

Materials and methods

The mouse (*Mus musculus*) tissues were obtained from CBAT6T6 X NMRI matings, (vaginal plug=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 become accustomed to each other in separate cages for a day before being mated overnight; the following day was counted as E0.

The tissues for radioactive in situ hybridization and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining were fixed overnight in 4% paraformaldehyde (PFA), dehydrated, embedded in paraffin and serially sectioned at 7 or 10 µm for histology. The tissues for whole-mount in situ hybridization were fixed overnight in 4% PFA, dehydrated, and stored in 100% methanol or 70% ethanol.

The radioactive in situ hybridization with [^{35}S]UTP-labeled (Amersham) single-stranded RNA was carried out as described by Kettunen and Thesleff (1998) and the TUNEL staining as described by Vaahtokari et al. (1996). Whole-mount in situ hybridization was performed as described by Jernvall et al. (1998), except that the prehybridized tissues were usually stored in –20°C for at least overnight before hybridization with 0.5–1.0 µg of digoxygenin labeled single strand RNA probe in 1 ml of hybridization buffer overnight at +55°C. The antibody blocking with alkaline phosphatase antidigoxygenin FAB fragment (concentration 1/2000) was carried out at +4°C overnight and the color reaction with NBT and BCIP for 30’ to 3 h in RT.

The probes used for radioactive in situ hybridization were murine *Bmp2* (240-bp cDNA fragment), murine *Bmp4* (285-bp cDNA fragment), murine *Fgf4* (620-bp cDNA fragment), murine *Fgf8* (full-length cDNA), murine *Lef1* (660-bp 3'-truncation of the GL1 clone at NdeI site), murine *Msx1* (600-bp cDNA fragment), murine *Msx2* (800-bp cDNA fragment), murine *Pitx2* (1.8-kb cDNA fragment), murine *Pax9* (1370-bp fragment) murine $p21^{CIP1/WAF1}$ (740-bp cDNA fragment), and rat *Shh* (2.6-kb cDNA fragment). For whole-mount in situ hybridization we used the same *Shh* and a longer *Bmp2* probe (1.2-kb cDNA fragment).

The bright-field and dark-field images of radioactive in situ hybridization results were digitized using a Macintosh PPC computer with Cohu 4912–5000 CCD (Cohu, Calif., USA) camera and Scion LG-3 Frame Grabber card (Scion, MD, USA). The TUNEL apoptosis stainings were digitized as bright field images. Digitizing was carried out using the public domain NIH Image 1.61 program (United States 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, colored 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.

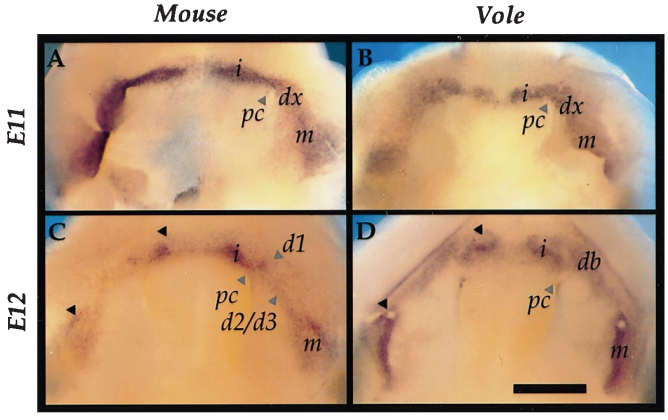

Fig. 2A–D Gene expression in the future dental lamina and the budding of tooth germs as visualized with whole-mount in situ hybridization analysis of *Pitx2* expression. Expression in the dental lamina at E11 in mice (**A**) and voles (**B**) was continuous, but at E12 it was being limited into the budding tooth germs both in mice (**C**) and in voles (**D**). *db* Vole diastema bud; *dx* diastema lamina; *d1* mouse first diastema bud; *d2/d3* presumptive mouse second and third diastema buds; *i* incisor; *m* molar; *pc* primary choana. *Black arrowheads* early epithelial signaling centers in molars and incisors; *gray arrowheads* primary choanae and the mouse diastema buds. *Bar* 0.5 mm

Results

Morphological development of mouse and vole diastema tooth germs

In careful analysis of frontal serial sections we found in mouse three upper diastema region rudimentary tooth germs (but see Peterková et al. 1998), while only a single rudimentary tooth germ was detected in the vole upper diastema (Fig. 1). We did not find rudimentary tooth germs in the lower jaws of either species (not shown). Thus, despite having the same final number of teeth, mouse and vole have different embryological dental formulas.

The first mouse diastema tooth germ (D1) developed by E12 near the incisors (Fig. 1K), but it was located in the maxillary instead of the frontonasal process. The second (D2; Fig. 1M) and third (D3; Fig. 1N) diastema tooth germs developed in the dental lamina posterior to D1 but clearly anterior to the first upper molar and they were connected to the palatal rugae (Fig. 1L–N). The vole diastema tooth germ was located in the maxillary process next to the primary choana in a similar location as D1 in the mouse, and hence it appears to be the vole

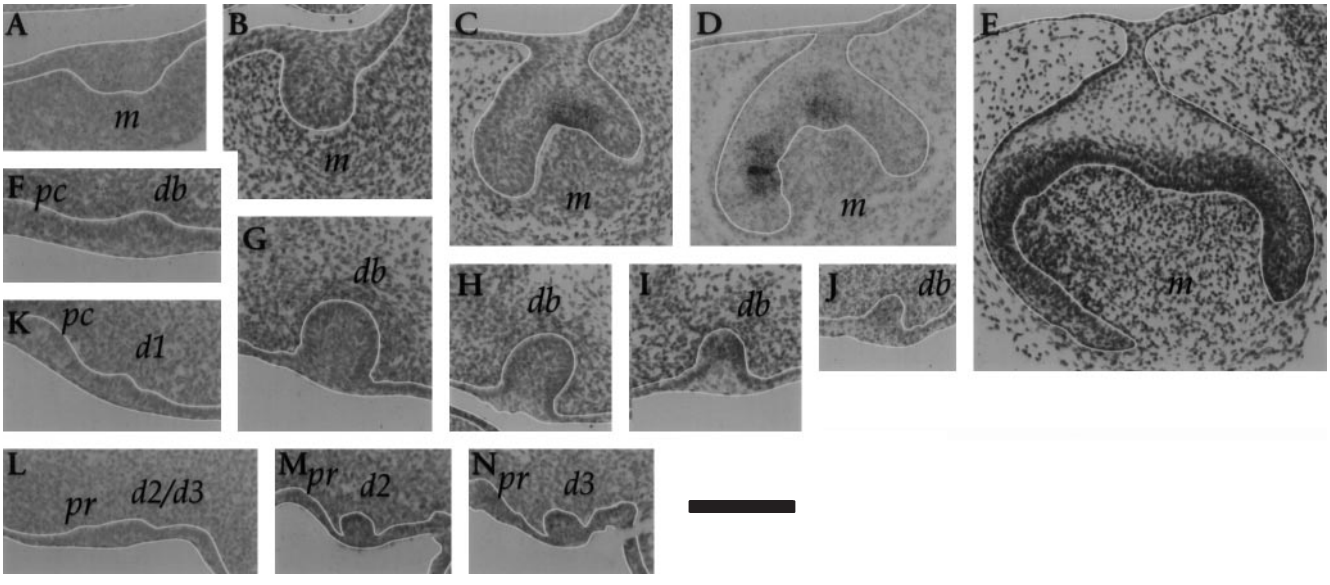

Fig. 1 Histological sections of developing first lower vole molars from E12 to E16 (**A**–**E**), vole upper jaw diastema tooth germs from E12 to E16 (**F**–**J**) and mouse upper jaw diastema tooth germs D1 at E12 (**K**), D2/D3 at E12 (**L**), D2 at E13 (**M**), and D3 at E13 (**N**). *db* Vole diastema bud; *d1* mouse first diastema bud; *d2/d3* presumptive mouse second and third diastema buds; *d2* mouse second diastema bud; *d3* mouse third diastema bud; *m* molar; *pc* primary choana; *pr* palatal ruga. *Bar* 150 μm

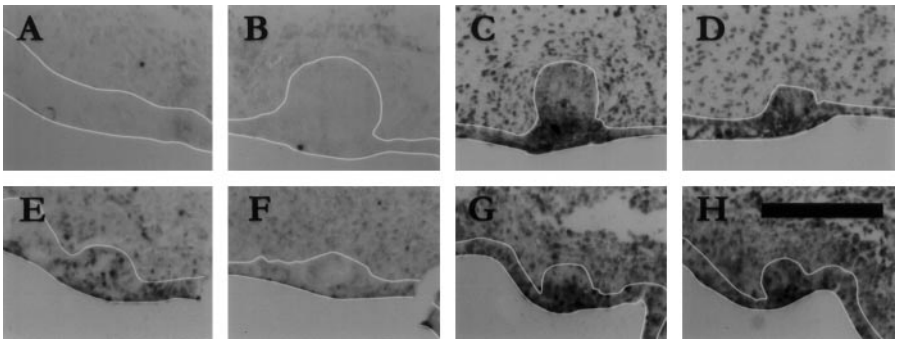

Fig. 3 Apoptosis in vole and mouse diastema buds as detected by TUNEL staining: E12 (**A**), E13 (**B**), E14 (**C**), and E15 (**D**) vole diastema buds; E12 (**E**) mouse D1, E12 (**F**) mouse D2/D3, E13 (**G**) mouse D2 and E13 (**H**) mouse D3 buds. *Bar* 150 µm

counterpart of mouse D1 (Fig. 1F). All diastema tooth germs originated from the dental lamina, which was clearly illustrated in the oral view of whole-mount in situ hybridizations (Fig. 2A, B). They did not, however, grow in mesiodistal direction as molars (Fig. 2C, D).

All mouse rudimentary tooth germs remained as small epithelial swellings, and they were quickly removed apoptotically at early bud stage. In D1 some apoptosis was detected at E12, and the germ disappeared at E13 (Fig. 3E). The D2 and D3 became clearly discernible from palatal rugae only after E12 (Fig. 1L), and we could not distinguish between D2 and D3 at E12. D2 and D3 were maximally developed at E13, but their apoptosis began at E12.5 (Fig. 3G, H), and they disappeared at E14 (not shown). (For more detailed descriptions of the development of mouse diastema tooth germs, see Peterková et al. 1995, Turecková et al. 1996.)

The epithelial thickening of the vole diastema tooth germ was seen at E11.5, at about the same time as in incisors but later than in molars. The bud became visible at E12, and the development was maximal between E13 and E14 (Fig. 1F–G). Instead of proceeding into the cap stage after the late bud stage, the bud degenerated apoptotically. Apoptosis began later in the vole bud, and it survived longer than the mouse diastema buds. First apoptotic cells were detected in the neck of late E13 diastema bud epithelium, and the bud was removed by E16 to E17 (Fig. 3A–D, Fig. 1H–J).

Although there was some epithelial cell rearrangement in the tip of vole E13 diastema bud (not shown), we did not find morphologically distinct enamel knots. The mesenchyma around the diastema buds did not condense properly as in molars (Fig. 1F–N). We did not detect mesenchymal apoptosis, although the rudimentary condensation disappeared slowly.

Gene expression patterns in the diastema tooth germs

The expression patterns of several developmental regulatory genes (*Bmp2, Bmp4, Fgf4, Fgf8, Lef1, Msx1, Msx2, p21, Pax9, Pitx2,* and *Shh*) were compared between the diastema buds and the normal tooth germs of the two species. We analyzed frontal serial sections of stages from E11 to E13 in mice and from E11 to E15 in voles and whole mounts of stages from E10 to E13 in both species.

We detected expression of all these genes in both molars and incisors (not shown). As most of the gene expression patterns have already been reported for molars (Keränen et al. 1998), we compared the gene activities primarily between diastema buds and molars.

Shh was present in the odontogenic epithelia of both species from E11 onwards. It was first expressed throughout the dental lamina (Fig. 4C, D). Then it became limited to the forming palatal rugae and to the budding tooth germs where expression was upregulated in the early epithelial signaling centers (Fig. 4G, H, K, L, U–Ä). It was lost from late bud stage molars at E13, but became again upregulated in the enamel knots by the cap stage (Keränen et al. 1998). In mouse the whole diastema buds expressed *Shh*, and the expression was continuous with either palatal rugae or primary choanae (Fig. 4Y–Ä). In voles the *Shh* expression, which was originally connected to the primary choanae, became limited into the tip of the E13 diastema bud (Fig. 4U–W). The tip of the E13 vole diastema bud also expressed *Bmp2, Lef1,* and *p21,* which are known to be coexpressed with *Shh* in early epithelial signaling centers (Keränen et al. 1998). Because the morphological development of the vole diastema buds was delayed 1 day compared to molars, it seems that the tip of the vole diastema bud corresponds to the E12 molar early epithelial signaling center.

As with *Shh, Bmp2* was expressed in odontogenic epithelium, in the forming tooth germs and the early epithelial signaling centers, but unlike *Shh, Bmp2* was not seen in the palatal rugae (Fig. 4A, B, E, F, I, J, M–T). *Bmp2* was upregulated later than *Shh*, especially in voles, and one of the differences between mouse and vole diastema buds was that mouse diastema buds expressed *Bmp2* early while in vole diastema buds it was seen only at E13. Based on *Bmp2* expression we detected D2/D3 at E12, before it could be distinguished morphologically (Fig. 4R).

As with *Shh, Pitx2* expression was first continuous in the dental lamina in both species and became limited into the budding tooth germs, including the diastema tooth germs in mouse and vole. *Pitx2* became downregulated in the early epithelial signaling centers of both incisors and molars in both species (Fig. 2C, D) while other re-

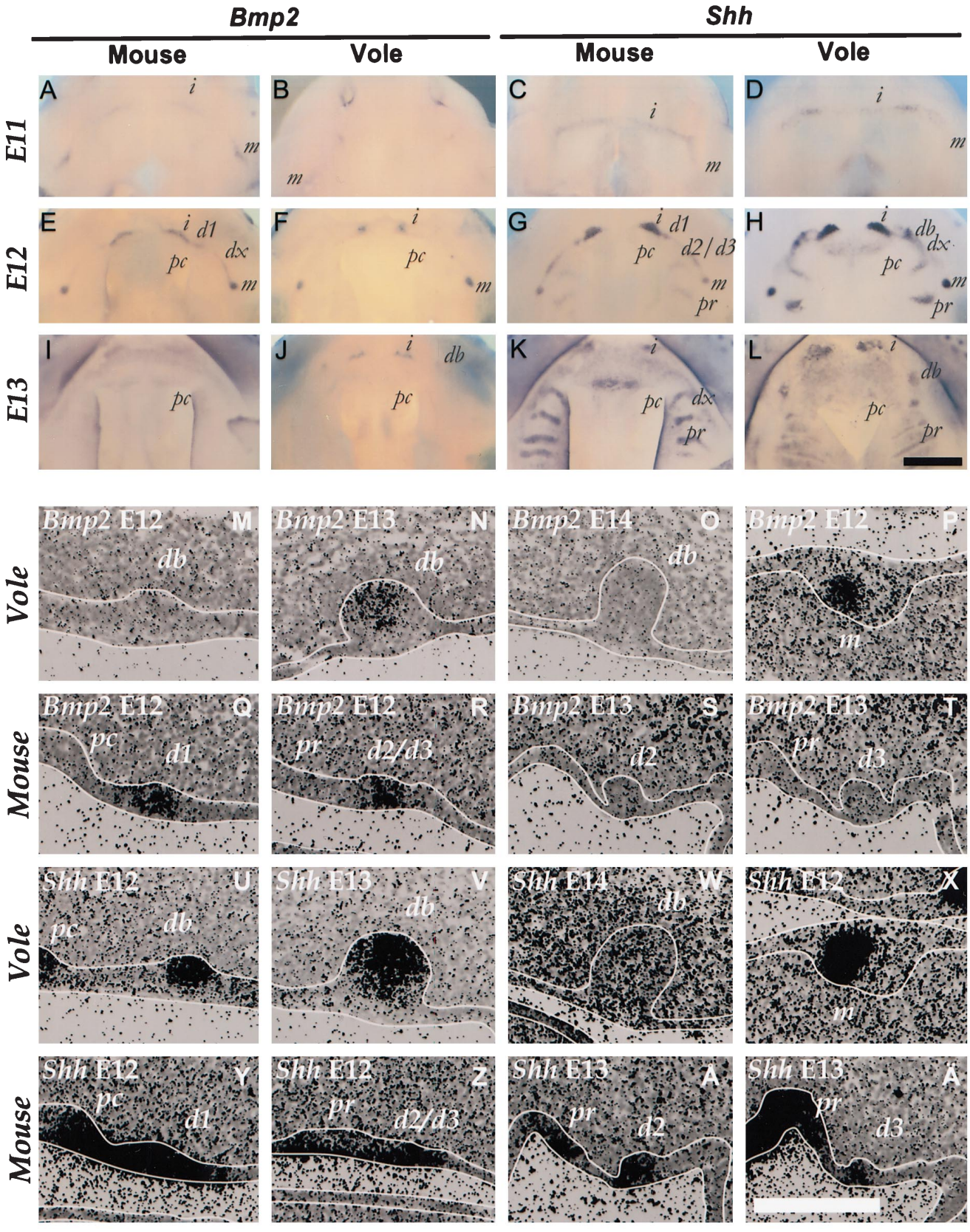

Fig. 4A–Z The development of the upper jaw dental pattern is seen in whole-mount in situ hybridization analysis of *Bmp-2* (**A**, **B**, **E**, **F**, **I**, **J**) and *Shh* (**C**, **D**, **G**, **H**, **K**, **L**) expression in mouse (**A**, **C**, **E**, **G**, **I**, **K**) and in vole (**B**, **D**, **F**, **H**, **J**, **L**). The weak and wide expression domains at E11 (**A**–**D**) became restricted into the dental lamina and tooth germs during E12 (**E**–**H**) and by E13 the molar expressions became downregulated (**I**–**L**). Unlike *Bmp-2*, *Shh* was also seen in palatal rugae. The frontal sections (**M**–**Ä**) show the localization of *Shh* (**U**–**Ä**) and *Bmp2* (**M**–**T**) within the molar (**P**, **X**) and diastema (**M**–**O**, **Q**–**W**, **Y**–**Ä**) tooth germs. *db* Vole diastema bud; *dx* diastema lamina; *d1* mouse first diastema bud; *d2/d3* presumptive mouse second and third diastema buds; *i* incisor, m molar; *pc* primary choana; *pr* palatal ruga. *Black bar* (in **L**) 0.5 mm for panels **A**–**L**; *white bar* (**Ä**) 150 µm for panels **M**–**Ä**

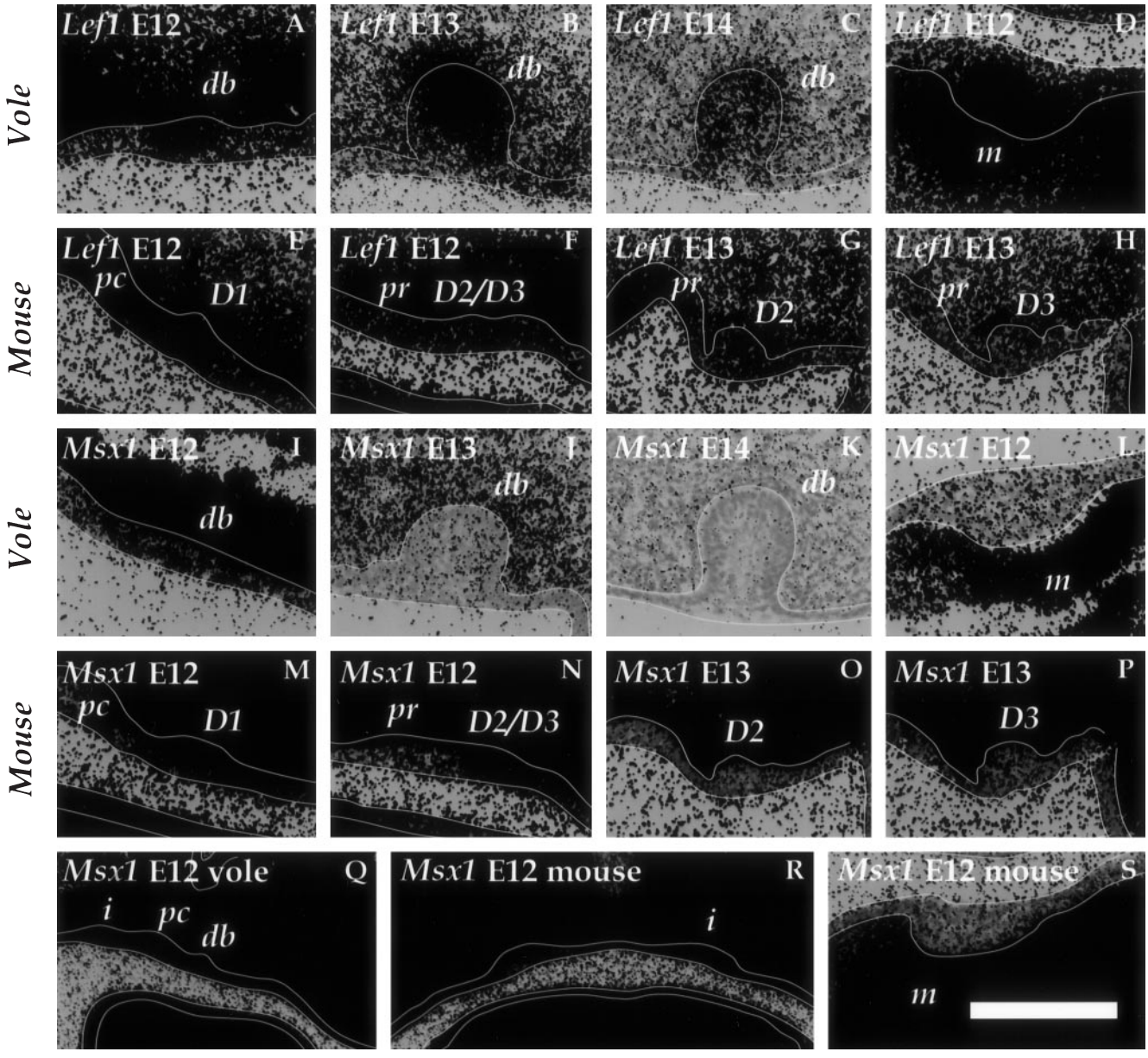

Fig. 5A–S *Lef-1* (**A**–**H**) and *Msx-1* (**I**–**S**) expression in frontal sections of vole (**A**–**D**, **I**–**L**, **Q**) and mouse (**E**–**H**, **M**–**P**, **R**, **S**). *db* Vole diastema bud; *d1* mouse first diastema bud; *d2/d3* presumptive mouse second and third diastema buds; *d2* mouse second diastema bud; *d3* mouse third diastema bud; *i* incisor; *m* molar; *pc* primary choana; *pr* palatal ruga. *Bar* 150 µm

gions of dental epithelium continued to express it intensely.

Epithelial *Lef1* expression resembled that of *Shh*. *Lef1* was expressed in the early epithelium (not shown) As well as in the forming tooth germs and palatal rugae and in the early epithelial signaling centers (Fig. 5A–H). In vole diastema buds *Lef1* was downregulated at E14 (Fig. 5C), while its expression continued in the tips of molar buds and in enamel knots (not shown, Keränen et al. 1998). The mesenchymal *Lef1* expression was downregulated in the diastema region (Fig. 5A–H) but maintained and upregulated in molar and incisor tooth germs (not shown).

As reported earlier by Turescová et al. (1995), *Msx1* expression was intense in the mouse diastema region up to E13 (Fig. 5M–P). In voles the initially high expression of *Msx1* at E11 became quickly downregulated in the diastema mesenchyma as compared to molars (Fig. 5I–L). We found that unlike in molars but as in incisors, the diastema bud epithelia in both species expressed *Msx1* (Fig. 5I–S).

The clearest early difference that we detected between the diastema tooth germs and the molars was the weaker mesenchymal *Pax9* expression in E11 diastema regions of both species (Fig. 6A, B, G, H). The *Pax9* expression was weaker in the E11 anterior maxilla or future diastema region of the serial sections than in future molar or incisor regions and was not upregulated there even later although its expression continued in molar (and incisor) mesenchy-

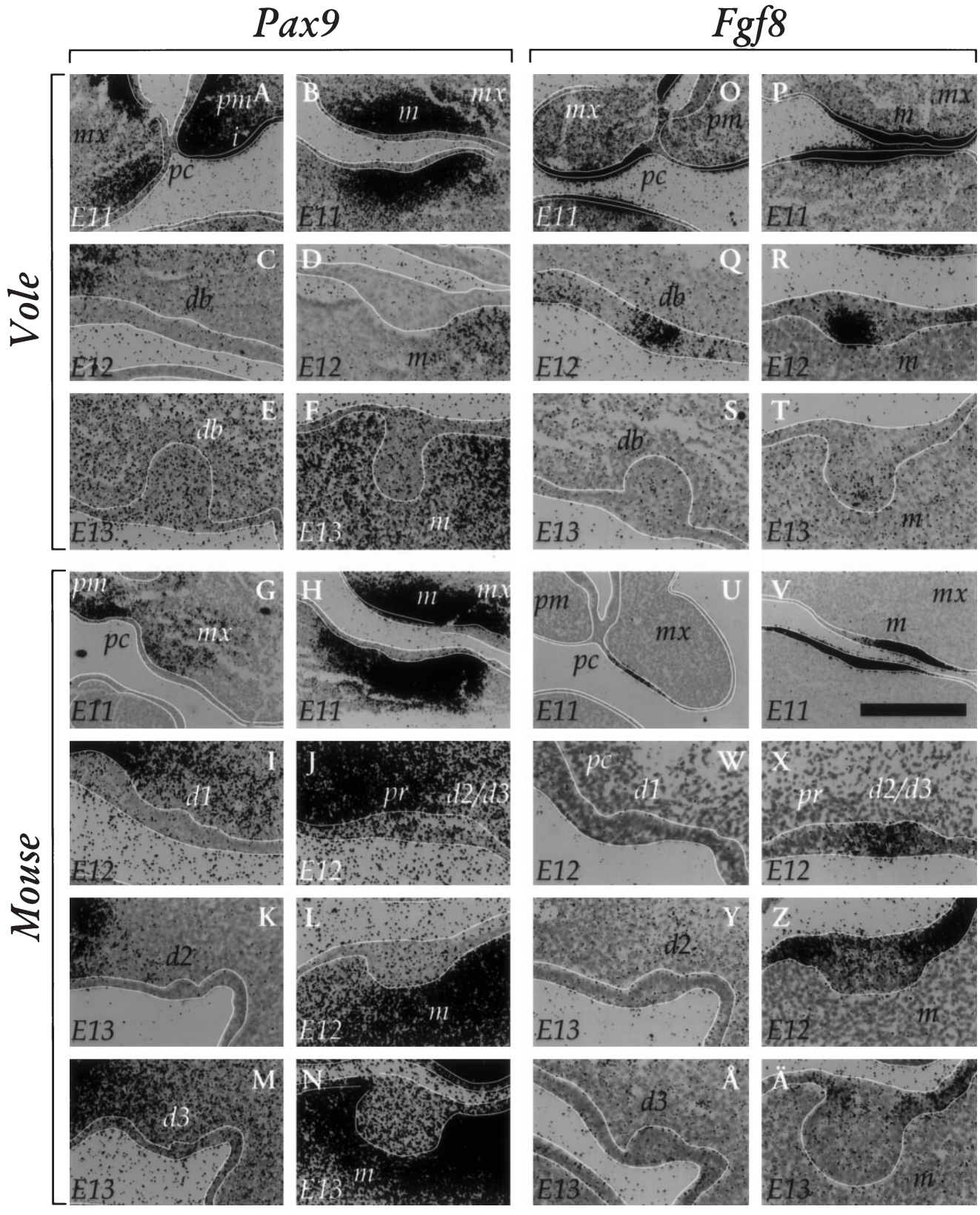

Fig. 6A–Z Expression of *Pax-9* (**A**–**N**) and *Fgf-8* (**O**–**Ä**) in mouse (**G**–**N**, **U**–**Ä**) and vole (**A**–**F**, **O**–**T**) diastema (**A**, **C**, **E**, **G**, **I**–**K**, **M**, **O**, **Q**, **S**, **U**, **W**–**Y**, **Å**) and molar (**B**, **D**, **F**, **H**, **L**, **N**, **P**, **R**, **T**, **V**, **Z**, **Ä**) regions. *Pax-9* was weaker in vole diastema region in anterior maxilla even at E11 (**A**), although *Fgf-8* downregulation in diastema region was clearly detectable at E12 (**Q**). *Pax-9* is also weaker in E11 mice in the anterior maxilla (**G**) than in molar, although *Fgf-8* was detected in both diastema and molar area (**U**, **V**). The *Pax-9* expression continued in E12 and E13 molars in both species (**D**, **F**, **L**, **N**). *Fgf-8* was downregulated in E13 vole (**T**) and mouse molar (**Ä**). For abbreviations, see Figs. 5 and 7. *Bar* 375 µm for panels of E11 mice and voles, 150 µm for the others

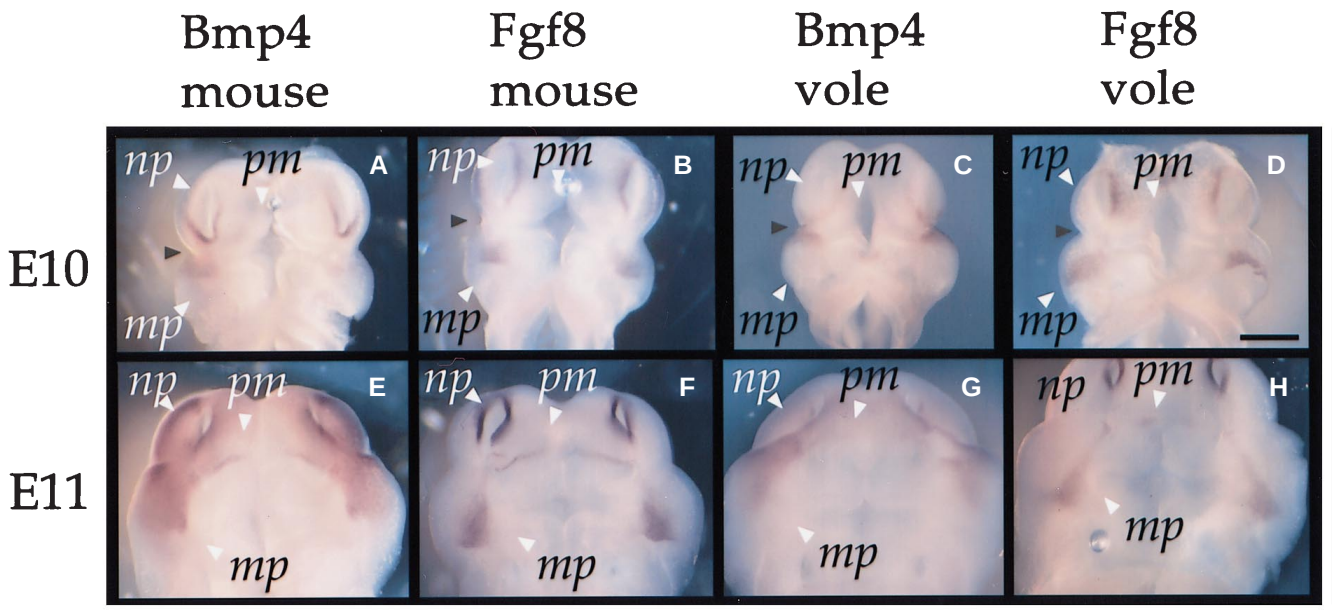

Fig. 7A–H *Bmp4* (**A**, **C**, **E**, **G**) and *Fgf8* (**B**, **D**, **F**, **H**) expression in E10 (**A–D**) and E11 (**E–H**) upper jaws in mouse (**A**, **B**, **E**, **F**) and vole (**C**, **D**, **G**, **H**). *mp* Maxillary prominence; *np* nasal prominence; *pm* premaxilla. *Dark arrowheads* border between nasal prominence and the premaxilla; *bar* 0.5 mm

ma (Fig. 6C–F, I–N). *Pax9* expression has been shown to be stimulated by FGF8 and inhibited by BMP4, and hence we investigated the patterns of *Bmp4* and *Fgf8* expression at E10 with whole-mount in situ hybridization.

Our whole-mount in situ hybridization analysis shows that at E10 the peak of *Fgf8* expression is posterior from the peak of *Bmp4* expression in the epithelium of maxillary prominence (Fig. 7A–D), and this trend is even more apparent in E11 maxilla (Fig. 7E–H). This suggests that the BMP4 signal is relatively stronger than the FGF8 signal in the putative future diastema region while the latter is stronger in the future molar region. The diastema *Fgf8* expression, although weakly present in E12 vole diastema bud epithelium and mouse D2/D3 epithelium (Fig. 6Q, X), was also downregulated earlier than in molar region in both species. In mice *Fgf8* expression was lost from D1 before D2 and D3, and in both species the *Fgf8* expression lasted longer in the posterior end of the molar region (not shown). Interestingly, *Fgf8* at E12 is strongly expressed in early epithelial signaling centers in vole but not in mouse molars (Fig. 6R, Z), which to-

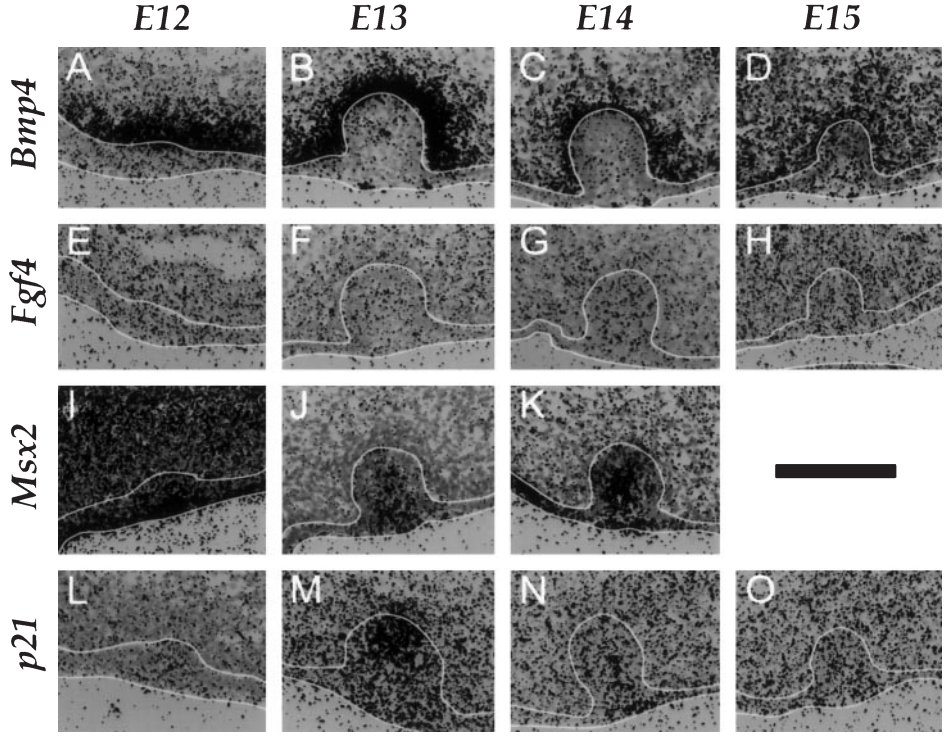

Fig. 8A–O *Bmp4* (**A**–**D**), *Fgf4* (**E**–**H**), *Msx2* (**I**–**K**), and *p21* (**L**–**O**) in vole E12 (**A**, **E**, **I**, **L**), E13 (**B**, **F**, **J**, **M**), E14 (**C**, **G**, **K**, **N**) and E15 (**D**, **H**, **O**) diastema buds. *Bar* 150 µm

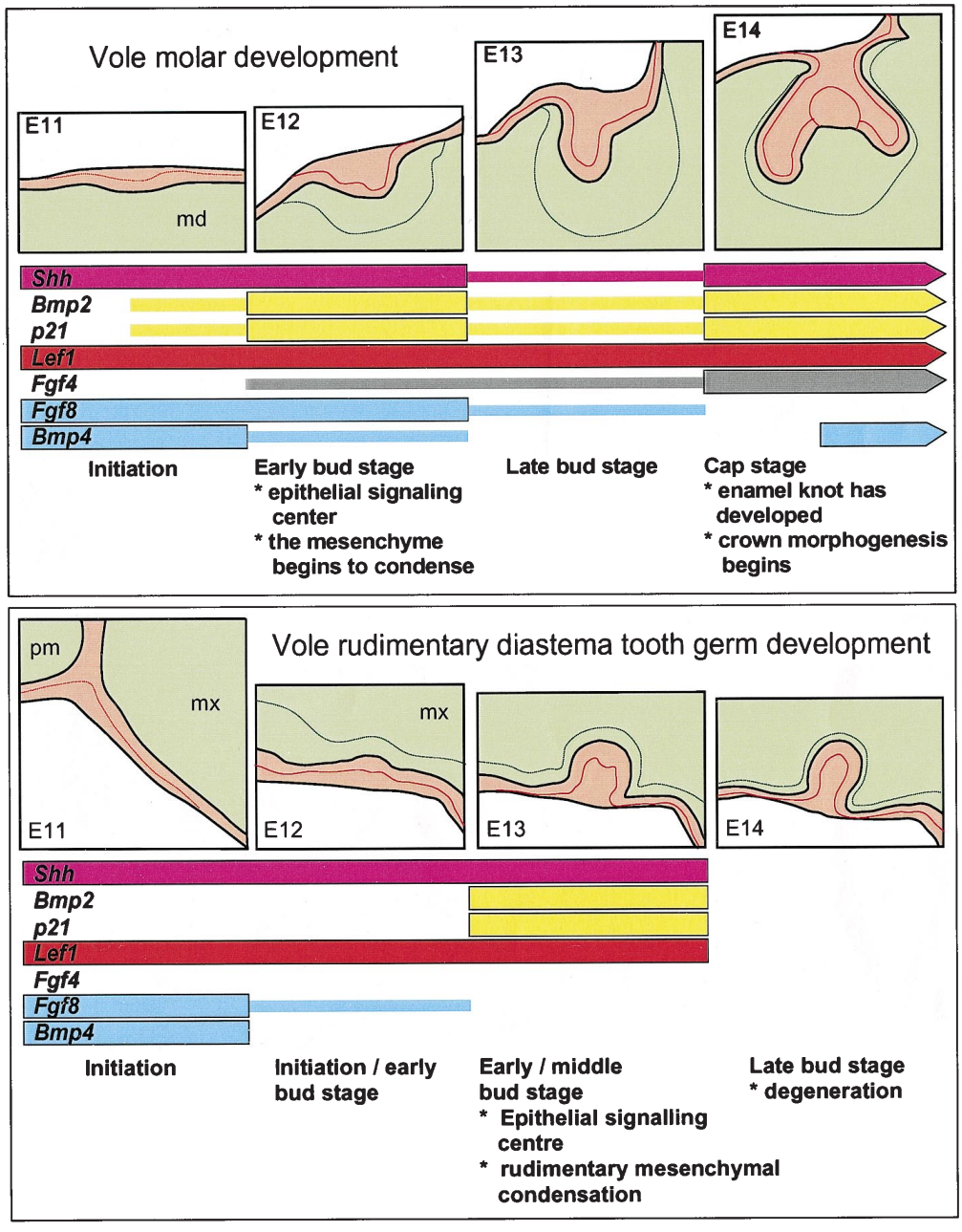

Fig. 9 The association of epithelial gene expression with the development of diastema buds and molars in vole. The morphological development of the diastema buds is delayed compared to the molars of same age and the expressions of the epithelial genes *Bmp2*, *Bmp4*, *Fgf4*, *Fgf8*, *Lef1*, *p21*, and *Shh* were correlated to the morphological stage in diastema and molar tooth germs. In molar buds these genes were all expressed in the early epithelial signaling center. Except for *Lef1*, all were downregulated at late bud stage until the enamel knot formation. Following the formation of enamel knot, which strongly expresses *Bmp2*, *Fgf4*, *Lef1*, *p21*, and *Shh*, the molar proceeds into cap stage. In diastema tooth germs some genes (*Bmp4*, *Fgf8*, *Lef1*, and *Shh*) are initiated normally, but *Bmp2* and *p21* expressions appear later in vole diastema buds than in molars and *Fgf4* is not seen in the diastema tooth germs. *Bmp4* and *Fgf8* are downregulated earlier in diastema buds than in molars. *Bmp2*, *p21* and *Shh* downregulation at late bud stage is normal, but *Lef1* downregulation is not. *Orange* epithelium; *green* mesenchyma; *md* mandible; *mx* maxilla; *pm* premaxilla

gether with *Fgf4* expression in E12 vole but not in mouse (Keränen et al. 1998), suggests that Fgf signaling is upregulated more strongly in early vole than in early mouse molars, although its developmental significance remains to be studied.

In voles *Bmp4* was downregulated in the diastema epithelium by E12, earlier than in the molars (Figs. 8A–D, 9) while in E12 mouse diastema buds *Bmp4* was located in central and superficial cells (not shown), as reported by Turecková et al. (1995). How this expression compares with the late vole epithelial *Bmp4* expression in single cells (Fig. 8B, C), and whether it is involved in the apoptotic removal of the diastema buds (Peterková et al. 1998) remains to be studied. *Bmp4* was also initially present in the subepithelial mesenchyma of both species, but was weaker than in molar areas even at E12 (not shown), and in voles it colocalized with the disappearing rudimentary mesenchymal condensation (Fig. 8A–D).

Unlike in molars, where both *Bmp4* and *Msx2* are biased to the buccal side of the tooth germ, in vole diastema buds their expression domains were similar in both buccal and lingual sides (Fig. 8A–D, I–K). These gene expression differences may be associated with the finding that the early epithelial signaling center in the vole diastema buds was located at the tip of the bud, not in its lingual aspect as in molars.

The early epithelial signaling center of the vole diastema bud coexpressed *Shh*, *Bmp2*, *p21*, and *Lef1* (Figs. 4M–O, U–W, 5A–C, 8L–O). However, other epithelial genes such as *Bmp4* and *Fgf8*, which are present in molar signaling centers, were downregulated (Fig. 9) and *Msx2* and *Fgf4* were not upregulated (Fig. 8E–K), indi-

cating that it is different from molar early epithelial signaling center. The lack of a morphologically distinguishable enamel knot with strong *Fgf4* expression in the vole diastema bud indicates that its development stops at the stage that corresponds to E13 bud stage molars before the transition to cap stage (Fig. 9).

Discussion

Although mouse and vole have the same number of molars and incisors, and the histological development of their teeth and the correlation of gene expression patterns to the developing histology (except for early Fgf signaling) are similar in the two species (Keränen et al. 1998), the diastema regions of the two species differ. Mouse has three small diastema buds which develop into early bud stage, whereas vole has one large diastema bud which develops into late bud stage. Mouse diastema buds do not contain separate signaling centers, but a clear early epithelial signaling center develops within the vole diastema buds. This center, however, is located at the tip of the bud unlike in molars, where it is located lingually, and in the tips of the diastema buds the timing of gene expression differs from that of the molar early epithelial signaling center. Subsequently, the vole diastema buds neither develop enamel knots nor proceed to the cap stage. All diastema buds in both species degenerate apoptotically. To investigate the genetic basis of the developmental arrest in the two species we compared the expression patterns of the developmental regulatory genes *Bmp2*, *Bmp4*, *Fgf4*, *Fgf8*, *Lef1*, *Msx1*, *Msx2*, *p21*, *Pax9*, *Pitx2*, and *Shh* between the rudimentary diastema tooth germs and the normal teeth.

Early odontogenic epithelium and rudimentary diastema tooth germs

Teeth are serially homologous organs (Stock et al. 1997). Hence the basic genetic mechanisms governing odontogenesis ought to be conserved between teeth of different identity as well as between the teeth in different species (Keränen et at 1998). Therefore the developmental differences between tooth germs should be reflected in local modifications in the expression patterns of shared developmental regulatory genes. The species-specific locations (and numbers) of tooth germs presumably depend on where the basic genetic program for tooth germ initiation is activated (Tucker et al. 1998). Classic recombination experiments have shown that the identity (and hence the morphological development) of an individual tooth germ depends on the early epithelium, which contains the necessary information for odontogenesis (Kollar and Mina 1991; Lumsden 1988; Mina and Kollar 1989), and recently *Bmp4* has been implicated in the determination of identity and tooth specific morphogenesis (Tucker et al. 1998).

All tooth germs, including the diastema buds, arose from the dental lamina in both species. The oral epithelium forming the future dental lamina could be visualized with the expression of *Pitx2* and *Shh* genes (Fig. 2A–D, 4C–D). These genes were first expressed as a continuous band through maxillary and premaxillary epithelium, and they became subsequently limited into budding tooth germs, including the diastema buds, where they were upregulated. The tooth germ epithelia thereafter became compartmentalized as the early epithelial signaling centers expressing, for example, *Shh, Lef1, Bmp2, p21,* were formed in molars and incisors of both species and in vole diastema buds. The restriction of expressions into individual tooth germs occurred at E12, i.e., after the odontogenic potential had been transferred into the mesenchyma. However, we did not note similar localized patterning in any of the mesenchymal genes that we studied, and the exact locations of the individual tooth germs may be determined within the epithelium, although a permissive signal from the mesenchyma is possible.

Although the developing tooth germs could be visualized with the expression of epithelial genes such as *Shh* and *Pitx2,* the developmental arrest of the diastema tooth germs was reflected by spatiotemporal differences in gene expressions as compared with molars. We detected all analyzed genes except *Fgf4* in the vole diastema tooth germs, and these were downregulated or upregulated at different times than with the molars (Fig. 9). Some of the genes (epithelial *Fgf8* and *Bmp4,* all the mesenchymal genes) were downregulated earlier the molars, some were not induced in the early signaling centers (*Msx2*, *Fgf4*) while others were expressed normally until late bud stage (*Shh, Lef1*), and the upregulation of yet other genes (*Bmp2, p21*) was delayed (Fig. 9). Since these genes may be involved in the early events of the odontogenesis, the differences between diastema buds and molars suggest both that the signaling cascades governing various components of early odontogenesis (e.g., the determination of the tooth location, epithelial invagination, determination of tooth identity, and transfer of odontogenic potential from epithelium to mesenchyma) are at least partially separate, and that only some of these are originally affected in the diastema buds.

Although the early odontogenesis depends on the oral epithelium, the earliest clear genetic difference that we found was weaker *Pax9* expression in diastema than in molar (or incisor) mesenchymas of both species at dental lamina stage (E11). *Pax9* is induced by epithelial FGF8, but at E10 BMP4 can inhibit *Pax9* expression (Fig. 9; Neubüser et al. 1997; Peters et al. 1998). The peak of *Fgf8* expression seemed to be posterior from the peak of *Bmp4* expression in E10 mouse and vole maxillary prominences (Fig. 7A–D), suggesting that the downregulation of *Pax9* in the anterior maxilla, or future diastema region may be associated with stronger expression of *Bmp4* than *Fgf8*.

Fgf8 and *Bmp4* have been suggested to participate in the transfer of odontogenic potential from epithelium to the mesenchyma by inducing mesenchymal *Pax9* and *Msx1* expression (Neubüser et al. 1997; Tucker et al. 1998; Vainio et al. 1993), both of which are necessary for the mesenchymal *Bmp4* expression. BMP4 is essen-

tial for tooth morphogenesis (Chen et al. 1996). It can induce epithelial *p21* and *Lef1*, and it has been speculated that mesenchymal BMP signals are necessary to induce enamel knot formation in tooth germs before they can proceed into cap stage (Kratochwil et al. 1996; Jernvall et al. 1998). In vole diastema buds both mesenchymal *Bmp4* and other mesenchymal genes became downregulated after *Pax9*. Together with the early downregulation of epithelial *Bmp4* and *Fgf8*, this suggests that the *Pax9* mediated transfer of odontogenic potential is disturbed specifically in vole diastema buds. The vole diastema tooth germs resemble morphologically the tooth germs of *Pax9*$^{-/-}$ mutant mice (Peters et al. 1998), and it is possible that early disturbances in the *Pax9*-dependent signaling pathway cause the late bud stage developmental arrest in the vole diastema tooth germs, even when other developmental processes are initially normal.

Indeed, the expression patterns of *Shh* and *Lef1* were quite similar in vole diastema buds and molars until late bud stage. Both *Shh* and *Lef1* have been implicated in epithelial invagination and early dental patterning (Hardcastle et al. 1998; Zhou et al. 1995). The species-specific differences in their expression patterns and their late disturbance in vole diastema buds suggest that these genes are involved in the early determination and/or budding of tooth germs. Moreover, the restriction of epithelial gene expression (*Bmp2, Lef1, Pitx2, Shh*) into forming tooth germs occurs after the early induction of *Pax9*. This restriction and budding occurs also in the areas which do not express *Pax9*, suggesting that the determination of exact locations of the tooth germs and their early development occurs parallel to and at least partly independently of the early induction of odontogenic mesenchyma by BMP4.

In addition to the differences between diastema tooth germs and molars (or incisors), there also were differences between the diastema tooth germs of the two species. Unlike the vole diastema buds, the mouse diastema buds degenerate at the early bud stage. Interestingly, we found that *Bmp2* expression was both upregulated and downregulated earlier in the mouse than in the vole diastema buds (Fig. 4E, F, M–T). Another difference between the two species was the continuing *Msx1* expression in the mouse but not in the vole diastema mesenchyma (Fig. 5I–P). Neither of these differences can explain the differences between the diastema regions of the two species, but it is reasonable to hypothesize that some early epithelial odontogenic signaling pathway may be differently affected in mouse than in vole, although the early expressions of *Bmp4, Fgf8,* and *Pax9* in the two species are similar.

Evolution of rudimentary diastema tooth germs

The most primitive rodent dental formula, also found in the earliest known rodent from the Eocene (Meng et al. 1994), consists of one incisor, two premolars, and three molars. Because mice have three diastema buds, at least one of them may have persisted for over 50 million years.

The persistence of rudimentary organs was been commented upon as early as Darwin (1856). Unlike the pseudogenes, which degenerate relatively soon under the random mutation load (e.g., Li and Graur 1991), the developmental programs of rudimentary organs seem to be more resistant to modifications. The reason for this is probably the modular use of the individual signaling pathways for various developmental processes. If even one gene within such signaling cascade were lost, the effects could be lethal or deleterious in multiple organs, including other teeth (Behrens et al. 1997; Feldman et al. 1995; van Genderen et al. 1994; Hogan 1996; Peters et al. 1998; Roessler et al. 1996; Satokata and Maas 1994; Semina et al. 1996). On the other hand, although the developmental programs themselves are conserved, the morphological evolution can be fast (Brunet-Lecomte and Chaline 1991), which suggests that the spatiotemporal application of the conserved programs can evolve flexibly.

Our data support the view that the early epithelial gene expression patterns are important in the patterning of the mesenchyma, depending on the identity of the tooth germs (Tucker et al. 1998). Because there are no diastema buds in the mandibles of either species, and because the reduction in tooth numbers in rodents tends to be more pronounced in the lower than the upper jaws (Luckett 1985), the loss of tooth germs is probably less favored in the upper than the lower jaw diastema regions. We found that both vole diastema buds and mouse D1 are associated with the primary choanae, where the maxillary processes fuse with the frontonasal process, while the mouse D2 and D3 were connected to the palatal rugae. The morphological associations were seen also as continuous expression areas of some genes such as *Lef1* and *Shh*. The morphological connections between tooth germs, nasal epithelium, and palatal rugae and the similarities in their differentiation support the notion that these structures develop during ontogeny from a common epithelial precursor (Peterková 1985). It is therefore possible that the more complex morphological development of the upper than the lower jaw also slows the evolutionary loss of upper diastema tooth germs by supporting the early epithelial patterning necessary for the spatial determination and initiation of the tooth germs.

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