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SHORT COMMUNICATION

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Expression of Dlx genes during the development of the murine dentition

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Abstract The mammalian Dlx homeobox gene family has been shown to play multiple roles in tooth development, but a detailed comparison of the expression pattern of all members throughout tooth development has been lacking. We provide such an analysis for the six known murine Dlx genes. The expression patterns for these genes allow a refinement of previously proposed models for the role of Dlx genes in tooth type specification and raise the possibility of roles for subsets of these genes in tooth initiation, morphogenesis (enamel navel formation, enamel knot induction, cervical loop growth), and enamel formation. The relationship of Dlx gene expression to their genomic organization suggests coordinate regulation of linked genes at early stages but regulatory differences at later stages.

Key words Dlx · Homeobox · Dentition · Tooth

Introduction

Mammalian teeth provide a useful model system for studying molecular control of fundamental aspects of pattern formation and organogenesis (Peters and Balling 1999; Weiss et al. 1998). Tooth shape varies along the mesial-distal axis of the jaws, resembling the sequential arrangement of other systems, such as the vertebral column (Weiss et al. 1998; Zhao et al., in press). Tooth initiation, morphogenesis, and cytodifferentiation are regulated by a series of interactions among epithelial and mesenchymal components (Ruch 1987), and a number of signaling and transcription factors involved in these interactions have been identified (Peters and Balling 1999; Zhao et al., in press). Fully formed teeth possess cell types and gene products found nowhere else in the body (Ruch 1987).

Among the genes likely to play important roles in the control of tooth development are members of the Dlx family of homeobox transcription factors. Six Dlx genes (*Dlx1*, *Dlx2*, *Dlx3*, *Dlx5*, *Dlx6*, and *Dlx7*) have been isolated from mammals and shown to be arranged in three closely linked pairs, each occupying different chromosomes (summarized in Stock et al. 1996). Functional analyses have suggested roles for Dlx genes at several stages of tooth development and provided evidence for functional redundancy among family members (Depew et al. 1999; Qiu et al. 1995, 1997).

Although expression of *Dlx1*, *Dlx2*, and *Dlx3* has been described in the developing murine teeth (Robinson and Mahon 1994; reviewed by Peters and Balling 1999; Weiss et al. 1998; Zhao et al., in press), systematic and dentition-specific analyses are not available for most members of the Dlx family. A detailed comparison of the expression patterns of all six murine Dlx genes throughout tooth development would provide important clues to likely roles not yet uncovered by functional experiments and aid in the design of further experiments. In addition, consideration of Dlx gene expression pattern in relation to their unusual genomic organization might suggest mechanisms of their regulation. Here we describe the expression of the six murine Dlx genes from the period prior to the appearance of tooth germs to the beginning of cytodifferentiation. We have concentrated on the expression of *Dlx5*, *Dlx6*, and *Dlx7*, as the existing literature is

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Additional documentary material has been deposited in electronic form and can be obtained from http://link.springer.de/link/service/journals/00427/index.htm.

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very limited for the first two of these genes and entirely lacking for the last.

Materials and methods

Murine embryos (Swiss Webster) were collected at different stages [the presence of a vaginal plug=embryonic day (E) 0.5]. Embryos (E10.5), dissected jaws (E11.5–E14.5), and dissected tooth germs (E15.5–postnatal day 2) were processed for whole-mount in situ hybridization (Wilkinson 1992). Specimens were also cryosectioned at 12–20 µm to analyze tissue distribution of the signals. Nomarski optics was applied to some sectioned specimens. The riboprobes used in this study are as follows: *Dlx1* (900 bp), *Dlx2* (567 bp), *Dlx3* (760 bp), *Dlx5* (400 bp), *Dlx6* (325 bp), and *Dlx7* (936 bp).

Results and discussion

First branchial arch development

The earliest morphological sign of tooth development in the mouse occurs at E11.5 (Ruch 1987). However, tooth location and tooth type are believed to be determined prior to this (Lumsden 1988). All six Dlx genes were expressed in the mesenchyme of the first branchial arch (data are presented as a supplementary material in electronic form and can be obtained from http://link.springer.de/link/service/journals/00427/index.htm), with *Dlx3* additionally expressed the mandibular epithelium in the most mesial region (Fig. 1D). The pattern of *Dlx1–Dlx6* expression in the first branchial arch, from which much of the dentition develops, was described by Qiu et al. (1997) for E9.5 embryos. We observed generally similar patterns at E10.5 in the following aspects. *Dlx1–Dlx6* were expressed in the mandibular arch (Fig. 1), and only *Dlx1* and *Dlx2* were expressed in the maxillary process (data not shown). *Dlx2*, *Dlx5*, and *Dlx6* were expressed in almost the entire mandibular arch along the distal-mesial axis, except for the most mesial region (Fig. 1B, E, F; axial definitions for the mandibular arch are given in Fig. 1). In contrast to previous analyses, we found that the expression domain of *Dlx1* in the mandibular arch was more distally restricted than those of *Dlx2*, *Dlx5*, and *Dlx6* (Fig. 1A). We also found expression of *Dlx3* and *Dlx7* in the distal-labial region of the mandibular arch, but clearly away from the tooth-forming area (oral side; Fig. 1C, D). None of these Dlx genes was expressed in the lingual side of the medial nasal process, the region from which upper incisors develop (e.g., Fig. 1D). The expression patterns of Dlx genes in the E10.5 first branchial arch are summarized in Fig. 1G.

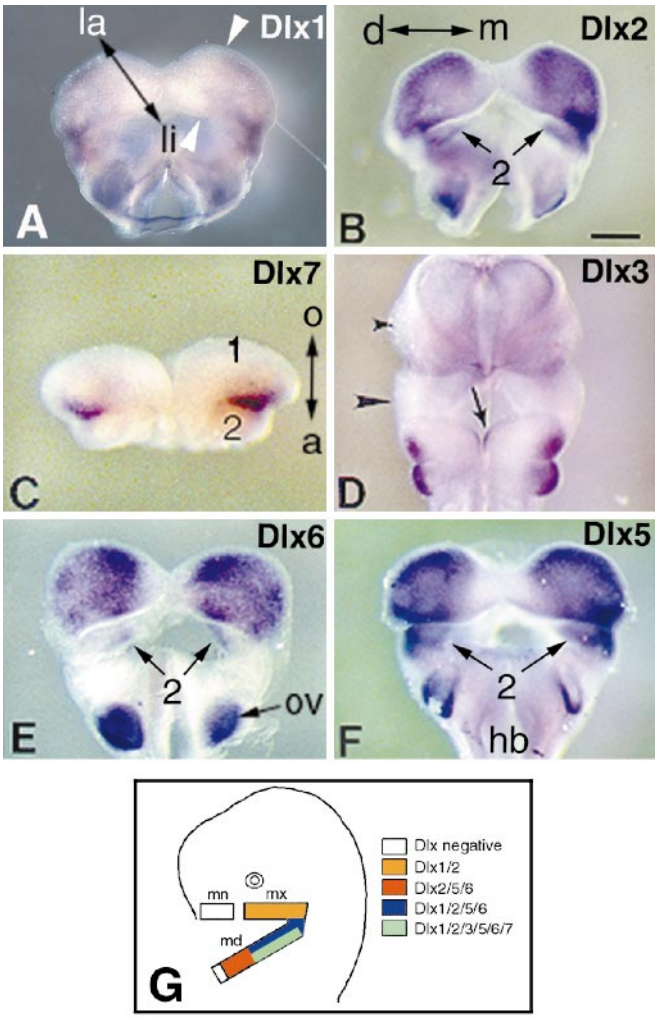

Fig. 1A–G Expression of Dlx genes in the mandibular arches of E10.5 murine embryos. **A–C** Axial definitions. *Dlx1* (**A**), *Dlx2* (**B**), *Dlx5* (**F**), and *Dlx6* (**E**) are viewed from the oral side (tooth-forming side). The second branchial arches are incompletely removed, and the remaining tissues (*2*, *arrows*) are visible in **B**,**E**,**F**. **A** *Arrowheads* Mesial boundary of *Dlx1* expression domain. *Dlx7* (**C**) and *Dlx3* (**D**) are shown in frontal view. Expression of *Dlx3* (**D**) is also seen in the epithelium of the mesial region of the mandibular arch (*arrow*) and the nasal pit (hardly visible in this picture). *Small arrowhead* Nasal pit; *large arrowhead* maxillary process. **G** Schematic summary of Dlx gene expression in the mesenchyme of the first branchial arch at E10.5. *1* First (mandibular) branchial arch; *2* second branchial arch; *a* aboral; *d* distal; *hb* hindbrain; *la* labial; *li* lingual; *m* mesial; *md* mandibular arch; *mn* medial nasal process; *mx* maxillary process; *o* oral; *ov* otic vesicle. *Scale bars* **A–C**,**E**,**F** 300 µm; **D** 350 µm. (Histological section data of the E10.5 first branchial arches are presented as supplementary material in electronic form and can be obtained from http://link.springer.de/link/service/journals/00427/index.htm)

The expression patterns of Dlx genes in the first branchial arch at E9.5 (Qiu et al. 1997) and E10.5 (this report) suggest a role in regionalization of the arch. Functional analysis has demonstrated such role for *Dlx1*, *Dlx2*, and *Dlx5* (Depew et al. 1999; Qiu et al. 1995, 1997). However, the involvement of Dlx genes in patterning the dentition requires more investigation. Dentition pattern is very similar between the upper and lower jaws in most mammals (Weiss et al. 1998 and references therein; Zhao et al., in press), suggesting similar molecular systems for controlling the upper and lower dentition. The Dlx family shows different expression patterns between the upper and lower jaws at the stages when tooth type and location are specified. For example, only *Dlx1* and *Dlx2* are expressed in both jaws, the

rest members of the family are expressed only in the lower jaw. Although *Dlx1* and *Dlx2* have similar early expression patterns in the maxillary process (presumptive upper molar region), the expression of *Dlx2* in the mandibular arch extends farther mesially than that of *Dlx1* to the incisor regions, which is indicated by the expression of the *Pax9* gene (Peters and Balling 1999 and references therein). These raise the possibility that Dlx genes are not involved in patterning the dentition, or that different members of the Dlx family are involved in upper or lower dentition patterning systems (Qiu et al. 1997; Thomas et al. 1998).

Thomas et al. (1998) proposed a homeobox code for specifying tooth type, in which *Dlx1* and *Dlx2* control maxillary molars, and *Dlx5* and *Dlx6* control mandibular molars. This proposal is based on regionalized expression of Dlx genes along the mesial-distal axis of the first branchial arch (Qiu et al. 1997) and the absence of maxillary molars in *Dlx1*/*Dlx2*-knockout mice (in which other teeth were unaffected; Qiu et al. 1997). The dental phenotype of the *Dlx1*/*Dlx2* knockout can be alternatively interpreted as a result of functional redundancy among genes in the lower molar region (such as *Dlx5* and *Dlx6*; Peters and Balling 1999). Inactivation of *Dlx5* does not affect tooth morphology or location (Depew et al. 1999). Double knockout of *Dlx5* and *Dlx6* will provide information on their roles in dentition patterning.

Tooth initiation

The earliest morphological sign of tooth initiation is a thickening of the oral epithelium to form the dental lamina. Expression of *Dlx2* and *Dlx3* was detected in the labial part of the dental lamina of molar and incisor primordia in both jaws at E12.5 (Fig. 2A, B). No other Dlx genes were found in the early epithelial thickenings. *Dlx2* was also detected in the dental mesenchyme at these stages (data not shown). Robinson and Mahon (1994), using radioactive probes, observed similar expression patterns for *Dlx2* at the similar stages. However, the expression of *Dlx3* that they described only in the dental mesenchyme is different from that that we observed only in the dental epithelium. This discrepancy could be due to the different techniques used, of which the radioactive in situ hybridization has lower cellular resolution.

Tooth development is initiated by signals from the epithelium (Lumsden 1988; Mina and Kollar 1987). *Dlx2* and *Dlx3* are the only Dlx genes expressed in the dental lamina, suggesting a possible role in tooth initiation. As mice lacking *Dlx2* show no dental phenotype (Qiu et al. 1995), and mice lacking *Dlx3* die before the stage of tooth initiation, due to defects in the placenta (Morasso et al. 1999), however, it may be necessary to specifically inactivate both genes in the dental tissues to test this possibility.

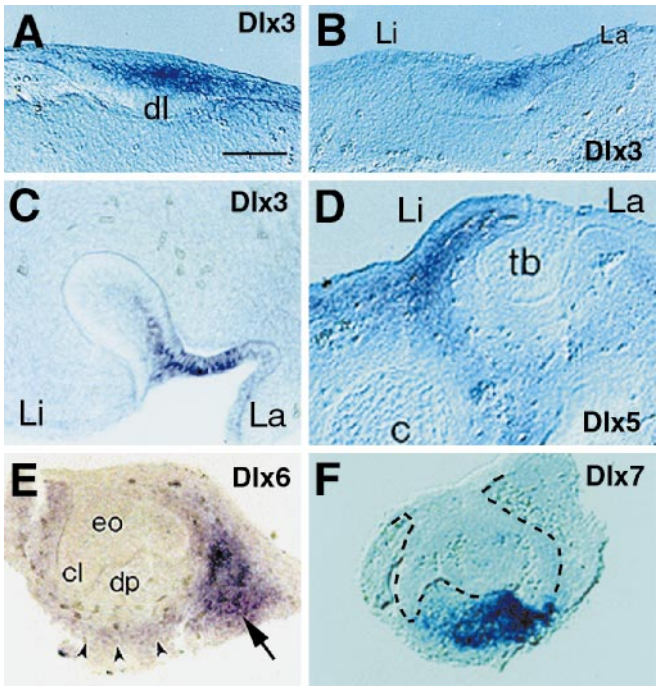

Fig. 2A–F Expression of Dlx genes in the early development of teeth. *Dlx3* is expressed in the dental lamina of lower incisors (**A**) and lower molars (**B**) at E12.5, and the labial enamel epithelium of upper molars at E13.5 (**C**). Note: *Dlx2* shows similar expression patterns at the above stages with additional expression in the dental mesenchyme (data not shown; see Fig. 5). **D** *Dlx5* expression in the lower jaw at E13.5. Signals are detected in the jaw mesenchyme but not in tooth germs. **E** *Dlx6* expression in lower molar at E14.5. Expression is observed in the jaw mesenchyme (*arrow*) and dental follicle (*arrowheads*). **F** Expression of *Dlx7* in lower molar at E15.5. *Dlx2*, *Dlx3*, and *Dlx5* show similar expression patterns at this stage (data not shown). *Dashed line* Outline of the enamel organ; *c* Meckel's cartilage; *cl* cervical loop; *dl* dental lamina; *dp* dental papilla; *eo* enamel organ; *La* labial; *Li* lingual; *tb* tooth bud. *Scale bars* **A**–**C** 200 μm; **D** 100 μm; **E**,**F** 150 μm

Tooth morphogenesis

After the dental lamina forms, it invaginates into the dental mesenchyme to form an epithelial bud (bud stage) and subsequently develops into cap- (cap stage) and bell- (bell stage) shaped tooth germs. Before the cap stage only *Dlx2* and *Dlx3* were expressed in the tooth germs, while expression of the other genes was detected in other tissues in the jaw (e.g., Fig. 2D). At the bud stage *Dlx2* and *Dlx3* were expressed in the labial dental epithelium of the tooth bud (Fig. 2C). *Dlx2* was also expressed in the mesenchyme (data not shown). At the cap (E14.5) and early bell (E15.5) stages, *Dlx1* and *Dlx6* were expressed in the dental follicle (Fig. 2E). Expression of *Dlx3*, *Dlx5*, and *Dlx7* was detected in the dental papilla (e.g., Fig. 2F). *Dlx5* was additionally expressed in the cervical loop (data not shown).

At the late bell stage (E16.5–E17.5), the six Dlx genes showed complex expression patterns in the developing molars. In the dental mesenchyme *Dlx1* was still expressed in the dental follicle (Fig. 3A). *Dlx3* and *Dlx7*

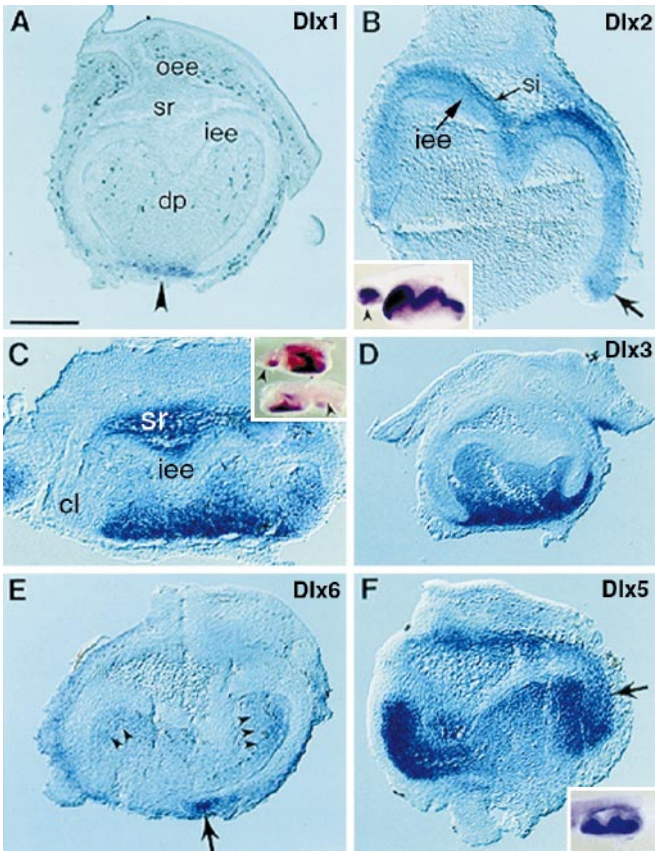

Fig. 3A–F Comparison of Dlx gene expression patterns in molar germs at the late bell stage (E16–17.5). *Insets* Whole-mount in situ hybridization of the tooth germs; *small arrowheads* second molars. **A** *Dlx1* expression in the dental follicle (*large arrowhead*). **B** *Dlx2* expression in the inner enamel epithelium, the stratum intermedium, and the cervical loop. **C** *Dlx7* expression in the dental papilla and the stellate reticulum. **D** *Dlx3* expression in the dental papilla and dental follicle. **E** *Dlx6* expression in the dental papilla (*small arrowheads*) and the cervical loop (*arrow*). **F** *Dlx5* expression in the dental papilla, the cervical loop (*arrow*), and the outer enamel epithelium. *cl* Cervical loop; *dp* dental papilla; *iee* inner enamel epithelium; *oee* outer enamel epithelium; *si* stratum intermedium; *sr* stellate reticulum. *Scale bars* **A**–**F** 100 µm; **C**,**F** *insets* 650 µm; **B** *inset* 550 µm

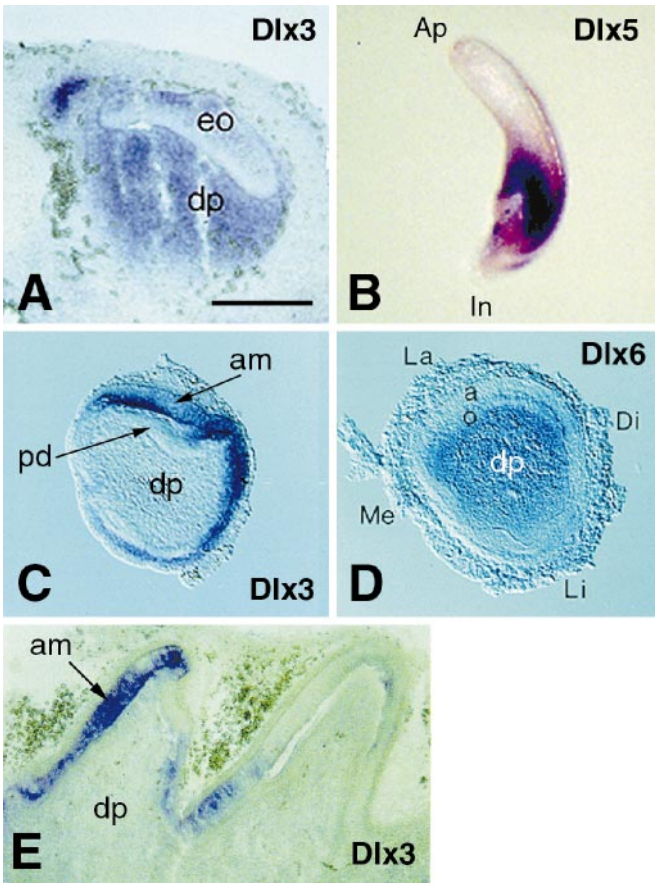

Fig. 4 Expression of Dlx genes in incisors (**A**–**D**) and postnatal molars **E**. **A** *Dlx3* in the lower incisors at E14.5. **B** Expression of *Dlx5* in lower incisor at E16.5 (whole mount). **C** Cross-section of E16.5 incisor in the apical region shows *Dlx3* expression in the ameloblasts. **D** Cross-section of E16.5 incisor in the incisal region shows *Dlx6* expression in the dental papilla. **E** Expression of *Dlx3* in the cusps of lower molars at the postnatal day 2. *a* Preameloblast; *am* ameloblast; *Ap* apical; *Di* distal; *dp* dental papilla; *eo* enamel organ; *In* incisal; *La* labial; *Li* lingual; *Me* mesial; *o* odontoblast; *pd* predentine. *Scale bars* **A** 100 µm; **B** 1 mm; **C** 120 µm; **D** 150 µm; **E** 60 µm

showed stronger signals in the region between the cervical loops (Fig. 3C, D). Expression of *Dlx3* extended to the dental follicle (Fig. 3D). *Dlx5* and *Dlx6* were expressed in the dental papilla, with higher levels in the developing cusps (Fig. 3E, F). In the epithelial components *Dlx2* was expressed in the inner and outer enamel epithelia, stratum intermedium, and cervical loop (Fig. 3B). *Dlx7* was expressed in the stellate reticulum (Fig. 3C). *Dlx5* and *Dlx6* were expressed in the cervical loop (Fig. 3E, F). The spatial-temporal expression patterns of the Dlx genes in molar development are summarized in Fig. 5.

Dlx gene expression was observed in the upper and lower incisors during development, and the patterns in the early morphogenesis of incisors (e.g., Fig. 4A) resembled those seen in molars. The lower incisor specimens were analyzed in sections and presented here. At E16–E17 all six Dlx genes were expressed in the incisal region of the former tooth type, a region of active tissue growth (Fig. 4B, D).

During the cap and bell stages all six Dlx genes show complex expression patterns in the developing teeth (Fig. 5), suggesting divergent roles for these genes in tooth morphogenesis. Formation of the cap-shaped tooth germ involves transient structures within the enamel organ known as the enamel knot, enamel cord, and enamel navel (Zhao et al., in press and references therein). *Dlx2* and *Dlx3* are expressed in the enamel navel (labial region of the enamel epithelium). Demonstrating a role for *Dlx2* and *Dlx3* in morphogenesis based on their enamel navel expression may also require simultaneous inactivation of these genes. The enamel knot is believed to function as an organizer in the developing tooth germ, and its formation requires signals from the dental mesenchyme (Peters and Balling 1999 and references therein). While

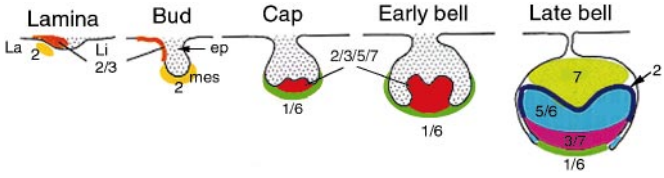

Fig. 5 Schematic summary of Dlx gene expression in the developing molars. *Above* Developmental stages; *numbers* Dlx genes; *ep* dental epithelium; *La* labial; *Li* lingual; *mes* dental mesenchyme

we did not find expression of any Dlx genes in the enamel knot, the expression of some of these genes in the underlying mesenchyme (*Dlx2* at the bud stage and *Dlx2*/*Dlx3*/*Dlx5*/*Dlx7* at the cap stage) raises the possibility of a role in enamel knot induction or maintenance. Development of the cervical loop, which is regulated by the interactions between the dental epithelium and mesenchyme (dental papilla and dental follicle) contributes to root formation (Ruch 1987). A possible involvement of Dlx genes in this process based on the expression of *Dlx5*/*Dlx6* in the cervical loop and *Dlx3*/*Dlx7* in the dental papilla and follicle requires further investigation. *Dlx1*, *Dlx3*, and *Dlx6* may be involved in the development of the periodontal ligaments and the cementum, which are derived from the dental follicle mesenchyme (Ruch 1987).

Dental histodifferentiation

Subsequent to morphogenesis, histological differentiation occurs in the tooth germ. The inner enamel epithelium differentiates to form the ameloblasts, which produce the enamel matrix, and the mesenchyme forms odontoblasts that produce components of the underlying dentine layer. Functional differentiation of ameloblasts occurs earlier in incisors (E16–E17) than in molars (after birth; Ruch 1987). Of the Dlx genes we found *Dlx2* and *Dlx3* to be associated with ameloblast differentiation. *Dlx2* is expressed in the preameloblasts of E16–E17 molars (Fig. 3B) and E16–E17 incisors in the incisal region (data not shown). *Dlx3* is expressed in the ameloblasts of E16–E17 incisors in the apical region (Fig. 4C) and the first molars after birth (Fig. 4E), in which expression of *Dlx2* was negative (data not shown). Expression of other Dlx genes in newborn teeth was not determined.

The expression of *Dlx2* and *Dlx3* in the late development of teeth suggests a role for these genes in ameloblast differentiation. This is consistent with the findings by Price et al. (1998) that mutations of human *DLX3* are associated with enamel hypoplasia. Inactivation of *Dlx5* affects maturation of the dental enamel (Depew et al. 1999). Production and mineralization of enamel is regulated by epithelial-mesenchymal interaction between the dental papilla and the inner enamel epithelium (Ruch 1987). *Dlx5* and *Dlx6* are expressed in the dental mesenchyme adjacent to the cuspal inner enamel epithelium. Inactivation of *Dlx5* may interrupt the mesenchymal signals that control the differentiation of the ameloblasts and/or mineralization of the enamel matrix.

The expression of Dlx genes in the first branchial arch prior to tooth initiation is directly related to their genomic organization; each member of a linked pair shares the same pattern. This suggests that common enhancers may regulate both genes in a pair. Following the initial diffuse, nested pattern of Dlx gene expression in the first arch, expression becomes restricted to various tissues in the presumptive jaws, consistent with a possible change in role from regional specification to regulation of organogenesis. Expression patterns within tooth germs (summarized for molars in Fig. 5) show no simple relationship to gene linkage or phylogeny (Stock et al. 1996) with respect to tissue layer, region, or tooth type. Dlx genes are likely to play multiple roles in tooth development spanning the period from tooth initiation to differentiation of dental tissues. Some of these roles may be shared among subsets of the Dlx gene family. Our data on the expression of Dlx genes provide a framework for designing experiments to further elucidate their roles in tooth development.

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