

Differential expression of laminin-5 subunits during incisor and molar development in the mouse

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ABSTRACT Rodent incisors are continuously growing teeth and enamel deposition is restricted to the labial side. In the present study, the expression of laminin-5 subunits ($\alpha 3$, $\beta 3$ and $\gamma 2$) has been analyzed by *in situ* hybridization in developing mouse lower incisors and compared to that reported in the molar. At the bud stage (E12), mRNAs for all subunits were detected in the whole epithelial thickening. At E14, when histogenesis had started, transcripts for $\alpha 3$ and $\gamma 2$ subunits were restricted to the outer dental epithelium (ODE), whereas the $\beta 3$ subunit was intensely expressed in the inner dental epithelium (IDE). A transient expression for $\alpha 3$ subunit was seen in the enamel knot area and disappeared at E15. Subsequently, all laminin-5 subunit genes were re-expressed in differentiating ameloblasts on the labial side. Similar patterns of transcription were observed in incisor and molar, suggesting that the differential expression of laminin-5 subunits in the IDE might be involved in the histogenesis of the IDE and ameloblast differentiation. At E16.5, cells of the IDE at the anterior extremity of the incisor and in the anterior part of the lingual IDE expressed transcripts for $\alpha 3$ and $\beta 3$ but not for $\gamma 2$ subunit. Similar expression patterns were observed in the enamel-free areas of the E18 molar. This specific expression might thus be related to cells that do not differentiate as functional ameloblasts. Throughout incisor development, intense expression for all laminin-5 subunits was restricted to the labial side of the cervical loop. The asymmetrical expression of laminin-5 might be related to incisor morphogenesis and to the differences in histogenesis and cytodifferentiation of the IDE that exist in the labial versus lingual aspect of the cervical loop.

KEY WORDS: mouse, incisor, morphogenesis, basement membrane, laminin-5

Tooth morphogenesis and cytodifferentiations are regulated by reciprocal interactions between the epithelial and mesenchymal tissues. The basement membrane plays a role in mediating these interactions (Ruch *et al.*, 1984; Ruch, 1985). Laminins are a family of basement membrane proteins consisting of α , β and γ subunit chains and play a role in cell adhesion, migration and differentiation (Timpl and Brown, 1994). So far, at least eleven laminin isoforms have been described. The expression patterns of the laminin isoforms are tissue specific. Laminin-5 ($\alpha 3\beta 3\gamma 2$) is restricted to basement membranes associated with the hemidesmosomes (Rousselle *et al.*, 1991; Jones *et al.*, 1994). Recently, laminin-5 has been suggested to be involved in epithelial morphogenesis (Stahl *et al.*, 1997).

We have previously shown that during mouse molar development, the expression of laminin-5 subunits in the dental epithelium is differentially and independently regulated, and that their stage-specific expression in the inner dental epithelium (IDE) is related to the ameloblast differentiation (Yoshida *et al.*, 1998a). Invaginating

dental epithelium at the bud stage expressed mRNAs for all laminin-5 subunits and also the corresponding proteins. During cap and early bell stages, transcripts for $\alpha 3$ and $\gamma 2$ subunits disappeared from the IDE, whereas transcripts for the $\beta 3$ subunit remained abundant and immunodetection remained negative for all laminin-5 subunits. At the late bell stage, transcripts and proteins for all subunits were re-expressed by the differentiating and functional ameloblasts, suggesting that laminin-5 is essential for ameloblast terminal differentiation (Yoshida *et al.*, 1998a, see Fig. 3). This agrees with other findings (Salmivirta *et al.*, 1997; Sahlberg *et al.*, 1998), and indeed, targeted disruption of the laminin $\alpha 3$ gene reveals abnormalities in ameloblast differentiation (Ryan *et al.*, 1999). Furthermore, we suggested that laminin-5 plays a role in the adhesion of dental epithelial cells to the mineralizing tissues (dentin and enamel) (Yoshida *et al.*, 1998b).

Abbreviations used in this paper: IDE, inner dental epithelium; ODE, outer dental epithelium.

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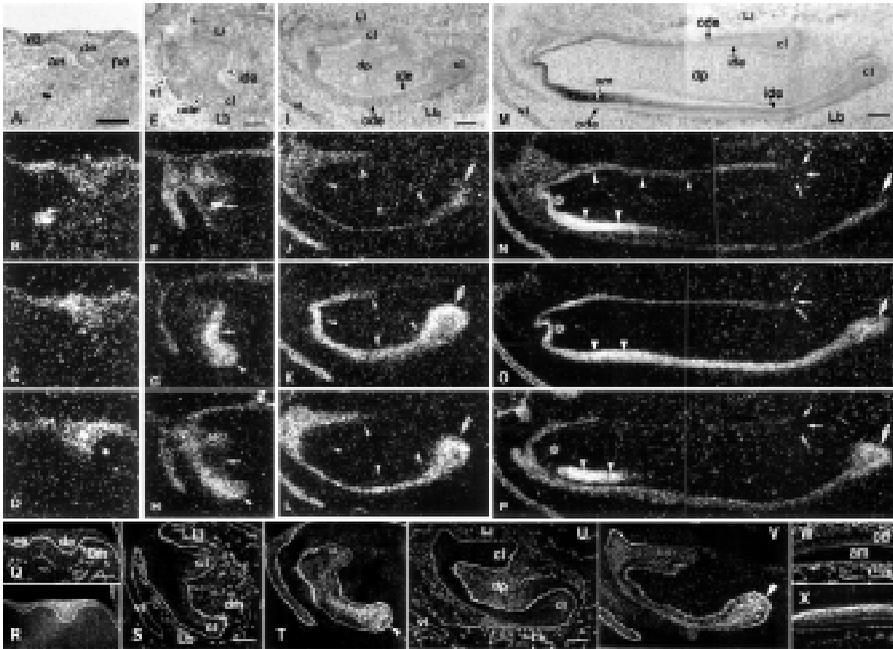


Fig. 1. *In situ* hybridization and immunolocalization of laminin-5 subunits in the developing mouse lower incisor at E12 (A-D, Q, R), E14 (E-H, S, T), E15 (I-L, U, V) and E16.5 (M-P, W, X). (A), (E), (I) and (M) show bright field images of (B), (H), (J) and (N), respectively. mRNAs for $\alpha 3$ (B, F, J, N), $\beta 3$ (C, G, K, O) and $\gamma 2$ (D, H, L, P) subunits. Immunostaining for EHS-laminin (Q, S, U, W) and laminin $\alpha 3$ subunit (R, T, V, X). Lb, labial side; Li, lingual side; an, anterior; po, posterior; am, ameloblast; bm, basement membrane; cl, cervical loop; de, dental epithelium; dm, dental mesenchyme; dp, dental papilla; ide, inner dental epithelium; od, odontoblast; ode, outer dental epithelium; ve, vestibular epithelium; vl, vestibular lamina. Bar, 100 μm . For explanation of arrows, arrowheads and asterisks see text.

Amongst several features that distinguish the incisor from molars in rodents, the incisor is characterized by the presence of an enamel-free lingual surface. This is due to the fact that the lingual IDE does not give rise to functional ameloblasts (Amar and Ruch, 1987; Amar et al., 1989; Nso et al., 1992). Furthermore, rodent incisors are continuously growing teeth and the labial part of the cervical loop is thought to consist of stem cells for all the epithelial cells (see Yoshiba et al., 1998b, and references therein; Harada et al., 1999). In the present study, we have examined the expression of laminin-5 subunits during mouse lower incisor development by *in situ* hybridization and immunohistochemistry, using RNA probes and antibodies specific for all three subunit chains ($\alpha 3$, $\beta 3$ and $\gamma 2$). In addition, we have compared the expression pattern of laminin-5 in the incisor with that occurring during molar development (Yoshiba et al., 1998a).

At E12, incisor morphogenesis is characterized by an invagination of the dental epithelium (Fig. 1A). mRNAs for all laminin-5 subunits were detected in the whole region of the dental epithelium (Fig. 1B-D).

At E14, histogenesis of the enamel organ had started and the dental epithelium differentiated into the inner and the outer dental epithelium (ODE) (Fig. 1E). Transcripts for $\alpha 3$ subunit were restricted to the ODE and absent from the IDE except for the most anterior central part (enamel knot) (Fig. 1F, arrow), while signals for $\gamma 2$ subunit disappeared from the central part of the IDE (Fig. 1H, arrow). On the other hand, $\beta 3$ transcripts accumulated in the IDE (Fig. 1G, arrow). The labial part of the cervical loop, which was more developed than the lingual side, expressed intense signals especially for $\beta 3$ and $\gamma 2$ subunits (Fig. 1G, H, arrowhead).

At E15, the labial and lingual parts of the cervical loop further elongated and surrounded the dental papilla mesenchyme (Fig. 1I). mRNA expression for $\alpha 3$ and $\gamma 2$ subunits was restricted to the ODE (Fig. 1J, L). In contrast, transcripts for $\beta 3$ subunit were strongly detected in the IDE (Fig. 1K, arrowheads). In the labial part of the cervical loop, intense signals for $\beta 3$ and $\gamma 2$ subunits were found almost in the whole region (Fig. 1K, L, arrow), whereas $\alpha 3$ mRNA was seen at the outer part (Fig. 1J, arrow).

At E16.5, the terminal differentiation of odontoblasts is initiated both on the labial and lingual sides of the incisor, in the anterior part. However, differentiating ameloblasts are confined to the labial side (Fig. 1M). Intense signals for all subunits of laminin-5 were detected in the anterior-IDE (differentiating ameloblasts) on the labial side (Fig. 1N, O, P, large arrowheads). The $\beta 3$ subunit was expressed in the entire IDE (Fig. 1O). On the lingual side, $\alpha 3$ mRNA was present in the anterior part of the IDE as well (Fig. 1N, small arrowheads). At the anterior extremity of the incisor, IDE cells expressed transcripts for $\alpha 3$ and $\beta 3$ subunits but not for the $\gamma 2$ (Fig. 1N, O, P, asterisk). In the lingual cervical loop region, transcripts for $\alpha 3$ and $\gamma 2$ subunits were sparse or undetectable (Fig. 1N, P, small arrows).

In spite of differential mRNA expression in the IDE, the immunostaining for $\alpha 3$, $\beta 3$ and $\gamma 2$ subunits showed a similar distribution pattern (Fig. 1R, T, V, X: $\alpha 3$; $\beta 3$ and $\gamma 2$, not shown). At E12, the immunostaining for corresponding proteins mainly concentrated in the basement membranes stained with antibody against EHS-laminin (Fig. 1Q, R). Cells of the invaginating epithelium were also positive (Fig. 1R). At E14, the proteins for all subunits of laminin-5 and EHS-laminin were present along the basement membrane underlying the IDE as well as the ODE (Fig. 1S, T). The labial part of the cervical loop demonstrated a strong immunoreactivity for all subunits when compared to the lingual side (Fig. 1T, arrowhead). At E15, the basement membrane along the ODE showed positive immunostaining for all subunits of laminin-5 (Fig. 1V). In contrast, weak or negligible staining for laminin-5 subunits was seen along the IDE, where EHS-laminin remained positive (Fig. 1U, V). Compared to the lingual cervical loop, the labial one was intensely stained with antibodies against all subunits (Fig. 1V, arrowhead). At E16.5, differentiating ameloblasts showed intense immunoreactivity for all subunits of laminin-5, whereas the immunostaining for EHS-laminin had become discontinuous (Fig. 1W, X).

In the E18 lower first molar, all subunits of laminin-5 were expressed in the ODE while no expression was seen in the IDE except for the future enamel-free areas, where $\alpha 3$ and $\beta 3$ subunits were detected (arrow) (Fig. 2A, B: $\alpha 3$; 2C, D: $\gamma 2$; $\beta 3$, not shown).

Immunostaining for all three subunits was negative along the IDE (Fig. 2F: $\alpha 3$; $\gamma 2$ and $\beta 3$, not shown), whereas EHS-laminin was positive (Fig. 2E).

The patterns of gene expression of laminin-5 subunits and the corresponding proteins have previously been investigated during molar development from the bud stage up to ameloblast differentiation (Yoshida *et al.*, 1998a) thus allowing a comparison with the situation in the incisor (Fig. 3). The mouse incisor and molar teeth share developmental similarities. Initially (E12), invaginating dental epithelium expressed all subunits of laminin-5. As soon as the histogenesis of the enamel organ had started (E14-), cells of the IDE expressed transcripts for $\beta 3$ but not for $\alpha 3$ and $\gamma 2$ subunits, whereas the ODE constantly expressed all subunits throughout the developmental stages investigated here. A transient expression for $\alpha 3$ subunit was seen in the enamel knot area. At E16.5 in the incisor, all subunits were re-expressed by the differentiating ameloblasts on the labial side. The differential expression pattern of laminin-5 subunits in the IDE during tooth development thus appears to be a common feature shared by mouse incisor and molar (Fig. 3). This differential expression may be involved in the histogenesis of the IDE and ameloblast differentiation (Yoshida *et al.*, 1998a). The regulatory mechanisms controlling laminin-5 expression are currently unknown. We have suggested that transcriptional and/or translational control exists in this process and that the dental papilla mesenchyme controls the expression of laminin-5 in the dental epithelium (Yoshida *et al.*, 1998a).

Incisors show interesting particularities: strong expression for all laminin-5 subunits was restricted to the labial part of the cervical loop and continued throughout development, while the lingual part showed very weak or negligible expression. The presence of multilayers or loops of basal laminae were observed in the labial part but never on the lingual aspect of the cervical loop in incisor tooth germs (Meyer *et al.*, 1995). This differential localization is thought to result from spatially localized differences in the interaction between the dental epithelial cells and the basement membrane. The lingual part of the IDE has been reported to be less labeled with BrdU when compared to the labial one in cultured E13 incisors (Coin *et al.*, 1999), suggesting a difference in the mitotic activity between the labial and the

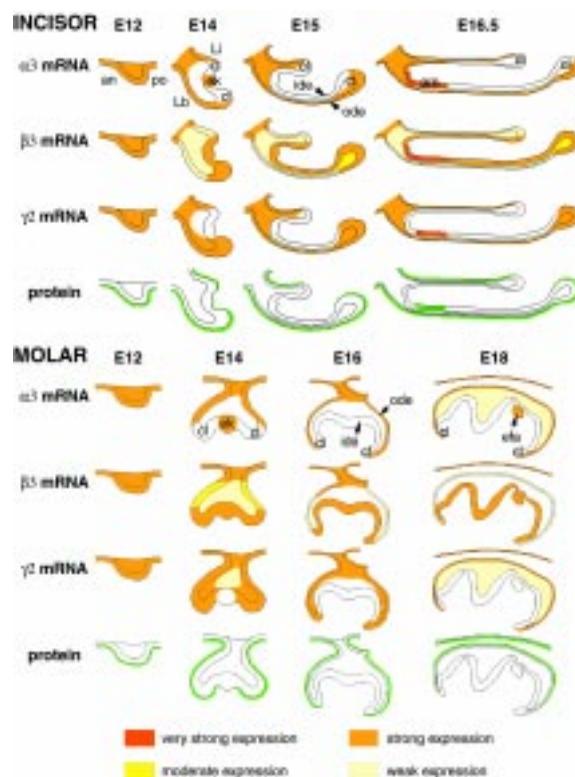


Fig. 3. Schematic illustration of the expression patterns of mRNAs and proteins for laminin-5 subunits in the developing mouse lower incisor and first molar. Sagittal sections of incisors and E18 molars and frontal sections of E12, E14, and E16 molars. All subunit proteins show a similar distribution pattern (shown in green) at the interface between the dental epithelium and the mesenchyme. *efa*, enamel-free area; *ek*, enamel knot. For explanation of other abbreviations see Fig. 1. (Expression patterns of molars from Yoshida *et al.*, 1998a).

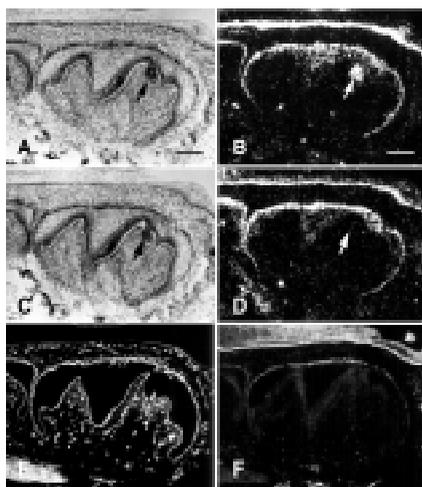


Fig. 2. *In situ* hybridization and immunolocalization of laminin-5 subunits in sagittal sections of the E18 first lower molar. (A) and (C) show bright field images of (B) and (D), respectively. mRNAs for $\alpha 3$ (B) and $\gamma 2$ (D) subunits. Immunostaining for EHS-laminin (E) and laminin $\alpha 3$ subunit (F). Arrows indicate the enamel-free areas. Bar, 200 μm .

lingual aspects of the cervical loop. Since the expression of laminin $\gamma 2$ chain has been shown to be upregulated in invasively growing cancer cells (Pyke *et al.*, 1994), a strong expression of laminin-5 in the labial part of the cervical loop might be associated with actively proliferating and migrating cells in the continuously growing incisors.

On the labial side of the incisor, the cells of the IDE differentiate into functional ameloblasts and deposit enamel matrix. In contrast, the epithelial cells of the cervical loop give rise to the epithelial root sheath on the lingual side. The IDE of the root sheath induces dentin formation, but lacks the ability to form enamel matrix (Yoshida *et al.*, 1998b, and references therein). The expression pattern of transcripts for laminin-5 subunits at the lingual aspect of the cervical loop in the incisor at E16.5, was similar to that observed in the cervical loop of the molar at E18 (Fig. 2). This finding confirms that the lingual part of the incisor is analogous to the root of molar. Taken together, our data suggest that the labio-lingual asymmetry of the expression for laminin-5 in the cervical loop apparently correlates with the histomorphogenesis and cytodifferentiations in the incisor. A different developmental origin of the epithelial component between the labial and lingual sides of the upper incisor has been suggested (Peterková *et al.*, 1993).

At E14 in the incisor, transcripts for the $\alpha 3$ subunit were transiently expressed by the IDE of the enamel knot area, where the $\beta 3$ mRNA was also detected but not for the $\gamma 2$ subunit. During molar develop-

ment, similar expression pattern has been observed (Yoshida et al., 1998a, see Fig. 3), and the $\alpha 3\text{B}$ variant has localized in the enamel knot (Salmivirta et al., 1997). The morphogenetic role of the enamel knot in tooth development is under debate (Thesleff et al., 1995; Lesot et al., 1996; Vaahtokari et al., 1996; Viriot et al., 1997; Lesot et al., 1999). The cells in the enamel knot have been shown to be non-dividing and some undergo apoptosis (Lesot et al., 1996; Vaahtokari et al., 1996). Attempts are being made to compare the analogies/differences in the enamel knot of the incisor versus molar (Coin et al., 1999; Kieffer et al., 1999) and to understand its role in the morphogenesis of the two classes of tooth. It has recently been speculated that in incisors, non-dividing IDE cells of the enamel knot area are not redistributed, which results in one cusp development, in contrast to molars where those cells segregate and several cusps develop (Coin et al., 1999). It has not been ascertained whether the gene deficiency of laminin-5 would affect tooth morphogenesis.

Interestingly, at E16.5 in the incisor, transcripts for $\alpha 3$ and $\beta 3$ subunits but not for the $\gamma 2$ subunit were expressed at the anterior extremity of the IDE and also in the anterior part of the lingual IDE. No enamel formation occurs in these regions. Similar expression pattern was found in the enamel-free areas located at the cusp tips in the mouse molar at E18 (Fig. 2). This unique expression pattern for laminin-5 genes appears to characterize a cell population that will not give rise to functional ameloblasts. The non-dividing cells of the enamel knot area, which are redistributed in the molar but not in the incisor, have been suggested to be as the origin of IDE cells of the enamel-free areas (Coin et al., 1999). The results of the present study may support this hypothesis, since the expression pattern for laminin-5 subunits in these cells is identical to that observed in the enamel knot area.

Experimental procedures

Tissue preparation

Swiss mouse embryos (vaginal plug: day 0) were used. The heads from mice at embryonic day (E) 12 to 18 were dipped in 2-methyl butane pre-cooled by dry ice, cut in 8- μm sagittal sections, and mounted on gelatin-coated glass slides.

In situ hybridization

In order to synthesize cRNA probes, MN97 (1.5kb), MN9.1E (2.0kb) and MN21 (3.0kb) cDNAs corresponding to the mouse $\alpha 3$, $\beta 3$ and $\gamma 2$ laminin-5 subunits (Aberdam et al., 1994) were used. The [α - ^{35}S]CTP-labeled sense and antisense riboprobes were prepared by *in vitro* transcription and used at a final concentration of 50,000 cpm/ μl in the hybridization buffer. *In situ* hybridization on cryosections was performed essentially as described (Yoshida et al., 1998a).

Immunohistochemistry

The antibodies used in this study were affinity-purified rabbit polyclonal antibody SE513 (diluted 1:300) specific for laminin $\alpha 3$, SEtdc3 (diluted 1:200) specific for laminin $\beta 3$, and SE144 (diluted 1:200) specific for laminin $\gamma 2$ (Aberdam et al., 1994). A rabbit polyclonal antibody against EHS-laminin (a kind gift from Dr. D. Hartmann, Institut Pasteur, Lyon, France) (diluted 1:1000) was used as a control to label the basement membrane. Indirect immunofluorescence on cryosections was done as previously described (Yoshida et al., 1998a).

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