



Role of the Notch signalling pathway in tooth morphogenesis

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Summary Notch receptors are involved in cell fate decisions through the process of lateral inhibition or inductive signalling. Jagged2 belongs to the family of transmembrane proteins that serve as the ligands for Notch receptors. We have analysed the expression of the *Jagged2* gene in developing mouse teeth. *Jagged2* expression is restricted in inner enamel epithelial cells that give rise to the ameloblasts. We have also examined the role of *Jagged2* in tooth development using mutant mice that lack the domain of the Jagged2 protein required for interaction with the Notch receptors (DSL domain). Homozygous mutant mice die after birth, exhibit abnormal tooth morphology and fusions between the palatal and mandibular shelves. These results demonstrate that Notch signalling plays an essential role in tooth development.

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Introduction

Teeth arise from progressive reciprocal inductive interactions between the stomodeal epithelium and the neural crest-derived mesenchyme. These interactions transform the tooth primordia into mineralised structures with various cell types. Signalling molecules such as fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), Wnts and sonic hedgehog (Shh) play an important role in tooth initiation, morphogenesis and cytodifferentiation.¹ Our previous data have suggested that the

Notch signalling pathway is also involved in odontogenesis.^{2–4}

The Notch signalling pathway enables adjacent cells to adopt different fates.^{5–8} In *Drosophila*, the *Notch* gene encodes a transmembrane receptor with an extracellular domain carrying epidermal growth factor-like repeats and a cytoplasmic domain required for signal transduction. The Notch receptor interacts with transmembrane ligands encoded by the *Delta* and *Serrate* genes. The signal induced by ligand binding is transmitted at the intracellular part of the receptor in a process involving interactions with cytoplasmic and nuclear proteins.⁶

Four genes encoding Notch receptors (*Notch1*, *Notch2*, *Notch3*, and *Notch4*) and five genes encoding ligands for the Notch receptors (*Jagged1*,

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Jagged2, *Delta1*, *Delta3*, and *Delta4*) have been identified in vertebrates.^{6–8} All these ligands are transmembrane proteins that, in their extracellular domain, contain multiple EGF-like motifs and the DSL domain (*Delta*, *Serrate*, *Lag-2*). The conserved DSL motif is essential for interaction with the Notch receptors.⁶ Studies on mammalian Notch ligands and receptors indicate that the Notch signalling is involved in phenomena as diverse as differentiation, apoptosis and cell proliferation, thus controlling organ formation and morphogenesis.⁶ Notch malfunction has been shown to disrupt aspects of neurogenesis, somite formation, angiogenesis, kidney and lymphoid development. In humans, mutations in the *Notch1*, *Notch3* and *Jagged1* genes are associated, respectively, with a neoplasia, CADASIL and Alagille syndrome.⁶

Here we examined the expression of the *Jagged2* gene in developing dental tissues of mouse embryos. Furthermore, we studied oral and tooth development in mice with a targeted mutation of the *Jagged2* protein. *Jagged2* homozygous mice die at birth. In the oral cavity, the maxillary shelves are fused with the mandibular shelves, while the teeth exhibit an abnormal morphology. These results demonstrate that Notch signalling plays an essential role during tooth development.

Materials and methods

Animals and tissue preparation

Swiss mice were used at embryonic stages. Embryonic age was determined according to the vaginal plug (day 0) and confirmed by morphological criteria. The heads from the embryonic day 10 (E10) to E18 mouse embryos were dissected in Dulbecco's phosphate-buffered saline (PBS), pH 7.4. The tissues were fixed in 4% paraformaldehyde for 24 h at 4 °C and prepared for sectioning.

Targeting vector construction and genotyping

To construct the targeting vector, a 2.2 kb *SacI* fragment of the *Jag2* gene was subcloned upstream of a PGK-*neo* expression cassette, and a 4.0 kb *SpeI-NotI Jagged2* fragment was subcloned downstream of the PGK-*neo* cassette. This resulted in the deletion of a 5.0 kb genomic fragment containing the exons encoding the DSL domain and half of the first EGF repeat of the *Jagged2* protein.

CJ7 ES cells were electroporated with 25 µg of linearized targeting vector injected into blastocysts

from C57BL/6J mice, and the resulting chimeras bred with C57BL/6J females.⁹ F1 animals heterozygous for the *Jagged2* mutant allele were intercrossed for analysis. Mice and embryos were genotyped by allele-specific PCR. PCR primers for the mutant *Jag2* allele were J2K02 (5'-GCACGAGAC-TAGTGAGACGTG-3'), located in the *neo* cassette and J2K03 (5'-GAGTGAGAGTGTTCATGCTGAG-3'), giving a 530 bp amplification product.

Histology and in situ hybridisation

Embryos were dissected and DNA was prepared from the yolk sacs or tails for genotyping by PCR analysis. Heads of embryos for histological analysis were fixed in Bouin's fixative. Fixed heads were dehydrated through graded alcohols, embedded in paraffin, sectioned at 6 µm, and stained with hematoxylin and eosin.

Whole mount in situ hybridization and in situ hybridization on cryostat-sectioned embryos heads (wild type and mutants) using a digoxigenin-labelled mouse *Jagged2* probe were performed as described previously.^{3,4}

Results

Jagged2 expression in developing teeth

To determine the role of *Jagged2* in tooth development, we first analyzed the expression pattern of the *Jagged2* gene in dental structures during mouse embryogenesis. Whole mount in situ hybridisation analysis showed that the gene was expressed in the oral epithelium (data not shown). Patterns of *Jagged2* expression in developing facial and dental tissues were reported earlier.^{9,10}

We then followed the expression of the *Jagged2* gene in sections of E11 to E18 embryos. From E11 to E13, when the dental epithelium thickens (bud stage), *Jagged2* mRNA were localized in dental epithelium. From E14, *Jagged2* expression in the developing teeth was restricted to the inner enamel epithelium. This epithelial expression pattern persisted throughout later embryonic stages of tooth development (late cap and bell stages) (Fig. 1).

Jagged2 mutant mice die at birth

While mice heterozygous for the *Jagged2* mutant allele appear normal, the homozygous mutants are dying at birth. *Jagged2* homozygotes were unable to breath. During normal embryogenesis, the maxillary shelves elevate and then fuse to form

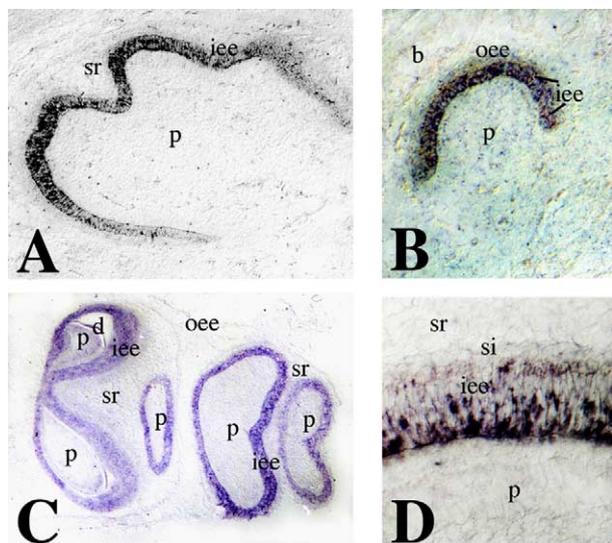


Figure 1 *Jagged2* expression during embryonic tooth development. In situ hybridization on cryosections of E17-E18.5 mouse embryos. (A) At E17, *Jagged2* is restricted in cells of the inner enamel epithelium (iee) of the bell-staged molar germs. (B) In E17 incisors, *Jagged2* expression shows a similar expression pattern as in molars. (C) At the late bell stage (E18.5), *Jagged2* expression persists to the inner enamel epithelial cells of the first molars. The figure shows a coronal section through the forming cusps. (D) A higher magnification of the lingual side of an E18.5 incisor shows *Jagged2* expression in inner enamel epithelial cells. Additional abbreviations: b, bone; d, dentin; de, dental epithelium; oee, outer enamel epithelium; p, dental papilla; si, stratum intermedium; sr, stellate reticulum.

the secondary palate, the future roof of the oral cavity. Fusions were observed between the maxillary and mandibular shelves in the *Jagged2* homozygous mutants (Fig. 2A). The fusion of the shelves prevents proper formation of the oral cavity, and is the apparent cause of the breathing difficulties and perinatal lethality of the *Jagged2* homozygous neonates.

Jagged2 mutant mice have teeth with abnormal morphology

Teeth of the *Jagged2* homozygous embryos exhibited an abnormal morphology. The developing teeth were affected by the fusion between the mandibular and maxillary processes (Fig. 2A and B), and in many instances appeared with abnormal cusps and cervical loops. Histological analysis revealed that the cervical loop of *Jagged2* homozygous embryos was hyperplastic (Fig. 2B). Whereas in wild type and heterozygous littermates the cervical loop was a relatively thin epithelial structure (Fig. 2C), in the *Jagged2* homozygotes the cervical loop was larger

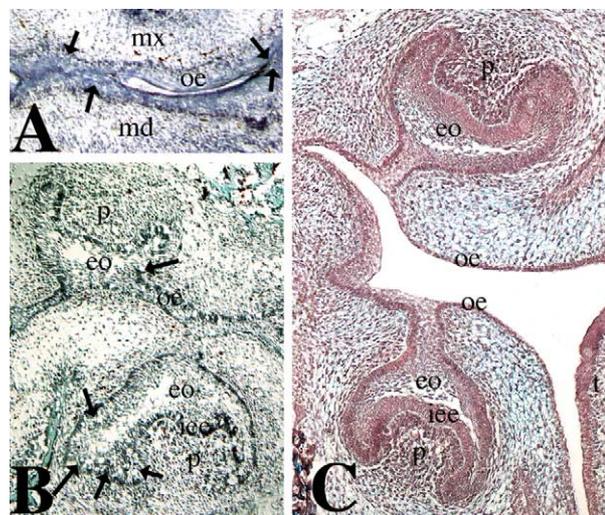


Figure 2 Fusions in the oral cavity and tooth defects in mutant *Jagged2* homozygotes. Histological staining (Masson's trichrome staining) in frontal sections of E16 embryos. (A) In the *Jagged2* homozygote the mandible (md) fusions with the unelevated maxillary (mx) shelves (arrows). (B) Mutant *Jagged2* homozygotes have a hyperplastic cervical loop. The extent of the cervical loop is indicated by the arrows. (C) Histological analysis of E16 littermate control shows the correct tooth morphology. Additional abbreviations: eo, enamel organ; iee, inner enamel epithelium; oe, oral epithelium; p, dental papilla; t, tongue.

and protruded into the underlying mesenchyme (Fig. 2B). The tooth cusps of the *Jagged2* homozygotes were not well defined as those of their littermates (Fig. 2B).

Discussion

The phenotype of the *Jagged2* mutant homozygotes demonstrates that *Jagged2*-mediated Notch signaling is essential for proper tooth morphogenesis. These phenotypes correlate well with domains of *Jagged2* expression in dental epithelium.

Cell fate decisions in dental epithelium and tooth morphogenesis in *Jagged2* mutant mice

Jagged2 is expressed at all stages of the developing mouse teeth (initiation, bud, cap, and bell stages). Expression of *Jagged2* in the epithelium of developing teeth is correlated with proliferation rather than differentiation. Previous studies have shown that in the developing teeth, the *Notch1* gene is expressed in the stratum intermedium.^{2,4} The *Jagged2* gene is expressed in the adjacent

cell layer of inner enamel epithelial cells, suggesting that the Jagged2 protein may function as the ligand for the Notch1 protein during tooth development. The Jagged2 ligand has been shown to activate the Notch1 receptor in mammalian cells.^{11,12}

It is possible that the determination of cell fates in the enamel organ is occurring via inhibitory interactions between adjacent dental epithelial cells. Initially, cells of the forming dental epithelium appear to constitute a developmental equivalent group in which inner enamel epithelial cells suppress differentiation in their immediate neighbours through lateral inhibition. These interactions may be mediated through the Notch signalling pathway, a molecular mechanism that is involved in the determination of a variety of cell fates.^{5–8}

In order to influence developmental decisions, molecules of the Notch signalling pathway must obviously interact with other signalling pathways. Notch-dependent cell fate acquisition between non-equivalent dental precursor cells could be influenced by extrinsic signals.⁶ BMP and FGF signalling molecules are important for tooth development.¹ BMPs and FGFs have opposite effects on the expression of Notch receptors and ligands in dental tissues,^{3,4} indicating that the dental cell fate choices are under the concomitant control of the Notch and BMP/FGF signalling pathways. Regulation of the Notch pathway is also important for maintaining the correct balance among cell proliferation, differentiation and apoptosis during embryonic tooth development. To determine whether Notch-mediated lateral inhibition has a role in the establishment of the tooth morphology, we examined the teeth of the *Jagged2* mutant mice. The overall structure and development of teeth in the *Jagged2* mutant mice are not consistent with teeth of wild type mice. These results demonstrate that Notch signalling mediated through the *Jagged2* gene is essential for normal tooth development.

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