Sequential expressions of Notch1, Jagged2 and Math1 in molar tooth germ of mouse

SILVIA S. BORKOSKY1,2, HITOSHI NAGATSUKA1, YORIHISA ORITA3, HIDETSUGU TSUJIGAWA4, JUNKO YOSHINOBU3, MEHMET GUNDUZ1, ANDREA P. RODRIGUEZ1, LILIANA R. MISSANA2, KAZUNORI NISHIZAKI3, AND NORIYUKI NAGAI1

1. Department of Oral Pathology and Medicine, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 2-5-1, Shikata-Cho, Okayama 700-8525, JAPAN.
2. Cátedra de Anatomía y Fisiología Patológicas, Facultad de Odontología, Universidad Nacional de Tucumán, Av. Benjamin Araoz 800. (4000) San Miguel de Tucumán, ARGENTINA.
3. Department of Otolaryngology-Head and Neck Surgery, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 2-5-1, Shikata-Cho, Okayama 700-8525, JAPAN.
4. Department of Virology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 2-5-1, Shikata-Cho, Okayama 700-8525, JAPAN.

Key words: Notch signaling, odontogenesis, cell differentiation, molar morphogenesis.

ABSTRACT: The Notch signaling pathway is an evolutionary conserved mechanism that plays an important role in cell-cell communication and cell fate in a wide range of tissues. The mammalian family of Notch receptors consists of 4 members: Notch1/2/3/4. The Notch ligand family consists of 5 members: Delta1/3/4 and Jagged1/2. Math1 encodes a murine basic helix-loop-helix (bHLH) transcription factor that acts as positive regulator of cell differentiation. Recently, links between Notch and Math1 pathways were demonstrated in various tissues. Expression of Notch1, Jagged2 and Math1 were analyzed in the mouse molar tooth germ during embryonic stage (E) 13 and E15 and during postnatal stage (PN) 1, PN3, PN5, PN10 and PN14 by using in situ hybridization. Positive Notch1 expression was found at the tooth bud during embryonic stages, but its expression was absent from the basal cells in contact with the dental mesenchyme. Jagged2 and Math1 were strongly expressed in differentiated ameloblasts and odontoblasts and Math1 strong expression was even maintained until PN14 stage. Math1 showed the strongest expression. Our results suggest that the Notch1 signaling pathway through Jagged2 could be importantly related to Math1, directing the process of odontogenesis toward cell differentiation.

Introduction

Odontogenesis or tooth development results from reciprocal and sequential interactions between the dental epithelium and mesenchyme (Thesleff et al., 1995a,b, 2003). As a result of these interactions, differentiation of mesenchymal cells into odontoblasts occurs first in response to epithelial induction. Subsequently, once predentin deposition has occurred, signals from differentiated odontoblasts induce dental epithelial cells to differentiate into ameloblasts. Several growth factors, transcription factors, cell surface molecules, and structural molecules of the extracellular matrix are implicated in this process (Thesleff et al., 1995a,b, 2003).

The Notch signaling pathway is an evolutionary conserved mechanism that plays an important role in cell-cell communication and consequently in determining cell fates in a wide range of tissues. In vertebrates, these include the organ of Corti (Zine and de Ribaupierre, 2002), the olfactory epithelium (Orita et al., 2006), hair follicle (Powell et al., 1998), central nervous system (Lütolf et al., 2002), adult gut (Sander
and Powell, 2004) and developing tooth (Mitsiadis et al., 1995). During development, Notch signaling regulates cell differentiation and specification through two types of regulatory signals: lateral inhibition and inductive signaling (Chitnis, 1995; Greenwald, 1998; Artavanis-Tsakonas et al., 1995, 1999; Lai, 2004; Fiúza and Martinez Arias, 2007).

The mammalian family of Notch receptors consists of 4 members: Notch1/2/3/4 (Fiúza and Martinez Arias, 2007). On the extracellular domain, Notch contains 36 epidermal growth factor (EGF)-like repeats, and three Notch/lin 12 repeats, while in the intracellular domain it contains six copies of the ankyrin repeat, a motif important for cell signaling (Mitsiadis et al., 1995; Chitnis, 1995; Artavanis-Tsakonas et al., 1995, 1999). The Notch ligand family consists of 5 members in mammals: Delta1/3/4 and Jagged1/2 (Fiúza and Martinez Arias, 2007). Similar to Notch receptors, Notch ligands are transmembrane proteins that carry EGF repeats in their extracellular domain.

On the surface of one cell, the extracellular domain of Delta or Serrate is expressed and binds to the extracellular domain of the Notch receptor expressed in an adjacent cell through the specific EGF repeats. After this binding, Notch undergoes a series of proteolytic processes and the Notch intracellular domain (NICD) is cleaved and translocated into the nucleus where it associates with a transcription factor, CSL (for CBF1 (C-promoter binding factor 1), RBP-Jk/Su(H)/Lag-1 in mammals/Drosophila/Caenorhabditis elegans) forming a complex that subsequently upregulates expression of primary target genes of Notch signaling (Lütolf et al., 2002; Artavanis-Tsakonas et al., 1995, 1999; Lai, 2004; Fiúza and Martinez Arias, 2007).

Math1 encodes a murine basic-helix-loop-helix (bHLH) transcription activator that is specifically expressed in developing auditory hair cells (Hawkins and Lovett, 2004) probably acting as positive regulator of the inner ear hair cell differentiation (Zine and de Ribaupierre, 2002). In addition, Math1 is required for the development of cerebellar granule cells (Ben-Arie et al., 2000; Gazit et al., 2004). Recently, links between Notch and Math1 pathways were demonstrated in various tissues (Zine and de Ribaupierre, 2002; Gazit et al., 2004; Yang et al., 2001).

Previous works have analyzed the expression and probable function of Notch signaling during tooth development (Mitsiadis et al., 1995, 1997, 1998, 2005; Harada et al., 1999, 2006; Pouyet and Mitsiadis, 2000; Mustonen et al., 2002; Valsecchi et al., 1997; Tummers and Thesleff, 2003). However the relation between Notch signaling and the proneural gene Math1 has not been reported in odontogenesis. The present study, describes the expression patterns of Notch1 receptor, the Notch ligand Jagged2 and the proneural gene Math1 in the molar tooth germ of wild type mice. Since most studies have focused on early odontogenesis, we also evaluated the expression patterns during late stages of odontogenesis.

FIGURE 1. Expression of Notch1 in the molar tooth germ at E13. Molar tooth germ at E13, hematoxylin-eosin (A). Expression of Notch1 at the tooth bud is restricted to the central cells and absent from the basal cells in contact with the dental mesenchyme (B). Bar represents 50 μm.
Materials and Methods

Animals

BALB/c mice were used as experimental animals. Evaluation was performed at embryonic days (E) 13 and E15; and at postnatal days (PN) 1, PN3, PN5, PN10 and PN14. The mice were housed and handled according to the Okayama University Medical School Guidelines for Care and Use of Laboratory Animals.

Histology and in situ hybridization

The specimens were fixed overnight in 4% paraformaldehyde-0.1 M phosphate buffer (pH 7.4) at 4°C. They were embedded in paraffin and sectioned with a thickness of 5 μm for hematoxylin and eosin staining and 4 μm for in situ hybridization. Digoxigenin (DIG)-11-UTP-labeled single-strand RNA probes for Notch1, Jagged2 and Math1 were prepared using DIG labeling kit (Roche Diagnostics GmbH, Penzberg, Germany). After RT-PCR, the cDNA of each gene was subcloned into pCR21 (Life Technologies, USA). Once transcription was completed, 40 units of RNase-free DNase (Roche Diagnostics) were added to the reaction mixture and incubated at 37°C for 10 min. Transcription products were recovered using 25 μg of RNase-free glycogen (Roche Diagnostics) as a carrier and the precipitate was washed with ethanol, air dried and resuspended in 50 μl of 10 mM Tris-HCl (pH 8) 1mM EDTA.

After deparaffinization, the samples were rehydrated and incubated with 3 μg/ml of proteinase K (Roche Diagnostics) in 10 mM Tris-HCl (pH 8.0), 1mM EDTA for 10-15 min at 37°C. Sections were fixed with

![FIGURE 2. Expression of Notch1, Jagged2 and Math1 in the molar tooth germ at PN1. Molar tooth germ at PN1, hematoxylin-eosin (A). Positive Notch1 expression is present in stratum intermedium, also weak Notch1 signals are detected in preameloblast and odontoblast layer (B). Jagged2 is weakly expressed in preameloblasts with strong expression in the cuspal odontoblasts (C). Math1 is strongly expressed in the whole tooth germ (D). Bars represent either 50 μm for the main micrographs or 100 μm for the insets.](image-url)
4% paraformaldehyde-0.1 M phosphate buffer, washed with 0.1 M phosphate buffer and equilibrated with 0.1 M triethanolamine-HCl buffer (pH 8.0). Acetylation was performed by incubating the sections in 0.25% acetic anhydride in 0.1 M triethanolamine-HCl buffer (pH 8.0) for 10 min at room temperature. Sections were then dehydrated by passage through ascending series of ethanol, air dried, and used for in situ hybridization. Hybridization was performed overnight at 50ºC. After hybridization, the slides were washed, incubated first with DIG buffer 1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) and then blocked with blocking reagent in DIG buffer 1. Anti-digoxigenin Fab fragment in DIG buffer 1 was mounted on the sections. Then, the sections were washed twice with DIG buffer 1 for 15 min, equilibrated with DIG buffer 3 (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl2) and treated with NTB-BCIP for color development and methyl green as counterstaining. Adjacent sections were stained with hematoxylin and eosin for topographical orientation.

**Results**

By E13, restricted expression of Notch1 was detected in the central cells of the dental epithelium within the tooth bud. However, neither the basal epithelial cells in contact with the dental mesenchyme nor the dental mesenchyme itself showed Notch1 signal (Fig. 1B). Jagged2 and Math1 expressions were not observed at this stage.

By E15, Notch1 positive signal was detected in dental epithelium and, similar to E13, its expression was restricted to stellate reticulum also showing high intensity at the cervical loops, with no expression in either the outer enamel epithelium, inner enamel epithelium

**FIGURE 3. Expression of Notch1, Jagged2 and Math1 in the molar tooth germ at PN3.** Molar tooth germ at PN3, hematoxylin-eosin (A). Notch1 positive signals can be detected in the stratum intermedium (B). Jagged2 shows positive signals in the cuspal ameloblasts and in the entire odontoblast layer, being particularly strong at the tip of the cusp (C). Math1 strong signals are found in the whole tooth germ (D). Bars represent either 50 μm for the main micrographs or 100 μm for the insets.
or dental papilla. Jagged2 positive and Math1 weak expressions were observed in both the outer and the inner enamel epithelium.

At PN1, positive expression of Notch1 was found in stratum intermedium. Notch1 signals were weakly expressed in the preameloblast and odontoblast layers at the cuspal areas (Fig. 2B). Jagged2 and Math1 strong signals were observed in odontoblasts, especially at the cuspal areas (Figs. 2C and D). At the preameloblast cell layer, Jagged2 was weakly expressed (Fig. 2C) while Math1 was clearly expressed (Fig. 2D). At this stage Math1 showed the strongest expression and, its signal was even observed in stellate reticulum, stratum intermedium and dental papilla (Fig. 2D).

At PN3, Notch1 expression remained positive in stratum intermedium. (Fig. 3B). Jagged2 and Math1 signals were detected in the ameloblasts at the cuspal areas, and along the entire odontoblast cell layer, with high intensity at the cuspal areas (Figs. 3C and D).

From PN5 to PN10 Notch1 expression in ameloblasts and odontoblasts became negative, whereas Jagged2 and Math1 remained strongly expressed (Figs. 4B and C). However, at PN14, Jagged2 became weakly expressed, whereas Math1 expression remained strong (Fig. 5B).

**Discussion**

From our results we observed that during embryonic stages, Notch1 positive signals were detected in the central area of the tooth bud but none was found in the basal cells in contact with the dental mesenchyme. This finding concurs with the report of Mitsiadis et al., who suggested that the absence of Notch1 expression in the basal cells of dental epithelium (future ameloblasts) depends on a negative regulation by the adjacent dental mesenchyme (Mitsiadis et al., 1995, 1997) and, that the mechanism for this downregulation could involve components of the basement membrane (Mitsiadis et al., 1995). Furthermore, Nagai et al. reported that basement membrane type IV collagen α1, α2, and α4 chains might function as a trapping and delivery system by sequestering factors involved in epithelial mesenchymal interaction during molar tooth germ development (Nagai et al., 2001). It is therefore probable that Notch1 downregulation in the basal cells adjacent to dental mesenchyme may be mediated by type IV collagen α chains that form the dental basement membrane.

In the present study, Jagged2 was not expressed at E13 but became notably expressed in dental epithelium by E15. The absence of Jagged2 expression at E13 could imply that Notch1 interacts with other ligands during this stage. On the other hand, during E15 expression patterns of Notch1 and Jagged2 were different and complementary in dental epithelium: Jagged2 was expressed in outer enamel epithelium and inner enamel epithelium and Notch1 was restricted to stellate reticulum. Expression of the Notch family receptors and their ligands play an important role in cell fate through a process called lateral inhibition or lateral specification (Chitnis, 1995; Greenwald, 1998;
FIGURE 5. Expression of Math1 in the molar tooth germ at PN14. Molar tooth germ at PN14, hematoxylin-eosin (A). Math1 is strongly expressed in ameloblasts and odontoblasts (B). Bars represent either 50 μm for the main micrographs or 100 μm for the insets.
**References**


