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

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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

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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).

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.

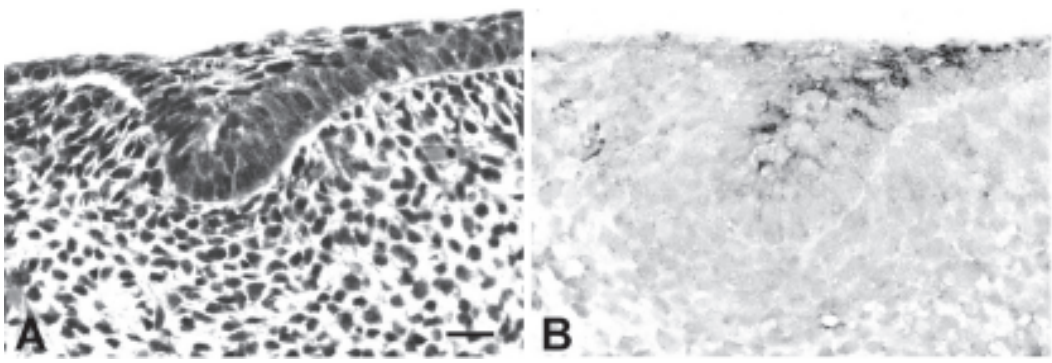

FIGURE 1. Expression of *Notch1* in the molar tooth germ at E13. Molar tooth germ at E13, hematoxylin-eosin (A). Expression of *Notch1* at the tooth bud is restricted to the central cells and absent from the basal cells in contact with the dental mesenchyme (B). Bar represents 50 μm.

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 4ºC. 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 37ºC 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/1ml of proteinase K (Roche Diagnostics) in 10 mM Tris-HCl (pH 8.0), 1mM EDTA for 10-15 min at 37ºC. Sections were fixed with

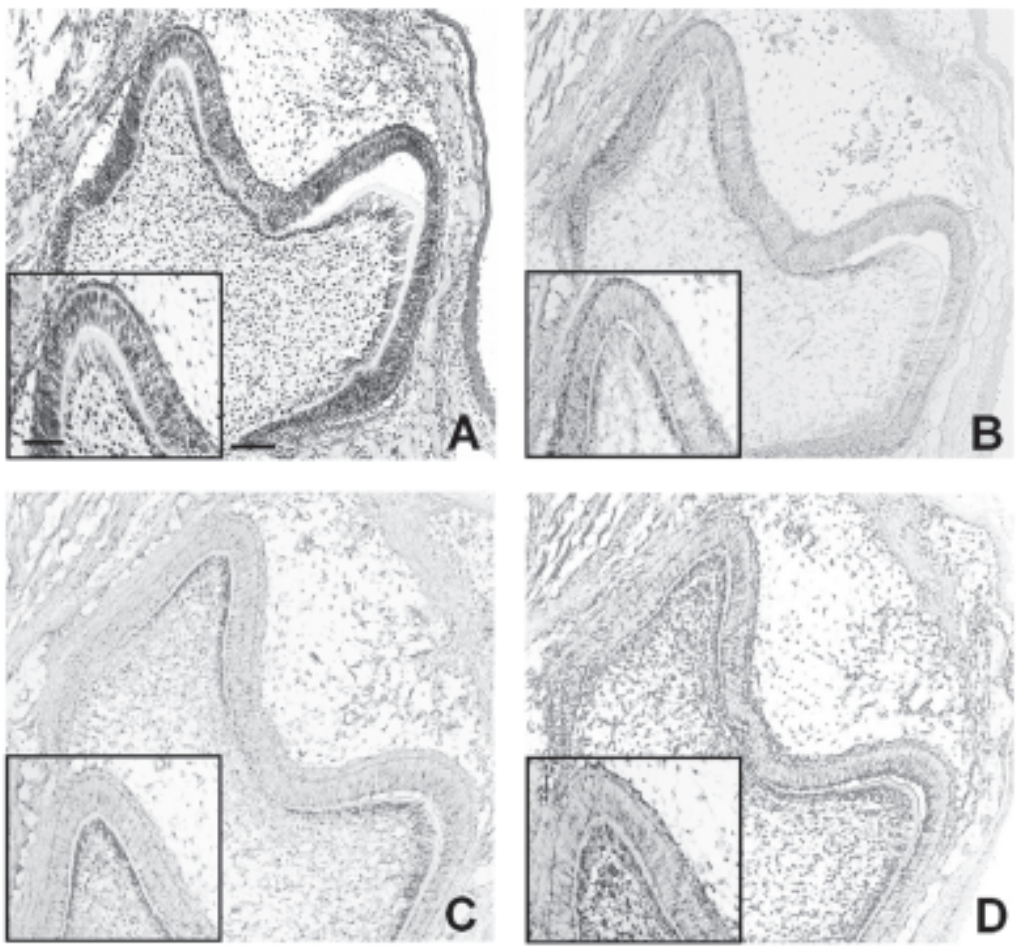

FIGURE 2. Expression of *Notch1*, *Jagged2* and *Math1* in the molar tooth germ at PN1. Molar tooth germ at PN1, hematoxylin-eosin (A). Positive *Notch1* expression is present in stratum intermedium, also weak *Notch1* signals are detected in preameloblast and odontoblast layer (B). *Jagged2* is weakly 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.

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, 100mM NaCl, 50mM 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

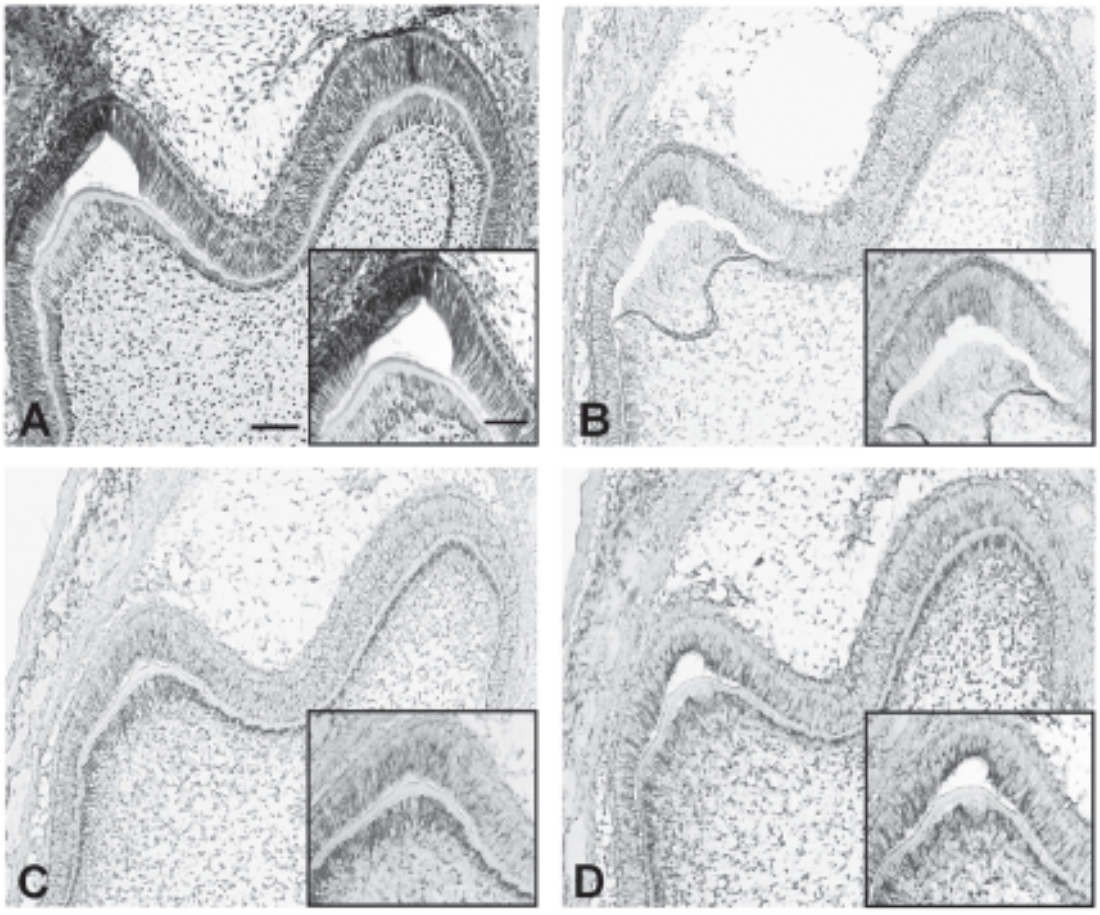

FIGURE 3. Expression of *Notch1*, *Jagged2* and *Math1* in the molar tooth germ at PN3. Molar tooth germ at PN3, hematoxylin-eosin (A). *Notch1* positive signals can be detected in the stratum intermedium (B). *Jagged2* shows positive signals in the cuspal ameloblasts and in the entire odontoblast layer, 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 $\alpha 1$, $\alpha 2$, and $\alpha 4$ 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;

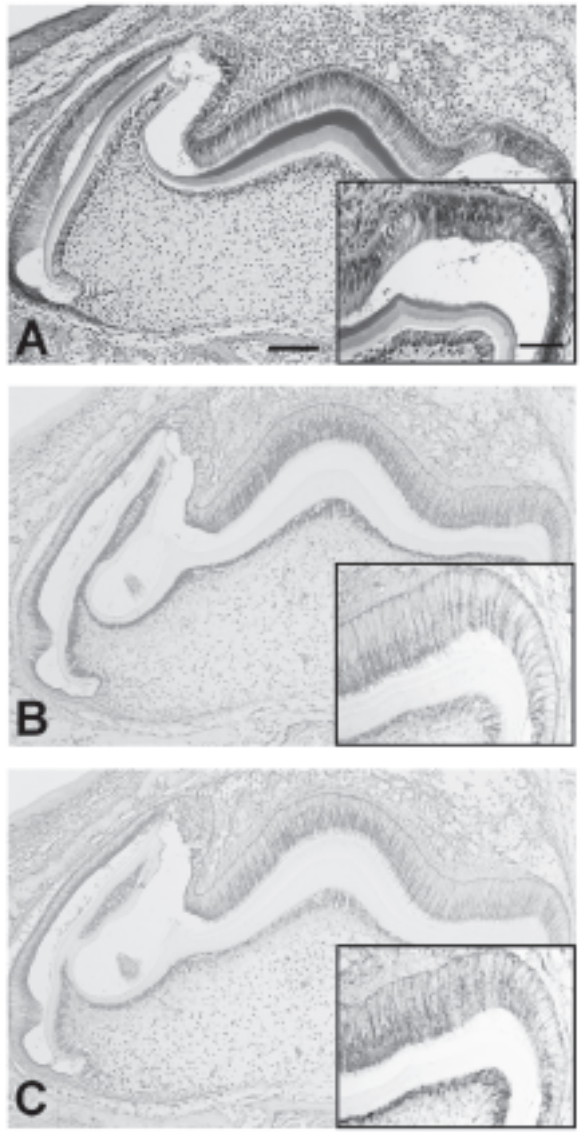

FIGURE 4. Expression of *Jagged2* and *Math1* in the molar tooth germ at PN5. Molar tooth germ at PN5, hematoxylin-eosin (A). *Jagged2* (B) and *Math1* (C) are both strongly expressed in ameloblasts and odontoblasts. Bars represent either 50 μm for the main micrographs or 100 μm for the insets.

Artavanis-Tsakonas *et al*., 1995, 1999; Lai, 2004). Through this mechanism, the complementary expressions of *Notch1* and *Jagged2* within dental epithelium, would regulate the commitment of the enamel organ cells. *Jagged2* expression in the inner enamel epithelium suggests it might also be implicated in proliferative growth of dental epithelium (Mitsiadis *et al*., 2005). *Notch1* expression in the central cells of the dental epithelium can be attributed to its role in maintaining the competence of undifferentiated cells.

During postnatal stages, our data also showed *Notch1* and *Jagged2* complementary expression patterns in the enamel organ: From PN1 to PN3 and concurring with previous reports, *Notch1* was expressed in stratum intermedium (Mitsiadis *et al*., 1995, 1998; Harada *et al*., 1999, 2006; Pouyet and Mitsiadis, 2000; Tummers and Thesleff, 2003), whereas *Jagged2* signals were detected in the adjacent differentiating ameloblasts. Thus, similar to Delta-Notch signaling (Mitsiadis *et al*., 1998), Jagged2-Notch1 signaling in the enamel organ may prevent the stratum intermedium cells from adopting the ameloblast fate. Hence, Notch1 would function as inhibitor of ameloblast differentiation, through lateral specification (Harada *et al*., 2006). Absence of *Notch1* signals during late stages would corroborate Notch1 classical function as inhibitor of differentiation.

With regard to *Math1*, its expression pattern in ameloblasts and odontoblasts was also similar to *Jagged2* expression, suggesting that *Math1* could be linked to the activation of *Jagged2*-mediated *Notch* signaling as previously reported (Zine and de Ribaupierre, 2002; Lanford *et al*., 2000). Moreover, in the organ of Corti, *Jagged2* has been reported to simultaneously express with *Math1* only in cells that will develop as hair cells (Hawkins and Lovett, 2004). In a similar way, in the tooth germ *Math1* and *Jagged2* expressions could be related to ameloblast and odontoblast differentiation. The fact that their expression was strongly maintained even until late stages of odontogenesis suggests that these two genes might act together playing a crucial role not only in cytodifferentiation but also maintaining these cells in a differentiated state, regulating molar morphogenesis and enamel and dentin matrix secretion. Several genes and molecules are implicated in these functions. For instance, the transcription factors Runx-2 and Sp3 (Specificity Protein 3) are importantly involved in tooth cytodifferentiation (Nagatsuka *et al*., 2004; Miletich and Sharpe, 2003). In addition, Shh signaling has been reported in ameloblast differentiation (Gritli-Linde *et al*., 2002). Notch-Shh interactions have been demonstrated during tooth development (Ohazama *et al*., 2004). Other studies showed interactions between Notch target genes and Runx-2 in osteogenesis (Zamurovic *et al*., 2004; Shen and Christakos, 2005). Therefore, we can speculate similar interactions controlling odontoblast and ameloblast differentiation. Some growth factors, such as TGF-β and BMP-2 are known to function in ameloblast and odontoblast differentiation (Coin *et al*., 1999; Fan *et al*., 1998). Furthermore, TGF-β, IGF-1 and-2, FGF-2 and various angiogenic factors have been identified in dentin (Goldberg and Smith, 2004). Since *Notch* signaling and *Math1* showed to be associated with some members of these growth factor families (Mitsiadis *et al*., 1997, 1998;

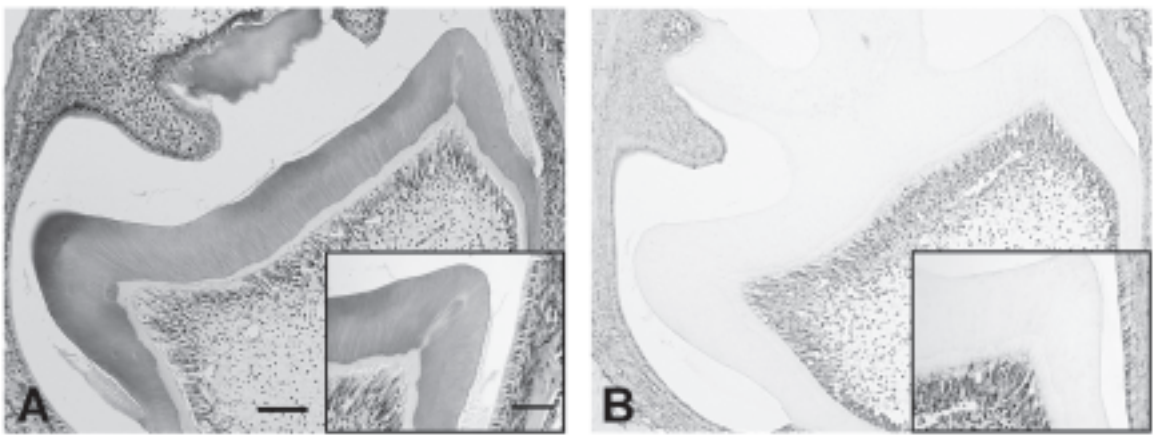

FIGURE 5. Expression of *Math1* in the molar tooth germ at PN14. Molar tooth germ at PN14, hematoxylin-eosin (A). *Math1* is strongly expressed in ameloblasts and odontoblasts (B). Bars represent either 50 μm for the main micrographs or 100 μm for the insets.

Mustonen *et al*., 2002; Alder *et al*., 1999), these associations might also occur during enamel and dentin matrix synthesis and secretion.

Finally, it is important to remark that *Math1* showed the strongest expression, maintained consistently until PN14, even when other genes were not expressed, suggesting that *Math1* might be essential for molar tooth development. Nevertheless, further studies are necessary to clarify the roles of *Math1* and *Notch* signaling in tooth development.

We conclude that the expressions of *Notch1* and *Jagged2* in different cells of the enamel organ during embryonic stages suggest its regulation in embryonic odontogenesis mainly through lateral specification. *Jagged2* and *Math1* are involved in advanced stages of odontogenesis determining the differentiation of odontoblasts and ameloblasts, molar morphogenesis and enamel and dentin matrix synthesis and secretion.

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