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***In situ* expression of 15kDa interferon alpha responsive gene in the developing tooth germ of the mouse lower first molar**

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Abstract We previously performed cDNA subtraction between the mouse mandibles at embryonic day 10.5 (E10.5) and E12.0 to make a profile of the regulator genes for odontogenesis. *Fifteen kDa interferon alpha responsive gene (Ifrg15)* is one of several highly-expressed genes in the E12.0 mandible. The current study examined the precise expression patterns of Ifrg15 mRNA in the mouse mandibular first molar by *in situ* hybridization to evaluate the possible functional roles of this gene in odontogenesis. Ifrg15 mRNA was expressed in the epithelial and mesenchymal tissues of the mandible at E10.5 and E12.0. The Ifrg15 *in situ* signal was detected in the epithelial bud and the surrounding mesenchyme at E14.0, and was present in the enamel organ including the primary enamel knot, and in the underlying mesenchyme at E15.0. The *in situ* signal was restricted in the inner and outer enamel epithelia and the stratum intermedium at E16.0. The signal of Ifrg15 mRNA was further restricted to the inner enamel epithelium and the adjacent stratum intermedium at E17.0 and E18.0. Consequently, the expression of Ifrg15 mRNA was localized in the ameloblasts and odontoblasts at postnatal days 1.0 to 3.0. However, the *in situ* signal was markedly weaker than at the embryonic period. The expression of Ifrg15 mRNA was coincidentally observed in various craniofacial organs as well as in the tooth germ. These results suggest that Ifrg15 is closely related to odontogenesis, especially the differentiation of the ameloblasts and odontoblasts, and to the morphogenesis of the craniofacial organs.

Keywords Interferon alpha responsive protein, Ifrg15, tooth germ development, *in situ* hybridization

Introduction

The tooth germ of the mouse lower first molar is formed as one of the mandibular components of the first branchial arch (D'Souza 2007). Tooth germ development occurs by coordinated multi-step molecular interactions between the ectomesenchymal and ectodermal cells (Thesleff et al. 1995). The molecular interactions are associated with more than 300 genes, and regulate the initiation and morphogenesis of the tooth germ (Thesleff 2006). However, the precise mechanisms of the molecular interactions are still unclear. Therefore, it is important to identify the regulator genes that orchestrate these molecular interactions.

To investigate the regulator genes in odontogenesis, we previously performed cDNA subtraction between the murine mandibles at embryonic day 10.5 (E10.5) in the pre-initiation stage of odontogenesis and E12.0 in the late initiation stage of odontogenesis. Thirty-five highly-expressing positive clones were obtained from the E10.5 mandible by colony array screening. In addition, 47 highly-expressed positive clones were also obtained from the E12.0 mandible (Yamaza et al. 2001a). Minute *in situ* expression patterns of the thymosin beta 4 (T β 4), phosphoglycerate kinase 1 (Pgk1), heat shock proteins (Hsc73, Hsj2, Hsp86), nucleolin, ribosomal protein L21 (Rpl21), and Set- α were the highly expressed genes in the developing lower first molar tooth germ (Akhter et al. 2005; Honda et al. 2008; Wada et al. 2002; Xie et al. 2009; Xie et al. 2007; Yamaza et al. 2001a; Yamaza et al. 2001b). Furthermore, we demonstrated that tooth germ development was disturbed by treatment with nucleolin anti-sense sulfur-substituted oligodeoxynucleotide (AS-S-ODN) in the cultured E11.0 mandible (Xie et al. 2007).

Fifteen kDa interferon alpha responsive gene (Ifrg15) is one of the highly

expressed genes that was detected by cDNA subtraction assay in the E12.0 mandible (Yamazaki et al. 2001a). *Ifrg15* is registered in the EMBL/GenBank/DDBJ databases (accession No. AJ251363). The Ifrg15 protein consists of 131 amino acids and has a molecular mass of 15.3 kDa. However, the functions of Ifrg15 are still unknown in the field of molecular biology. In a recent study, the novel interferon(IFN)-responsive protein, of which the amino acid sequence corresponds almost exactly to the sequence of murine Ifrg15, is highly expressed in the inner-cell mass of the rabbit blastula (Qi et al. 2007). Qi *et al.* concluded that the interferon-responsive protein may play an important role in embryonic development and tissue differentiation.

The current study investigated the precise expression patterns of Ifrg15 mRNA in the developing tooth germ using *in situ* hybridization because no embryological studies have documented the Ifrg15 gene expression during the development of the mouse tooth germs. The *in situ* signal patterns of Ifrg15 mRNA may therefore reveal a close relationship between the roles of Ifrg15 and tooth germ development.

Materials and methods

Animals

BALB/c mice at E10.5, E12.0, E14.0, E15.0, E16.0, E17.0 and E18.0, and postnatal days (PNs) 1.0, 2.0, and 3.0 were used in this study. Parental mice were obtained from Charles River Japan Inc. (Yokohama, Japan). All experimental procedures using mice were performed according to the Animal Care and Use Review Committee at Kyushu University (Fukuoka, Japan). Adult female mice (10–15 weeks of age) were caged together with male mice. After allowing 12 h for mating, successful insemination was

determined based on the presence of a post-copulatory plug in the vagina. The embryonic day was defined as E0.5 after such a plug was recognized.

In situ hybridization of Ifrg15 mRNA

Specific probes for Ifrg15 mRNA were designed according to the cDNA sequence provided by the GenBank (accession number AJ251363, 301-1355, 1055 bases long). The antisense and sense RNA probes were synthesized with the pGEM-3Z vector (Promega, Madison, WI, US). The probes were labeled with digoxigenin (DIG)-11-UTP using the DIG RNA Labeling Kit (Sp6/T7) (Roche Molecular Biochemicals, Mannheim, Germany). *In situ* hybridization using the antisense RNA probe was performed according to our previous studies (Akhter et al. 2005; Honda et al. 2008; Kobayashi et al. 2006; Wada et al. 2002; Xie et al. 2009; Xie et al. 2007; Yamaza et al. 2001a; Yamaza et al. 2001b). The specific day-old embryos were fixed in 4% paraformaldehyde (PFA) in diethylpyrocarbonate (DEPC)-treated phosphate buffered saline (PBS, pH 7.4) for 12 h. Heads dissected from the fixed embryos were embedded in the OCT compound (Sakura Finetechnical Co. Ltd, Tokyo, Japan). Serial frontal cryosections of the heads were cut in 8- μ m thick slices and mounted on silane-coated glass slides. The sections were fixed with 4% PFA in DEPC-treated PBS for 10 min, treated with 20 μ g/ml proteinase K for 2 min at room temperature (RT), and then immersed in 0.25% (v/v) acetic anhydride for 10 min at RT to avoid any background signals. The treated sections were immersed in the hybridization mixture containing 50% deionized formamide, 10% dextran sulphate, 1% Denhardt's solution, 250 μ g/ml yeast tRNA, 0.3 mM NaCl, 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 10 mM NaH₂PO₄, 1% *N*-lauroylsarcosine, and 1 ng/ μ l DIG-labeled antisense

RNA probe. The hybridization was carried out overnight in a humidified chamber at 55 °C. After hybridization, the sections were washed twice in 2 x standard saline citrate (SSC) containing 50% formamide for 30 min at 65 °C, followed by incubation with 20 µg/ml RNase A for 30 min at 37 °C to avoid any nonspecific binding of the probe. They were then washed twice in 2 x SSC containing 50% formamide for 20 min at 65 °C, followed by 2 x SSC for 15 min at 37 °C, and 0.1 x SSC for 15 min at 37 °C, and were washed in PBST (PBS+0.1% [v/v] Tween 20 [polyoxyethylene sorbitan monolaurate; Nakarai Tesque, Kyoto, Japan]) for 15 min at RT, and thereafter treated with 10% normal goat serum for 1 h at RT. The DIG-labeled antisense probe was visualized by an alkaline phosphatase-conjugated anti-DIG antibody using BM Purple (Roche Molecular Biochemicals). No hybridization signals were detected in the control tissue specimens where a sense probe was applied at any of the investigated developmental stages (data not shown).

Results

Expression patterns of *Ifrg15* mRNA in the developing tooth germ

The developmental stages of the tooth germ were classified into the pre-initiation stage (E10.5), the thickened epithelium stage (i.e., late-initiation stage, E12.0), the bud stage (E14.0), the cap stage (E15.0), and the bell stage (E16.0-18.0 and PN1.0-3.0) in order to describe the expression patterns of *Ifrg15* mRNA (Jernvall et al. 1994). *Ifrg15* mRNA was diffusely expressed in the epithelial and mesenchymal tissues of the mandibule at E10.5 (Fig. 1A). At E12.0, *Ifrg15* mRNA signals were detected in the thickened epithelium and in the underlying mesenchyme (Fig. 1B). The

expression of Ifrg15 mRNA was observed in the budding epithelium and in the surrounding mesenchyme that consists of focally condensed cells at E14.0 (Fig. 1C). Signals of Ifrg15 mRNA were detected in both the inner and outer epithelial cells of the enamel organ including the area of the primary enamel knot (PEK) at E15.0 (Figs. 1D and 1E). Faint Ifrg15-positive signals were also demonstrated in the mesenchymal cells condensing to the enamel organ. The signal was restricted to the inner and outer enamel epithelia and the stratum intermedium at E16.0 (Fig. 1F). Strong Ifrg15-positive signals were also demonstrated in the buccal and lingual cervical loops of the invaginating enamel organ. Faint signals of Ifrg15 mRNA were also detected in the underlying mesenchymal cells in the dental papilla (Fig. 1G). Strong Ifrg15 mRNA signals were restricted to the inner enamel epithelium and the adjacent stratum intermedium at E17.0 and E18.0 (Figs. 1H, 1I and 1J). The expression of Ifrg15 mRNA was localized to the ameloblasts and odontoblasts at postnatal days PN1.0 to PN3.0. However, the *in situ* signal was markedly weaker than the restricted signals at E17.0 and E18.0 (Figs. 1H-1N). Signals of Ifrg15 were also observed in the mesenchymal cells of both the buccal and lingual cusp sites in the dental papilla (Figs. 1K, 1L and 1N).

Expression patterns of Ifrg15 mRNA in other craniofacial organs

The expression of Ifrg15 mRNA was coincidentally observed in various craniofacial organs as well as in the tooth germ. Ifrg15 mRNA signals were found in the cells of the neuroepithelial cells of the lateral ventricles and olfactory epithelium (Figs. 2A and 2B), and in the retinal cells of the eye (Fig. 2C) at E14.0. Ifrg15 mRNA signals were detected in the acinar cells of the lacrimal gland at PN2.0 (Fig. 2D). The Ifrg15

mRNA was expressed in the cells of the epidermis and the hair follicles at PN3.0 (Fig. 2E). The *in situ* signal was also observed in muscular cells of the tongue and the cells of sublingual duct (Figs. 2F, 2G and 2H), and osteoblasts of membranous bone and the chondrocytes of Meckel's cartilage (Fig. 2I) at E16.0.

Discussion

The current study presented the expression patterns of *Ifrg15* mRNA during mouse lower first molar development. The results indicated that the expression of *Ifrg15* mRNA was associated with the morphogenesis of the murine tooth germ. This is the first report to show a relationship between the expression of *Ifrg15* mRNA and the morphological development of the tooth germ.

Ifrg15 as an IFN- α -responsive gene was newly identified by Michel Dron, *et al.* (EMBL/GenBank/DDBJ databases, accession No. AJ251363). The IFN- α expression is regulated by transcription factors termed IRFs (interferon regulatory factors). The expression of IRF-6 is observed in the incisor and molar tooth germs, hair follicles, palatal rugae, and medial edge of the secondary palate immediately before (E13.5) and during fusion (E14.5) of the mouse embryos by means of whole-mount *in situ* hybridization (Knight et al. 2006; Kondo et al. 2002). The IRF-6 gene is located in the critical region for the Van der Woude syndrome (VWS; Online Mendelian Inheritance in Man No. 119300) characterized by cleft lip, cleft palate and pits and/or sinuses of the lower lip (Murray et al. 1990; Schutte et al. 2000). Ranta and Rintala reported that VWS with cleft palate has hypodontia in 25-40% frequency (Ranta and Rintala 1983). Odontogenesis located in palatal cleft with VWS may be disrupted by IRF-6 disorders.

It is considered that Ifrg15 mediated by IRF-6 through IFN- α could be involved in odontogenesis.

IFN- α , which is a pleiotropic cytokine classified as a type I IFN, has roles in cellular resistance against virus infection, and in the regulation of cell proliferation and apoptosis (Caraglia et al. 1999; Gamero and Lerner 2001; Vilcek 2006). The expression of IFN- α gene is found in the oocytes to pre-implantation embryo (Riego et al. 1995). IFN- α functionally promotes the development of the bovine embryo from the morula to blastocyst stages *in vitro* (Takahashi et al. 2003). IFN- α secreted in an autocrine or paracrine manner binds to the IFN- α receptor (IFNAR) to activate the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signal transduction cascade (Platanias 2005). IFN- α mediates the transcription of many interferon-responsive genes through the activation of the JAK-STAT signaling pathway (Der et al. 1998; Friedman et al. 1984; Nicholl et al. 2000). The post-transcriptional effects of IFN- α consequently have pleiotropic activities on Ifrg15 and the other IFN-stimulated proteins in embryonic development. The possible roles of Ifrg15 may be deduced from IFN- α expression patterns and activities in the embryo development. Duc-Goiran *et al.* demonstrated that IFN- α mRNA was expressed in the murine embryo at E10.0 to E21.0, and its expression signals was highly detectable in the developing epithelia: skin, ependyme and intestine glandular epithelium (Duc-Goiran et al. 1994). IFN- α expressed in the developing epithelia could protect the embryo against viral infections based on the its central role in immune responses (Stetson and Medzhitov 2006). Duc-Goiran *et al.* suggested that IFN- α is involved not only in protection against viral infections but also in differentiation and physiological functions in developing embryos. The expression pattern of IFN- α is

also consistent with the *Ifrg15 in situ* signals in the developing craniofacial organs of the murine embryo in our present study. *Ifrg15 in situ* signals were specifically localized to the inner enamel epithelium and the underlying pulpal cells in a single cell layer at E16.0 to E18.0, and the signal intensity then decreased in mature ameloblasts and odontoblasts at postnatal days PN1.0 to PN3.0. These results support the involvement of *Ifrg15* in ameloblastic and odontoblastic differentiation in the early bell stage. Signal intensity decreases at PN1.0 to PN3.0 suggest that the enamel and dentin matrices have been already formed at these postnatal days, and therefore the signal of *Ifrg15* decreases in association with the terminal differentiation of ameloblasts and odontoblasts.

Possible roles of *Ifrg15* in the tooth germ development may also be deduced from well-known functions of the other IFN- α -stimulated genes. Many IFNs-stimulated genes were identified as highly mRNA-expressed profiles using oligonucleotide arrays in human fibrosarcoma cells (Der et al. 1998). Phospholipid scramblase (PLS) upregulated by IFN- α or - β is a calcium-dependent enzyme, and is involved in the translocation of phospholipids between the inner and outer leaflets of the cell membrane (Zhou et al. 1997). PLS1 in ovary cells stimulates caspase-dependent phosphatidylserine in response to enhanced outward movements to the cell surface during apoptosis (Yu et al. 2003). The previous study examined the temporal distribution of apoptotic cells in the developing tooth germ by terminal deoxynucleotidyl transferase-mediated deoxyuridine-5'-triphosphate (dUTP)-biotin nick end labeling (TUNEL) double-staining, and demonstrated that apoptosis occurs in the PEK at the bud-cap stages (Shigemura et al. 1999; Vaahtokari et al. 1996). The PEK in enamel organ at E15.0 was characterized by *in situ* signals of *Ifrg15* mRNA. Together with the IFN-stimulated protein PLS, which is involved in apoptosis, *Ifrg15*

may be concomitantly implicated in cellular apoptosis in the PEK at the bud-cap stages.

The predictable roles of Ifrg15 in tooth germ development are supported by previous studies for *in situ* signals of *Tβ4*, *Pgk1*, *Hsc73*, *Hsj2*, *Hsp86*, *nucleolin*, *Rpl21* and *Set-α* because these genes with *Ifrg15* were identified as significantly expressed genes at the pre-initiation or late intitiation stage of the murine tooth germ development (Yamaza et al. 2001a). The signal patterns of Ifrg15 were very similar to those of these genes from the thickened epithelium to the bell stages of the tooth germ development (Akhter et al. 2005; Honda et al. 2008; Wada et al. 2002; Xie et al. 2009; Xie et al. 2007; Yamaza et al. 2001a; Yamaza et al. 2001b). The Ifrg15 might therefore participate in site- and stage-specific molecular networks of these genes throughout the development of the tooth germ. In the bud-cap stages of the tooth germ, *in situ* signals for Ifrg15 mRNA were found at the tip of the dental epithelial bud invaginating into the underlying mesenchyme. The epithelial tip corresponding to the distribution area of BrdU-positive cells site-specifically appears to indicate cellular proliferative activity at the front of the epithelium (Shigemura et al. 1999). Ifrg15 might be implicated in molecular networks proliferating cells at the epithelial tip.

In conclusion, Ifrg15 mRNA was site- and stage-specifically expressed in the developing tooth germ. These results suggest that Ifrg15 participates in cellular apoptosis at the PEK, and pivotally in cellular proliferation at the front of the invaginating epithelial bud as well as in ameloblastic and odontoblastic differentiations in the developing tooth germ.

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Figure legends

Fig. 1

In situ expression of *Ifrg15* in the developing tooth germ of the mouse lower first molar. **A:** *Ifrg15* mRNA was diffusely expressed in the epithelial and mesenchymal tissues at E10.5. **B:** At E12.0, the *Ifrg15 in situ* signal was detected in the thickened epithelium (arrow) and the underlying mesenchyme. **C:** The expression of *Ifrg15* was found in the budding epithelium (arrow) and the surrounding mesenchyme at E14.0. **D:** Signals of *Ifrg15* mRNA were observed in the epithelial cells of the enamel organ (arrows) including primary enamel knot (PEK, boxed area) at E15.0. **E:** The boxed area of Fig. 1D was shown at higher magnification in Fig. 1E. *Ifrg15* mRNA signals were noted within the cytoplasm in PEK (arrows). **F:** *Ifrg15* mRNA signals were found in the inner and outer enamel epithelial cells, as well as the dental papilla cells at E16.0. Relatively strong *Ifrg15*-positive signals were also demonstrated in the buccal and lingual cervical loops (arrows). **G:** The enclosed area of Fig. 1F was shown at higher magnification in Fig. 1G. *Ifrg15* mRNA signals were noted within the cytoplasm of the inner enamel epithelium (large arrows) and the dental papilla (small arrows). **H, I, J:** The *in situ* signals of *Ifrg15* mRNA were restricted to the inner enamel epithelium and the adjacent stratum intermedium at E17.0 and E18.0 (arrows). The boxed area of Fig. 1I was shown at higher magnification in Fig. 1J. *Ifrg15* mRNA signals were noted within the cytoplasm of the inner enamel epithelium (large arrow heads) and the stratum intermedium (small arrow heads). **K-N:** The signals localized to ameloblasts and odontoblasts were distinct at PN1.0-3.0 (large and small arrows, respectively). The enclosed area of Fig. 1L was shown at

higher magnification in Fig. 1M. *Ifrg15* mRNA signals were noted within the cytoplasm of ameloblasts and odontoblasts (large and small arrow heads, respectively). The left side in Figs. 1A-D, 1F, 1H, 1I, 1K, 1L and 1N corresponds to the lingual side, and the right side is the buccal side in frontal planes. Scale bars: 30 μm (E, G, J and M), 130 μm (A-D, F and I) and 200 μm (H, K, L and N).

Fig. 2

The expression patterns of *Ifrg15* mRNA in other craniofacial organs. **A:** *Ifrg15* was expressed in the cells of the neuroepithelial cells of the lateral ventricles (large arrow) and olfactory epithelium (small arrows) at E14.0. **B:** The boxed area of Fig. 2A was shown at higher magnification in Fig. 2B. *Ifrg15* mRNA signals were noted within the cytoplasm of the neuroepithelial cells (arrows). **C:** *Ifrg15* mRNA signals were found in the retina of eye at E14.0 (arrows). **D:** Signals of *Ifrg15* mRNA were observed in the lacrimal gland at PN2.0 (arrows). **E:** Relatively strong *Ifrg15*-positive signals were also demonstrated in the epidermis (large arrows) and the hair follicles (small arrows) at PN3.0. **F:** The *in situ* signal was also observed in muscular cells of the tongue (large arrow) and the cells of sublingual duct (small arrow) at E16.0. **G, H:** The left and right enclosed areas of Fig. 2F were shown at higher magnification in Figs. 2G and 2H, respectively. *Ifrg15* mRNA signals were noted within the cytoplasm of the muscle (Fig. 2G, arrows) and the sublingual duct (Fig. 2H, arrows). **I:** The *Ifrg15 in situ* signal was detected in the membranous bone (large arrows) and Meckel's cartilage (small arrows) at E16.0. Scale bars: 30 μm (B, G and H), 150 μm (D, E and I), 300 μm (C) and 500 μm (A and I).

Figure 1

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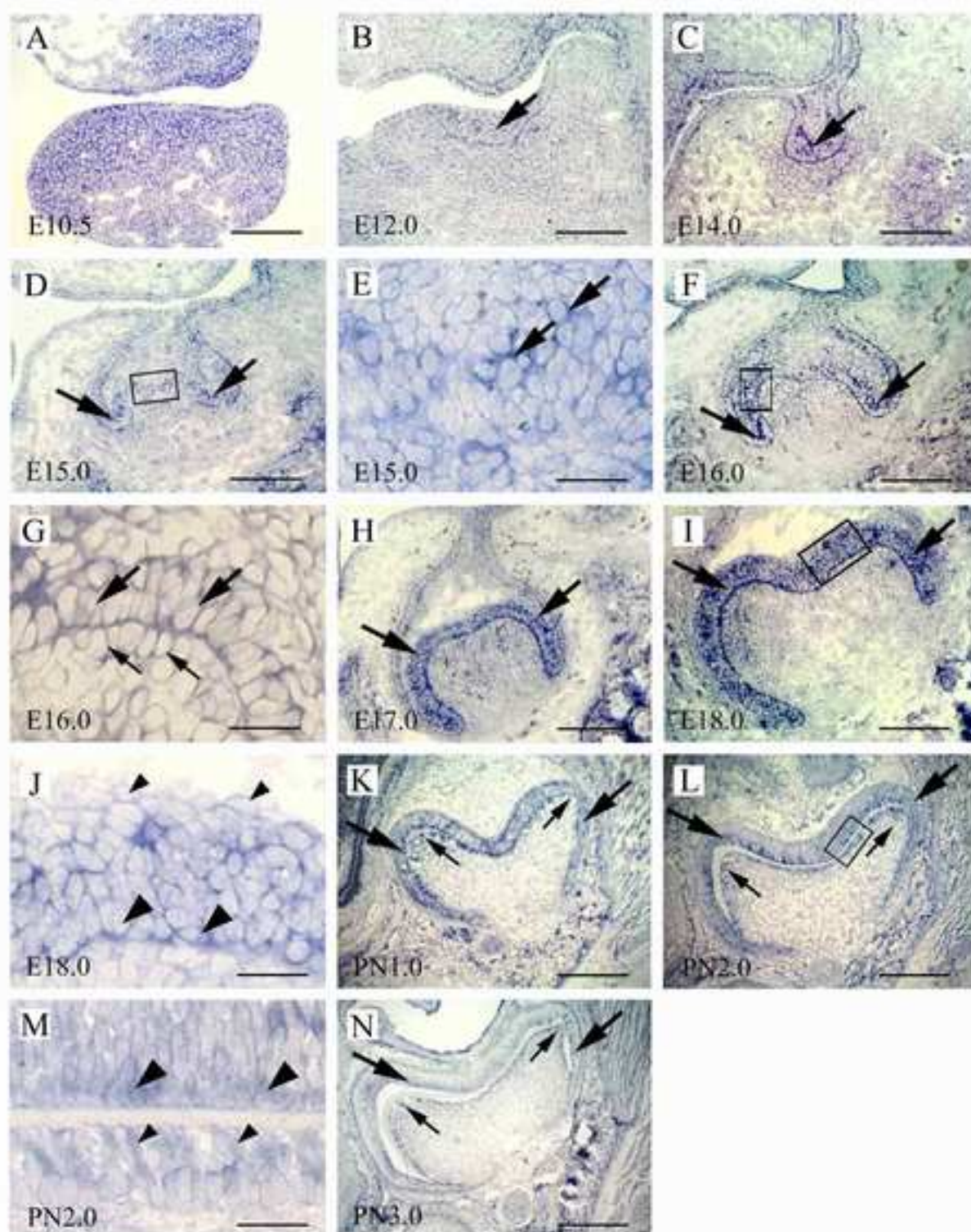


Figure 2

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