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The enamel knot-like structure is eternally maintained in the apical bud of postnatal mouse incisors

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ABSTRACT

Objectives: The boundary where inner and outer enamel epithelia meet is referred to as the cervical loop (CL) in molar tooth germs. In contrast, rodent incisors are continuously growing: the labial side of the teeth is covered with enamel (crown-analog), and the lingual side is covered with cementum (root-analog). These results in the appearance of CL in the frontal sections apart from the apical end. However, many researchers have used the term "labial CL" to indicate the apical end of incisors.

Design: This study investigated the gene expression patterns for the enamel knot signaling center in tooth morphogenesis to clarify the difference between "labial CL" and molar CL. We examined the three-dimensional expression patterns of markers for the enamel knot including *fibroblast growth factor 4* (*Fgf4*), *sonic hedgehog* (*Shh*), *Msx2*, and *P21* in frontal sections of murine mandibular incisors.

Results: Serial frontal sections from the apical end of the postnatal incisor clearly demonstrated the existence of enamel knot-like structures composed of supra-inner enamel epithelium and stellate reticulum in the "labial CL". This structure includes the expression of all examined markers for enamel knots such as *Fgf4*, *Shh*, *Msx2*, and *P21*.

Conclusions: The molar tooth germ-like structure is maintained indefinitely in the "labial CL". Therefore, the "labial CL" is not equivalent to the molar CL. The existence of an EK-like structure in the apical end of incisors implies that the usage of "labial CL" may be inappropriate for indicating this region. The "apical bud" could be used as an alternative term.

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1. Introduction

The biological process of tooth development via epithelial-mesenchymal interactions involves sequential steps of tooth initiation and morphogenesis, cell differentiation, and matrix deposition. Tooth germs are formed at their correct positions in the jaw, and they represent sequential morphological transitions such as bud-, cap-, and bell-like morphological features (bud, cap, and bell stages).[1] The enamel knot (EK) that is a cluster of non-dividing epithelial cells plays a crucial role for tooth morphogenesis. The EK is classified into the primary enamel knot (PEK) and the secondary enamel knot (SEK). During bud to cap stage the PEK appears at the tip of the tooth bud where the folding of epithelia commences. The PEK functions as a signaling center where several signaling molecules including bone morphogenetic protein (BMP), Wnt family proteins, sonic hedgehog (Shh), and fibroblast growth factor (FGF) are expressed.[2] BMP4 induces the expression of *P21* in the EK that is a cyclin-dependent kinase inhibitor and contributes to the EK formation through inhibition of the cell cycle progression of EK cells.[3,4] BMP4 also induces *Msx2* that may be involved in the apoptosis of EK cells.[2] FGFs stimulate epithelial growth around the EK and subsequently cause the down-growth of cervical loops (CL) to form the tooth crown shape.[2,5] The CL is the boundary where the inner and outer enamel epithelia meet.[6] Once the CL changes its developmental fate into a root, the inner and outer enamel epithelia form a bilayered structure known as the Hertwig's epithelial root sheath (HERS).[7] The SEKs appear after the PEK disappears due to apoptosis in the teeth with multiple cusps (like murine molars).[2] The cusp formation is induced by the SEKs through a similar mechanism as the PEK leads epithelial proliferation.

Rodent incisors are continuously growing teeth. Thus, all stages of odontogenesis including amelogenesis and dentinogenesis can be surveyed by preparing sections of the tooth from the apical end to the incisal edge.[8,9] This phenomenon is maintained by both cell proliferation at the apical end and the attrition of the incisal edge. Recent molecular biology studies have clearly demonstrated the existence of a niche for self-renewing adult stem cells in these rodent incisors. We have used the term "apical bud" for the labial epithelial bulge of the apical end of incisors because this specific labial epithelial compartment includes stem cells and generates a number of signaling molecules. The maintenance and cell fate decisions of adult stem cells and the epithelial-mesenchymal interaction are controlled by FGF signaling.[10,11] The epithelial stem cell niche of incisors was identified using *in vivo* genetic lineage tracing.[12,13] Shh signaling is not required for stem cell survival. However, it is essential for the generation of ameloblasts from the stem cells.[12] Cells expressing the transcription factor *Sox2* are restricted to the epithelial cell niche and contribute to the renewal of ameloblasts and all other cells of the enamel organ. *Sox2* expression is regulated by the tooth initiation marker FGF8 and specific miRNA.[13] FGF10 is a survival factor that maintains the stem cell population in developing incisor germs.[11] Stem cell proliferation is stimulated by FGF3 and BMP4 represses *Fgf3* expression. Thus, activin, strongly expressed in the labial mesenchyme, inhibits the repressive effect of BMP4. In addition, TGF-β inhibitor Follistatin is expressed in the lingual epithelium and antagonizes the activity of activin.[14] Sprouty genes encode antagonists of receptor tyrosine kinase signaling and regulate ameloblast generation on only one side of the incisor by preventing the establishment of an epithelial-mesenchymal FGF signaling loop.[15] FGFR2b signaling regulates both the establishment of the incisor stem cell niches in the embryo and the regenerative capacity of incisors in the adult.[16] Furthermore, the overexpression of BMP inhibitor Noggin results in stimulated growth of the incisor and causes the total absence of enamel.[17] Thus, there is a delicate balance of signaling pathways that regulate enamel formation and the development of the asymmetric incisor.

The rodent incisor exhibits a unique form. It grows continuously and the enamel is deposited asymmetrically so that only the labial side is covered with enamel, but the lingual side is covered with cementum.[18,19] In sagittal sections, the incisor looks like a banana shape with curvature toward the lingual side in the apical end region. As it resembles a rotated molar shape, the terms "labial CL" and "lingual CL" have been commonly used to represent the apical end of the dental epithelia.[7,10,11,20] However, the enamel epithelium exhibits a U-shape in frontal sections of the apical end region due to the topographical location of the apical foramen that opens not in the exact apical end but in the lingual side. This shape is evocative of molar-tooth germ having ameloblasts and enamel in one side (crown-analog) and the CL in the other side (root-analog).[8,9,21,22] As introduced above, the CL is located distantly from the EK and the CL formation is induced by the EK in molar development. Thus, the so-called "labial CL" in murine incisors would be different from the CL in molars, if the existence of an EK-like structure is identified in the apical bud of incisors. To clarify this hypothesis, the present study aimed to investigate the gene expression patterns in relation to the EK in the apical bud.

2. Methods

2.1. Ethical approval

All experiments were reviewed by the Committee on the Guidelines for Animal Experimentation of Niigata University and were performed according to the recommendations or under the conditions proposed by the committee.

2.2. Animals and tissue preparation

Prenatal and postnatal ICR mice were used at embryonic Day 14 (E14) ($n = 3$), E15 ($n = 2$), E16 ($n = 3$), E17 ($n = 3$), E18 ($n = 3$) and postnatal Day 3 (P3) ($n = 22$). The heads were dissected from embryos after deep anesthesia was induced by an intraperitoneal injection of chloral hydrate (350 mg/kg) into a pregnant mother. The tissues were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 days. The postnatal mice were perfused via the heart with physiological saline and then 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under deep anesthesia. The mandibles including incisors were removed *en bloc* and immersed in the same fixative for an