New perspectives on tooth development and the dental stem cell niche*

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Summary. Adult stem cells have the capacity to self-renew and differentiate along multiple lineages in addition to contributing to ongoing tissue maintenance and regeneration after injury. They reside in specific locations called stem cell niches. In biology of the tooth, the discovery of dental epithelial stem cells in continuously growing teeth has been a recent breakthrough. The niche for the adult stem cells of these teeth is formed at the region of the apical end in tooth development. The region possesses a commonly specialized histological structure for the maintenance of adult stem cells and the production of various progenitor cells producing dental tissues. The molecular signals regulating the maintenance and cell fate decision of adult stem cells, such as Notch1, Lunatic fringe, fibroblast growth factor (FGF)-10, are expressed in the epithelial structure and the surrounding mesenchyme. Based on histological and molecular biological studies, we propose a new concept that the dental papilla produces various dental progeny are formed at the apical end in the development of continuously growing teeth, and coin a new term of "apical bud" for indicating this specialized epithelial structure. Furthermore, the relationship between signaling centers and the expression of FGF-10 mRNA as the determinant of morphogenesis is discussed with an emphasis on tooth and limb development, taking note that the expression pattern of FGF-10 is an important key for understanding the mechanisms for the diversity of cusp patterns and between continuous and limited growth.

Introduction

Adult stem cells are present in many vertebrate regenerative tissues including the hematopoietic system, nervous system, gut, gonads, skin, olfactory epithelium, and teeth (Morrison et al., 1997; Harada et al., 1999; Fuchs and Segre, 2000; Grontos et al., 2002). The stem cells are generally defined as cells that have the capacity to self-renew as well as to give rise to differentiated progeny. Adult stem cells have been shown to undergo asymmetric cell division resulting in one daughter cell remaining in the stem cell compartment and another undergoing further cell divisions to give rise to differentiated cells (Morrison et al., 1997; Harada et al., 1999). It is generally thought that stem cells respond to an environmental cue which stimulates cell division both in stem cells and their multipotent progeny referred to as the transit-amplifying cells. Recent studies of adult stem cells have revealed their location in various tissues, and the molecular mechanism for their niche has come to be elucidated (Watt and Hogan, 2000; Marshman et al., 2002; Doetsch, 2003; Zhang et al., 2003). Some conserved signal molecules, receptors, and transcriptional factors have been proposed as serving to function in the regulation of the self-renewal of the stem cells (Fuchs and Segre, 2000).

Teeth are epithelial appendages located at the entrance of the digestive tract and possess a complex morphology consisting of different arrangements, shapes and numbers of cusps, sizes of crowns, and fashion of growth evolutionally.
dependent on dietary custom. The exquisitely functional form of a developing tooth is the result of coordination between the processes of cell proliferation, differentiation, and death (Salazar-Ciudad et al., 2003). The processes are regulated by the sequential and reciprocal interaction between the oral ectoderm and neural crest-derived mesenchyme (Jernvall and Thesleff, 2000). The tooth bud folds at its tips and forms a cap-resembling structure surrounding the mesenchymal dental papilla (Ten Cate et al., 2003). Continuous growth and folding in the epithelium results in acquisition of the shape of the tooth crown during the bell stage of tooth development. Furthermore, it has been proposed that the primary and secondary enamal knots work as a signaling center in the regulation of a cusp pattern during the bud-bell stage. In mammalian dentition, the diversity of teeth is recognizable in not only the shape of the crown but also the fashion of growth. In the development of teeth showing limited growth such as mouse-, rat- and human-molars, formation of the root with periodontal tissue starts after the morphogenesis of the crown. In contrast, the rodent incisors and molars of voles, guinea pigs, and rabbits exhibit continuous growth throughout life. Cells that have the capacity to self-renew as well as to give rise to differentiated progeny are present at the apical end of their incisors and molars.

This review comparisons with limb development to discuss the acquisition of the fashion of growth during tooth development, which is deeply associated with the formation of the niche to maintain dental stem cells. Subsequently, to understand properly continuously growing teeth, a new term indicating specialized epithelial structures including the stem cell niche will be introduced. Moreover, from the aspect of regenerative medicine in dentistry attention is focused on adult stem cells and their niche in continuously growing teeth.

Common morphological features of the stem cell compartment in continuously growing teeth

The incisor of rodents represents a special tooth type since it grows continuously throughout the lifetime of the animal, and all stages of odontogenesis including amelogenesis and dentinogenesis can be analyzed if we observe the tooth from the apical end to the incisal edge (Smith and Warshtawsky, 1975a, 1976; Ohshima and Yoshida, 1992) (Fig.1). The dental epithelium of the continuously growing tooth has a special structure at the apical end which includes the cell-proliferative region. In three-dimensional
views by scanning electron microscopy, the specific bulbous protrusion of the incisor epithelium, which can be obtained from the lower mandibles of 2- or 3-day-old mice by treatment with 2% collagenase, is observed as a human head-like structure at the apical edge of the labial epithelium (Fig. 2). The localization of adult stem cells in the specific epithelial structure has been demonstrated by studies of cell kinetics using 5'-bromo-2'-deoxyuridine (BrdU) or Dil labeling analysis (Harada et al., 1999, 2002b).

It is very interesting that the morphological transition of the epithelial-mesenchymal compartment by the serial transverse section from the apical end to the incisal edge is likely to reflect the development of the tooth germ in the prenatal stage. Morphological features at about 30 μm from apical end of the epithelium are similar to those of the early bud stage of the molar tooth germ (Fig. 3a, e). At 90 μm, the stellate reticulum clearly appears in the epithelial mass; the epithelium possesses similar morphological features of the late bud stage (Fig. 3b, f). When cut incisally, the shape of the epithelium shows a cap-like form at 120–180 μm and a bell-like form at 210–300 μm (Fig. 3c, d, g, h). These structures are composed of the cells of the inner and outer enamel epithelium, and stellate reticulum; these distinct epithelial compartments of the enamel organ have also been confirmed by transmission electron microscopy (data not shown). When further cut incisally (about 500 μm from the apical end), the mesial and lateral Hertwig’s epithelial root sheaths (HERS) can be seen to elongate toward the lingual side and finally encircle the dental pulp totally (about 600 μm) (Fig. 4). The rodent incisors erupt throughout life, and the wear at the incisal edge is compensated by renewal in the apical end of the tooth, in which the proliferation and
differentiation of progenitor cells and their differentiation, the matrix deposition, and subsequent mineralization appear. Smith and Warshawsky (1975b) reconstructed outstanding three-dimensional views of the epithelial tissue at the apical end of the rat lower incisor, and referred to this epithelial compartment as the "odontogenic organ". They anticipated our new concept that the eternal tooth buds producing various dental progeny are formed at the apical end in the development of continuously growing teeth. Continuously growing teeth are represented not only by rodent incisors but also molars in certain other species, including rabbits, guinea-pigs, and the sibling vole (Microtus rossiae-melidonias). In these animals, the structural similarity of the epithelium has been detected (Starkey, 1963; Harada et al., 2002b; Tunmers and Thesleff, 2003). Basically, these specific structures are composed of a large amount of the stellate reticulum and basal epithelium, the latter becoming the inner and outer enamel epithelium.

In addition to the previous studies by in vitro and in vivo labeling analyzing in rodent incisors (Smith and Warshawsky, 1975a, 1976, 1977; Smith, 1980; Harada et al., 1999), our recent cell kinetic studies by the double staining of BrdU and Ki67 as markers of dividing cells have clearly shown the presence of adult stem cells, which demonstrate slow-cycling and asymmetric cell division, in the apical bud (Fig. 5). When cells labeled by BrdU are examined at 24h after BrdU injection, they are localized in the inner enamel epithelium and basal epithelium of the apical bud, which are also labeled by Ki67. However, at 120h after injection, the labeled cells reside only at the border between the stellate reticulum and the basal epithelium, and disappear from the inner enamel epithelium as transit-amplifying cells. Moreover, cells that behave like stem cells are also present at the apical end of the molar tooth germs of rabbits (Starkey, 1963). It is also true that immortalized cells derived from the epithelium of the rat incisor can differentiate a variety of dental epithelial cells—in outer enamel epithelial cells, stratum intermedium cells, stellate reticulum cells, and outer enamel epithelial cells (Kawano et al., 2004). Taken together, these findings show that the special epithelial component for the stem cell niche is obviously different from the cervical loop epithelium, which alters HERS (Kaneko et al., 1999), in mouse and human molar tooth germs. However, there has been no proper term indicating the specialized epithelial structure. Based on the studies of histology and molecular biology mentioned in the following sections, we propose a new anatomical term of "apical bud" for indicating this specialized structure and shall employ this term from here on.

**Molecular mechanisms for the stem cell niche in continuously growing teeth**

For most stem cells there are no specific markers that clearly show the tissue localization. However, evidence from a variety of studies indicates that the cells reside in specific locations called the stem cell niche (Watt and Hogan, 2000; Nishimura et al., 2002). The microenvironment in these niches supports the maintenance of stem cells as well as their self-renewal. Moreover, the specific environment for determining the fate of stem cells is thought to be present around their niches. The apical bud and the surrounding mesenchyme show the typical distinct gene-expression pattern of continuously growing teeth (Fig. 6). The gene expression has been examined exclusively in the mouse incisor (Harada et al., 1999) and vole molar (Tunmers and Thesleff, 2003). They exhibit two general sets of determinative factors: transmembrane or secreted signals are present in the local cellular environment, which is thought to be important for the regulation of the epithelial stem cell niche. The Notch signaling pathway is evolutionarily conserved as a cell-cell transmembrane interaction mechanism (Artavanis-Tsakonas et al., 1991, 1995; Lewis 1998; Hogan, 1999). In apical buds of the mouse incisor and vole molars, Notch1 mRNA have been shown to be expressed in the

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**Fig. 3.** Transverse semithin sections of the tooth germ of mouse molars at different stages (a–d) and the apical end of mouse incisors at different positions (e–h). a: At E13 (early bud stage), the oral epithelium proliferates and invaginates into the mesenchyme to form the bud-like structure. b: At E13.5 (late bud stage), the epithelial structure comes to include the stellate reticulum. c: At E14 (cap stage), the tooth germ assumes a cap-shaped conformation including the inner and outer enamel epithelium, stellate reticulum and dental papilla. d: At E15 (bell stage), the tooth germ has increased in size, resulting in the establishment of the tooth shape. e: The apical end of the dental epithelium (at about 30μm) shows a mass of epithelial cells which are similar to that of the early bud stage of the molar tooth germ. f: At about 90 μm, the epithelial mass comes to contain the stellate reticulum. g: The inner enamel epithelium can be distinguished from the outer enamel epithelium at 120–180μm from apical end of dental epithelium, showing a cap stage-like structure. h: At 210–300μm, the epithelial component shows bell stage tooth germ-like structure. DP: dental pulp, IEE: Inner enamel epithelium, OEE: outer enamel epithelium, SR: stellate reticulum, Bars = 50μm
Fig. 3. Legend on the opposite page.
Fig. 4. A diagram showing three-dimensional and transverse views at different positions (a–g) and a sagittal semithin section of the apical bud. The morphological transition by the transverse view from the apical to incisal edge (30–300 µm: a–d) is very similar to that of the tooth germ from bud to bell stage, respectively. Cutting toward the incisal edge (about 500 µm: e), the mesial and lateral HERS elongate toward lingual side and finally encircle the dental pulp (about 600 µm: f). HERS of the lingual epithelium is converted to epithelial cell rests of Malassez at the more incisal region (g). The specialized epithelial structure for continuous growth is seen as a human head like structure at the edge of the apical end of the labial epithelium and is known as the dental epithelial stem cell niche.
stellate reticulum, with especially strong expression in the border between the stellate reticulum and basal epithelium facing the basement membrane (Fig.6) (Harada et al., 1999; Tummers and Thesleff 2003). The strong expression of Notch1 mRNA is identical to that of HES1 (Hairy/Enhancer of Split) mRNA, which is a basic helix-loop-helix type transcriptional factor located as the downstream signal of Notch1. The basal epithelium expresses Lunatic fringe mRNA. In the inner enamel epithelium derived from basal cells, the expression of Lunatic fringe mRNA changes to Jagged1 mRNA, which continues to be expressed in the differentiated ameloblasts. The expression of fibroblast growth factor (FGF)-10 mRNA is restricted to the mesenchyme surrounding the apical bud. In the case of mice molars, on the other hand, mRNA of FGF-3 and FGF-10 are intensely expressed in the dental papilla mesenchyme during the cap stage when the tooth grows rapidly and the epithelium undergoes folding morphogenesis. The expression of these genes ceases gradually according to the progress of the tooth development (Keranen et al., 1998; Kettunen et al., 2000). The expression of FGF-10 mRNA in the vole continues in the mesenchyme surrounding the apical bud throughout life (Tummers and Thesleff, 2003). Our recent studies have shown that FGF-10 plays an important role in the formation and maintenance of stem cells in the development of mouse incisors (Harada et al., 2002a). Incisors obtained from FGF-10 gene deficient mice exhibit a limited growth pattern in vitro. Taken together, these findings indicate that the apical bud corresponding to the bud stage in the developing tooth germ is eternally maintained by the continuous expression of FGF-10. However, the regulatory mechanisms for the gene expression of FGF-10 have not yet been elucidated.

**Development of continuously growing teeth and limb bud**

The early morphogenesis of various organs shares similar early morphogenetic and molecular mechanisms. In the development of a variety of tissues, signaling centers such as the zone of polarizing activity (ZPA) in limb buds and
Fig. 6. Schematic illustrations showing gene expression patterns of apical buds and the surrounding mesenchyme. There are common gene-expression patterns of apical buds in the continuously growing teeth. Notch1 mRNA (pink) is intensely expressed in the stellate reticulum in the border of the basal epithelium facing the basement membrane. The expression of Notch1 mRNA overlaps that of HES1 mRNA (red), which is a basic helix-loop-helix type of transcriptional factors. Lunatic fringe mRNA (light green) is expressed in the basal epithelium, the inner enamel epithelium at the apical side, and Jagged1 mRNA (blue) in the region from the remainder of the inner enamel epithelium to differentiated ameloblasts. The expression of FGF-10 mRNA (yellow) is restricted to the condensed mesenchyme surrounding the apical bud. BE: basal epithelium, DA: differentiated ameloblasts, IEE: inner enamel epithelium, OEE: outer enamel epithelium, SR: stellate reticulum.

The FGF superfamily of proteins have been shown to be key players in the regulation of morphogenesis (Hogan, 1999; Ohuchi et al., 2000; Moorl oose et al., 2000). These signals are also likely to decide the pattern of growth in the development of continuously growing teeth. The tooth bud folds at its tips and form a cap-like structure surrounding the mesenchymal dental papilla. The growing and folding of the epithelium during the bell stage result in the acquisition of the shape of the tooth crown. The transition from the bud to the cap stages appears to be a critical step in tooth morphogenesis, and it marks the onset of the development of the tooth crown. The site at the tip of the tooth bud where the folding of the epithelium starts marks the formation of the enamel knots as a putative signaling center (Jernvall and Thesleff, 2000).

FGF-10 is one of factors secreted from mesenchymal cells, and the invagination of epithelium into dental papilla in tooth development is also deeply associated with it. The expression of FGF-10 mRNA starts at the bud stage of the tooth germ (Kettunen et al., 2000; Keranen et al., 1998; Tummers and Thesleff, 2003). In later stages, the expression comes to be restricted to the dental papilla facing the apical end of the elongated epithelium, including the enamel organ. In the development of mouse molars, FGF-10 deficiency inhibits the epithelial invagination into the dental papilla (Harada et al., 2002a), resulting in the hypoplastic
Fig. 7. Comparison between the relative position of signaling centers and the mesenchyme which expresses FGF-10 mRNA in development of continuously growing teeth and limbs. a: An apical bud of the rodent incisor is formed against a single enamel knot. b: In the development of the vole molar germ, plural secondary enamel knots appear and the plural apical buds are produced at the apical end of the invaginated epithelium. The formation of these apical buds is closely associated with the expression of FGF-10 mRNA in the mesenchyme. c: The mesenchyme underlying AER expresses FGF-10 and facilitates the growth of limb buds. ZPA plays a key role in the determination of the anterior-posterior axis in the limb. d: Application of exogenous signals at the opposite site leads to another limb bud.
On the other hand, the continuous expression of FGF-10 comes to maintain all parts of the enamel organ at the apical end of the epithelium throughout life in the continuously growing incisors and vole molars. Finally, the crown of vole molars or crown analog of mouse incisors continues to be produced by progenitor cells derived from the apical bud.

Interestingly, the relative position between enamel knots and the mesenchyme which express FGF-10 mRNA is extremely similar to that between ZPA and the apical ectodermal ridge (AER) in the development of the limb bud (Fig.7) (Gilbert, 2003). In development of the limb buds, a regulatory loop between the FGF-8 and FGF-10 has been shown to play a key role in the control of the formation of the limb buds, the induction of AER, and the activity of AER itself. ZPA located asymmetrically in the mesenchyme near the posterior end of AER regulates the growth towards anterior-posterior axis by secreting Sonic hedgehog (SHH). Implantation of ZPA tissue or application of beads absorbing recombinant SHH at the anterior end of AER induces a mirror-image ectopic limb structure (Riddle et al., 1993). In the development of rodent incisors (Kieffer et al., 1999), the single enamel knot is closely associated with the formation of the single apical bud. On the other hand, in the development of vole molars, plural secondary enamel knots may induce complicated structures which are composed of the continuous plural apical buds and implicate the intricate crown shape covered with enamel.

A variety of processes of morphogenesis and cellular differentiation ultimately result in the diversity of tooth types. Similar interactions using the same genes which are expressed in early tooth and limb development mediate patterns of cellular proliferation and differentiation through the activity of signaling centers within the developing tooth germ and limb bud. However, in the tooth germ, it remains unknown whether the gene expression in the enamel knots implicates FGF-10 mRNA in the mesenchyme.

**Prospects for the regenerative medicine in dentistry using dental stem cells**

The regeneration of the tooth as a whole organ is certainly much more demanding than that of other tissues such as the nerve, muscle, and bone. Tooth morphogenesis is characterized by sequential and reciprocal interactions between the dental epithelium and mesenchyme. Numerous signal molecules and growth factors, as mentioned above, have been implicated in the mediation of these interactions (Jernvall and Thepsleff, 2000; http://bite-it.helsinki.fi/). Studies on the function of signals and tissue interactions in cultured tissue explants and in mutant mice have shown inductive signaling and hierarchies in downstream transcriptional factors. The future, accumulation of molecular information should contribute to treatment for the regeneration of tooth. However, even with a more advanced understanding of the molecular mechanisms of a tooth development proceeds, the generation of tooth by tissue engineering would be most difficult since at least 200 genes are involved in the regulation of the position, shape, or number of teeth. At present our focus is on better practical material for tooth regeneration. Gene expression of the apical bud of the continuously growing teeth would be simpler than that of the developing tooth germ during the prenatal stage. The elucidation of molecular mechanisms for the maintenance and differentiation of apical bud cells would be more informative for the regenerative treatment of teeth. However, information on their gene expression has been lacking in our understanding of the mechanisms. Therefore, studies on the molecular mechanisms for adult stem cells, stem cell niche, determination of cell fate, and growth and differentiation of cells in these teeth are underway.

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**References**


