Journal of Oral Biosciences 57 (2015) 65–68

Contents lists available at ScienceDirect

Journal of Oral Biosciences

journal homepage: www.elsevier.com/locate/job

Review

Live imaging to elucidate cell dynamics in tooth organogenesis and regeneration

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ARTICLE INFO

Article history:
Received 14 January 2015
Received in revised form
2 February 2015
Accepted 6 February 2015
Available online 19 March 2015

Keywords:
Imaging
Tooth
Cell dynamics

ABSTRACT

Background: Tooth development involves epithelial–mesenchymal interactions, and its molecular mechanisms have been elucidated in various studies of growth factor signaling. However, there are discordances between the inferred molecular mechanisms and histological observations of shape changes of the growing tooth germ. To clarify the mechanisms of tooth morphogenesis, direct observations of cell dynamics are essential.

Highlight: A technique for direct observations of cell dynamics and morphological changes in the developing tooth germ has not been developed. Here, we present our observations of cell dynamics during tooth morphogenesis and review new imaging techniques used in recent studies. We also describe the importance of live imaging and its potential applications in the field.

Conclusion: Time-lapse imaging is a useful tool for understanding cell movement and cell lineages, and will contribute the elucidation of the mechanisms of tooth development.

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1. Introduction

Tooth germs develop via epithelial–mesenchymal interactions, and the molecular mechanisms of this process have been elucidated in various studies of growth factor signaling. A specific advantage of the tooth as a model is that the basic mechanisms regulating morphogenesis and differentiation have been analyzed in detail both in classical experimental embryological studies as well as in recent studies using molecular methods. Several conserved signaling molecules have been implicated in the mediation of the epithelial–mesenchymal interactions that regulate morphogenesis and cell differentiation in teeth. These include FGFs, BMPs, Wnts, Shh, and Notch, and there is a wealth of data on the expression patterns and functions of developmental regulatory molecules (Gene expression in tooth: http://bite-it.helsinki.fi/, 2015). However, previous studies have not focused on direct observations of cell dynamics, and none has directly observed cell movement in the tooth germ. An organ culture system of tooth

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http://dx.doi.org/10.1016/j.job.2015.02.005
1349-0079/

development had been designed and two-photon laser microscopes have been used to observe cell division and cell fate. Although there is no universally accepted technique for observing cell fate at present, imaging has increased our understanding of tooth organogenesis and will contribute to future elucidation of the mechanisms. We discuss the benefits and limitations of live cell imaging with respect to previous studies.

2. Understanding dental stem cell fate using live cell imaging in a continuously growing mouse incisor

Cell chase experiments have been useful for examining cell direction and/or cell fate during differentiation. Fluorescent dyes have been extensively used to label cells. Fluorescent 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate(DiI) is microinjected into dissected tissues and subsequently cultured. The movement and fate of cells can be observed by chasing the dye during organ culture. This technique has been used to visualize the fate of dental epithelial stem cells in the apical bud (labial cervical loop [CL] of mouse incisors) of a continuously growing mouse incisor [1,2]. The apical bud cells were labeled with fluorescent DiI at the onset of culture, and the localization of labeled cells was examined after 5 days of culture. Fluorescent cells extended from the CL to the differentiated ameloblasts, indicating that apical bud cells gave rise to differentiated dental epithelial cells, and in particular, ameloblasts. Cell movement has recently been observed in higher detail using time-lapse imaging.

The identification of dental epithelial stem cell marker proteins has facilitated the development of a technique for stem cell lineage analysis using mice expressing a fluorescent protein under the control of the promoter of the marker protein. Juuri et al. showed that dental epithelial stem cells express the Sox2 protein, and then used a chase experiment of Sox2-expressing cells using a combination of incisor organ culture of Sox2-GFP (green fluorescent protein) mice and three-dimensional (3D) live cell imaging [3]. Their results showed that the stem cells of the labial CL (apical bud) move toward the incisal side and expand to the labial and lingual sides. However, organ culture is technically limited in terms of the duration and difficulty associated with chasing single cells, so stem cell pathways during differentiation could not be completely monitored. Recently, this method has contributed to new findings regarding the dental pulp mesenchymal stem cell lineage [4,5].

3. Imaging of tooth germ development

The morphogenesis of tooth germs has mainly been studied by histological observation. Classical 3D reconstructions using serial sections have facilitated our understanding of tooth germ morphology [6]. Using 3D reconstruction software, various images of gene and protein expression patterns have recently been reported. Furthermore, 3D fluorescent images can be constructed using confocal microscopy without the need to generate serial sections. Because morphological changes occur sequentially during tooth organogenesis, we cannot fully observe the process of cusp formation using methods that involve fixation. In tooth development, the enamel knot is a known signaling center that regulates the cusp pattern [7]. A single enamel knot is thought to induce an incisor with a single cusp, and plural enamel knots produce a molar with a multicusp pattern. During molar development, the primary enamel knot initially develops during the bud stage, and plural secondary enamel knots develop during the transition from the cap to bell stages. The relationship between the existence of a primary enamel knot and the appearance of secondary enamel knots has been not established. Chase experiments using a DiI label have shown that the primary enamel knot remains in the same position and becomes buccal secondary enamel knots corresponding to the future paracone in the maxilla and protoconid in the mandible [8]. The secondary enamel knots contribute to the formation of other cusps. Live imaging allows the direct observation of processes involved in secondary enamel knot formation. Fucci mice, which were developed to monitor the cell cycle stages, have been used for the live imaging of tooth germ development [9]. Because cells of enamel knots do not divide and are confined to the G1/G0 stage, they strongly express red fluorescent signals. Accordingly, we were able to examine the relationship between primary enamel knots and the appearance of secondary enamel knots from the bud to bell stages by observing red fluorescent signals. Time-lapse imaging has shown that secondary enamel knots appear without the disappearance of the primary enamel knot. A primary enamel knot remains at the center of secondary enamel knots and produces the center cusp, presumably the protoconid (Fig. 1). Live imaging using Fucci and EDA1-GFP mice has shown a clear relationship between the primary enamel knot and secondary enamel knots [10]. These experiments have been conducted using enamel knot marker genes. Time-lapse imaging has shown changes in cusp pattern formation from single enamel knots to plural enamel knots during tooth germ development, indicating an increase in cusp number [10,11].

4. Development of stratum intermedium

Dental enamel epithelial stem cells differentiate into ameloblast cells via inner enamel epithelial (IEE) cells, stratum intermedium cells, stellate reticulum cells, and outer enamel epithelial (OEE) cells, as shown by chase experiments using the stem cell marker promoter-*Cre* and *loxP* (*flox*) system [3]. However, details regarding the differentiation process of dental epithelial stem cells have not been studied because differentiation markers for stratum intermedium cells, stellate reticulum cells, and OEE cells have not been identified. For example, the origin of stratum intermedium cells is not clear. Based on a chase experiment with DiI labeling and the discovery of transitional cells between stratum intermedium cells and IEE cells, Harada et al. inferred that stratum intermedium cells originate from IEE cells derived from stem cells [12]. However, this finding has been controversial owing to time-lapse imaging data using organ culture, which have captured the movement of a cell from the IEE cells to the stratum intermedium cells (Fig. 2). Although a direct path from stem cells to stratum intermedium

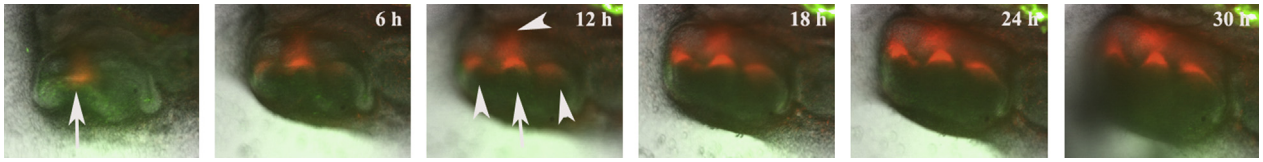

Fig. 1. Time-lapse imaging of the lower first molar of Fucci mice. The strong red color indicates the area in which cells are arrested at the G0/G1 stage, and consists of enamel knots. A primary enamel knot was observed at the center of the tooth germ (indicated by an arrow), and plural secondary enamel knots (indicated by arrowheads) appeared over time at the mesial, distal, and labial sides of the primary enamel knot. Bars: 500 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)