The regulation of tooth morphogenesis is associated with epithelial cell proliferation and the expression of Sonic hedgehog through epithelial–mesenchymal interactions

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ABSTRACT
Ectodermal organs, such as the tooth, salivary gland, hair, and mammary gland, develop through reciprocal epithelial–mesenchymal interactions. Tooth morphologies are defined by the crown width and tooth length (macro-morphologies), and by the number and locations of the cusp and roots (micro-morphologies). In our current study, we report that the crown width of a bioengineered molar tooth, which was reconstructed using dissociated epithelial and mesenchymal cells via an organ germ method, can be regulated by the contact area between epithelial and mesenchymal cell layers. We further show that this is associated with cell proliferation and Sonic hedgehog (Shh) expression in the inner enamel epithelium after the germ stage has formed a secondary enamel knot. We also demonstrate that the cusp number is significantly correlated with the crown width of the bioengineered tooth. These findings suggest that the tooth micro-morphology, i.e. the cusp formation, is regulated after the tooth width, or macro-morphology, is determined. These findings also suggest that the spatiotemporal patterning of cell proliferation and the Shh expression areas in the epithelium regulate the crown width and cusp formation of the developing tooth.

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1. Introduction

All organs arise from their respective germs through reciprocal interactions between the epithelium and mesenchyme during organogenesis in the developing embryo [1–4]. Organs develop according to predetermined programs, which include the regulation of their location, cell number and morphology. The induction of organ development at the appropriate future location requires both regional and genetic specificity [5]. It is well known in this regard that many cytokines, such as the fibroblast growth factor (FGF), hedgehog, Wnt, and transforming growth factor (TGF)/bone morphogenetic protein (BMP) families, play essential roles in epithelial and mesenchymal interactions during organogenesis [1,2].

Ectodermal organs, such as the tooth, salivary gland, hair, and mammary gland, also develop through reciprocal epithelial and mesenchymal interactions [1,2]. The number and morphology of the teeth in the tooth forming field, which are specified by the expression of homeobox genes in the underlying neural crest-derived mesenchyme in the embryonic jaw, have previously been determined during developmental process [3]. Tooth development begins with epithelium thickening and innervation of the underlying mesenchyme [1,2]. At the dental placode stage, the dental epithelium induces the condensation of the surrounding mesenchymal cells through the expression of signaling molecule genes such as Shh, Fgf8, Bmp4 and Wnt10b, which can induce the expression of a large number of transcription factors such as Msx1, Pax9 and Gli in the mesenchyme [2]. These interactions between these signaling molecules and transcription factors induce the formation of an enamel knot, which acts as a signaling center to coordinate tooth germ development [2]. Shh plays a particularly important role in tooth germ induction and formation, including the primary enamel knot formation, and thereafter functions in the growth and differentiation of epithelial cells into the ameloblast [6].

Following tooth germ formation, the epithelial and mesenchymal cells in the tooth germ differentiate into tooth-tissue forming cells and secrete hard tissues such as enamel dentin, cementum, and alveolar bone [7]. The tooth types that result, such as incisors (monocuspid) and molars (multicuspid), are thought to be
regulated by regional gene expression which controls the tooth-forming region at the mesenchyme during embryonic development [3]. It has also been reported that the tooth type and morphology is determined by the balance of endogenous inhibitors and mesenchymal activator [8] and by regulatory mechanisms that operate in the tooth forming field [9]. Tooth morphology is defined by both the crown size and tooth length at the macro-morphology, and by the number and position of the cusp and roots at the micro-morphology [10]. Although the crown size, as a determinant of macro-patterning during tooth morphogenesis, is based on the reaction-diffusion model [10], the underlying molecular and cellular mechanisms, such as cell growth and cell movement, have remained unexplored. The regulation of the cusp number and position, which underlies the micro-patterning of the tooth, is thought to be closely involved in the formation of the secondary enamel knot. This is regulated spatiotemporally by the reciprocal activation and inhibition of cell proliferation in the epithelium and mesenchyme via the reaction-diffusion mechanism, and determines the cusp pattern formation through cell growth and movement [11,12]. However, it remains to be undetermined how the regulation of cell proliferation and the underlying molecular mechanisms are involved in crown size determination through epithelial–mesenchymal interactions.

In our current study, we analyzed the mechanisms that determine the crown width and cusp number of a bioengineered tooth via the regulation of the contact area between the epithelial and mesenchymal cell layers. We provide evidence to suggest that the spatiotemporal regulation of epithelial cell proliferation and Shh expression in the tooth germ-epithelium is involved in determining the crown and cusp morphologies during tooth development.

2. Materials and methods

2.1. Animals

C57BL/6 mice were purchased from SLC Inc. (Shizuoka, Japan). B6.Cg-Shh<sup>tm1(EGFP)/cre/Cry</sup> mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mouse care and handling complied with the NIH guidelines for animal research and all experimental protocols involving animals were approved by the Tokyo University of Science Animal Care and Use Committee.

2.2. Reconstitution of a bioengineered tooth germ from single cells

Molar tooth germs were dissected from the mandibles of ED14.5 mice in order to reconstitute a bioengineered tooth germ by a three-dimensional cell manipulation method, the previously described organ germ method [13]. To regulate the contact length between the epithelial and mesenchymal cell layers, the epithelial and mesenchymal columnar cell layers were arranged contiguously using a micro-syringe with a 0.330 µm inner diameter (Fig. 1A). After one day of organ culture, we classified the bioengineered tooth germs into three-groups by measuring the contact length [13]. The bioengineered tooth germs, which were prepared using various contact lengths between the epithelial and mesenchymal cell layers, were reconstructed with a micro-syringe of a 0.330 µm inner diameter (Fig. 1A). After one day of in vitro organ culture, we classified the bioengineered tooth germs into three-groups by measuring the contact length using a side-view as follows: short-contact length (short), up to 450 µm; middle-contact length (middle), 450–900 µm, and long-contact length (long), 900–1500 µm. The mean widths were also calculated as follows: short, 366 ± 103 µm; middle, 584 ± 103 µm; and long, 934 ± 239 µm. All of the bioengineered tooth germs reached the early bell developmental stage at the same time as a natural tooth germ following 3–5 days in culture (Fig. 1B). To examine the correlation between the contact length and the tooth width of the bioengineered teeth, the germs were transplanted into a subrenal capsule. At 21 days post-transplantation, the entire bioengineered tooth germ developed into a tooth unit with the correct structure comprising enamel, ameloblast, dentin, odontoblast, dental pulp, alveolar bone, and blood vessels (Fig. 1C). Typical images of these teeth classified into the three-groups above are shown in Fig. 1C. The mean crown widths of the bioengineered molar that developed from the short, middle and long germ groups were 497 ± 118 µm, 727 ± 271 µm, and 1073 ± 186 µm, respectively. All of the crown widths of the samples following subrenal capsule

2.4. Histochemical and immunohistochemical analysis

Histochemical tissue analyses were performed as described previously [13]. Briefly, tissue sections (10 µm) were stained with hematoxylin and eosin and observed using Axiosimager A1 (Carl Zeiss) with an AxioCAM MRc5 (Carl Zeiss) microscope. Tissues were prepared for immunohistochemistry as described previously [14]. For fluorescent immunohistochemistry, the tissue sections (10 µm) were incubated with an anti-Ki67 primary antibody (1:100; Abcam, Cambridge, MA) and Hoechst33342 (1:500; Invitrogen, Carlsbad, CA) for 2 h at room temperature. Immunoreactivity was detected using an Alexa Fluor® 594-conjugated Goat Anti-rabbit IgG secondary antibody (1:500, Invitrogen). Fluorescence microscopy images were captured under a confocal microscope (LSM 510; Carl Zeiss) and processed with AxioVision software (Carl Zeiss).

2.5. In situ hybridization

In situ hybridizations were performed using 10 µm frozen sections as described previously [13]. Digoxigenin-labeled probes for specific transcripts were prepared by PCR with primers designed using published sequences (Shh; GenBank ID: NM_009170, Fgf4; GenBank ID: NM_010202, Fgf3; GenBank ID: NM_008007).

2.6. Statistical analysis

Statistically significant differences were determined by the unpaired student’s t-test. The analysis was performed using the Common Gateway Interface Program (twk, Saint John’s University).

3. Results

3.1. The crown width of a bioengineered tooth correlates with the length of the contact area between the epithelial and mesenchymal cell layers

We first investigated whether the contact area between the epithelial and mesenchymal cell layers affect the eventual morphology, such as the crown width and cusp number, of a bioengineered tooth germ reconstituted from ED14.5 molar tooth germ-derived single cells using the organ germ method [13]. The bioengineered tooth germs, which were prepared using various contact lengths between the epithelial and mesenchymal cell layers, were reconstructed with a micro-syringe of a 0.330 µm inner diameter (Fig. 1A). After one day of in vitro organ culture, we classified the bioengineered tooth germs into three-groups by measuring the contact length using a side-view as follows: short-contact length (short), up to 450 µm; middle-contact length (middle), 450–900 µm, and long-contact length (long), 900–1500 µm. The mean widths were also calculated as follows: short, 366 ± 103 µm; middle, 584 ± 103 µm; and long, 934 ± 239 µm. All of the bioengineered tooth germs reached the early bell developmental stage at the same time as a natural tooth germ following 3–5 days in culture (Fig. 1B). To examine the correlation between the contact length and the tooth width of the bioengineered teeth, the germs were transplanted into a subrenal capsule. At 21 days post-transplantation, the entire bioengineered tooth germ developed into a tooth unit with the correct structure comprising enamel, ameloblast, dentin, odontoblast, dental pulp, alveolar bone, and blood vessels (Fig. 1C). Typical images of these teeth classified into the three-groups above are shown in Fig. 1C. The mean crown widths of the bioengineered molar that developed from the short, middle and long germ groups were 497 ± 118 µm, 727 ± 271 µm, and 1073 ± 186 µm, respectively. All of the crown widths of the samples following subrenal capsule...
transplantation were then plotted and statistical analysis indicated a reliable correlation between the contact lengths of the bioengineered tooth germs and the crown widths of the resulting teeth ($R = 0.83$; Fig. 1D). These results indicate that the crown width is controlled by the contact area between the epithelial and mesenchymal cell layers.

### 3.2. The cusp number of the bioengineered teeth correlates with their crown width

We next examined whether the crown width affects the determination of the cusp number in the bioengineered teeth. At 21 days post-transplantation into the subrenal capsule, the cusp numbers of the bioengineered teeth were analyzed by Micro-CT (Fig. 2A). Statistical analysis indicated a reliable correlation between the contact length of the bioengineered tooth germs and the resulting cusp numbers of the bioengineered teeth ($R = 0.87$; Fig. 2B). These observations suggest that the eventual cusp number is indeed dependent on the crown width.

### 3.3. The Shh expression area correlates with the crown width in developing tooth germ

Sonic hedgehog (Shh) and fibroblast growth factor 4 (Fgf4) play important roles in the early development of tooth germ [1,2]. We next investigated the expression patterns of these factors during the early developmental stages of both natural molar and bioengineered tooth germ. During natural tooth germ development, Shh and Fgf4 mRNA-positive cells can be observed in the enamel knot, which is the epithelial signaling center, at ED13.5 and ED14.5 (Fig. 3A). Interestingly, the Shh expression area was found to extend from the enamel knot to the inner enamel epithelium, in accordance with the progression of crown development, but was restricted in the inner enamel epithelium at the prospective occlusal region, but not at the lateral region, at ED15.5 and ED16.5 (Fig. 3A). In contrast, Fgf4 mRNA-positive cells were found to be restricted locally throughout the early period of crown development and these transcripts were detectable in the enamel knot at ED13.5–ED15.5 and in the secondary enamel knot at ED16.5 (Fig. 3A). In the case of the bioengineered molar germ, the expression patterns of Shh and Fgf4 mRNAs were identical to those of the natural tooth germ (Fig. 3A). Shh expression was restricted to the enamel knot after 2 days and to the inner enamel epithelium at the prospective occlusal region after 3–7 days of organ culture (Fig. 3A). Fgf4 expression was detected in the enamel knot after 3–5 days and in the secondary enamel knot after 7 days organ culture (Fig. 3A). The expression of Fibroblast growth factor 3 (Fgf3), which is also thought to be an important mesenchymal signaling molecule, was detectable in the dental papillae adjacent to the Shh-expressing inner enamel epithelium throughout the early period of development in both natural and bioengineered tooth germ (Fig. 3B). Hence, although the development of bioengineered tooth...
germ is delayed compared with natural germ, the molecular mechanisms regulating early tooth germ development are similar for both bioengineered and natural tooth germ.

We further examined the correlation between the width of Shh-expressing area in the inner enamel epithelium at the prospective occlusal region and the crown width of the bioengineered tooth germ. We generated bioengineered molar tooth germs from the three contact area groups over three days in organ culture. The Shh-expression area was found to be extended from the enamel knot to the inner enamel epithelium at the prospective occlusal region, but not at the lateral region, over the culture period, and the width of this area was observed to be equivalent to the width of prospective crown of bioengineered tooth germ (Fig. 3C). These results suggest that the Shh-expression pattern in the inner enamel epithelium is closely associated with the mechanisms that regulate crown width.

3.4. The spatiotemporal epithelial cell growth that correlates with the Shh expression pattern is involved in the regulation of crown size

We finally investigated whether the spatiotemporal Shh expression pattern shown in Fig. 3 is involved in the patterning of epithelial cell proliferation during tooth germ development. We therefore analyzed Shh expression using a transgenic mouse expressing green fluorescent protein (GFP) at the Shh locus (ShhGFP mice). We measured cell proliferation by immunohistochemical analysis of Ki67 in natural and bioengineered tooth germs. In a natural ED14.5 molar tooth germ, Shh expression in an in vitro organ culture shows the same expression pattern as that detected by in situ hybridization in vivo (Figs. 3A and 4A). Cell proliferation in natural tooth germ detected by Ki67 was evident at the cervical loop after one day and the lateral region of the epithelium after 2–4 days, in which Shh expression was not found (Fig. 4A). The bioengineered molar tooth germ showed expression of Shh at the first enamel knot on day 3 and in the inner enamel epithelium in the prospective occlusal region at day 6 in an organ culture. Cell proliferation of the bioengineered germ was also observed in the Shh expression-negative regions at the cervical loop and inner enamel epithelium in the lateral region after 6 days of organ culture (Fig. 4B). These results suggest that the crown width is determined by the spatiotemporal area patterning of the Shh expression-positive and cell proliferation-negative regions of the inner enamel epithelium during the early bell stage.

4. Discussion

We demonstrate herein that the crown width of a bioengineered tooth is regulated by the contact area between the epithelial and mesenchymal cell layers and associates with cell proliferation and Shh expression in the inner enamel epithelium. We also demonstrate that the cusp number is significantly correlated with the crown width of the bioengineered tooth. These findings also suggest that the spatiotemporal patterning of the cell proliferation and Shh expression areas in epithelium regulates the crown width and cusp formation of the tooth.

At the initiation phase of tooth germ development, the tooth-forming field and the basic pattern of dentition (molar and incisor) are regulated by the coordination of gene expression patterning [3]. Rostral–caudal patterning determines the tooth-forming field through the expression patterns of Lhx6/7 and Gsc [3]. On the other hand, proximal-distal patterning determines the molar and incisor fields through the formation of gene expression patterning between Bmp4 and Fgf8 in the epithelium, and these gene products then induce Msx1/2, Barx1 and Dbx2 in the mesenchyme [3]. It is thought that the number of teeth, the sizes of which are also determined by the size of the tooth germ, is proportional to the size of the tooth-forming field [3]. A previous study has indicated that the final crown size is memorized in the dental mesenchyme and regulated by the reaction-diffusion model [10]. In our current study, we provide evidence that the crown width is determined by the contact area between the epithelial and mesenchymal cell layers. We also demonstrate that the Shh-expressing region in the dental epithelium of not only natural but also the bioengineered tooth germ gradually enlarges to the final size of the crown width, and that cell proliferation does not occur in the Shh-expression region. These findings indicate that cell proliferation is essential for the determination of the crown width in the inner enamel epithelium at the prospective occlusal region, in which the cells express Shh, and also for tooth root formation in the cervical loop region. It has been reported previously that Shh regulates epithelial proliferation, cell survival, and tooth size [15]. Our present results suggest the possibility that the spatiotemporal regulation of
epithelial cell proliferation and Shh expression are closely involved in the determination of tooth macro-morphology and represent the molecular basis of tooth size determination.

A reaction-diffusion model has been predicted and analyzed in the patterning of micro-structures such as digits in limbs, feathers in skin, and cusps in tooth [11,16,17]. Previous studies have suggested that the number of digits in the mouse limb is regulated by not only the width of the mesoderm but also the length of the Fgf4-expressing apical ectodermal ridge, which is the signaling center in limb development [18]. In tooth development, it is thought that the cusp patterning is regulated by the secondary enamel knots, which is one of the signaling centers for cusp

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**Fig. 3.** The width of the Shh expression area correlates with the crown width in the developing tooth germ. (A) In situ hybridization analyses of the Shh and Fgf4 expression profiles in natural tooth germ at ED13.5–ED16.5 and in bioengineered tooth germ after 2, 3, 5, and 7 days of organ culture. (B) Expression patterns of Shh and Fgf3 in natural tooth germ at ED14.5–ED16.5 and bioengineered tooth germ after 2, 5, and 7 days of organ culture. (C) Phase contrast images and expression analysis of Shh mRNA in the three contact area groups of bioengineered tooth germ after three days of cultivation. Dotted lines indicate the boundaries between the epithelium and mesenchyme. The area inside the line is the epithelium. Scale bars, 200 μm.
formation, and might be regulated by the interaction between FGF4, as an activator of cusp formation, and the BMPs and SHH as inhibitors [19]. Based on an earlier computational model of mammalian tooth development, there may be a simple basis for the variations in cusp patterning which can be explained by changes in single model parameters [12,20]. Although there are several reports regarding the determination of tooth size and cusp number, the mechanisms suggested in these studies remain controversial. It was previously reported that each tooth and cusp size is determined by each mesenchyme and epithelium, respectively, through species-specific biological programs [10]. It has also been suggested that the number of developing cusps of a tooth reconstituted from dental epithelium and dissociated mesenchymal cells was enlarged by increasing the mesenchymal cell number [21]. Furthermore, it has been suggested that the number of cusps, a micro-patterning event, is regulated by waves which are determined by the tooth size i.e. macro-patterning, in a reaction-diffusion model [10]. Our present findings indicate that the cusp number in a bioengineered tooth is significantly correlated with the crown width, which is regulated by the contact area between the epithelial and mesenchymal cell layers. These findings suggest that the micro-patterning of cusp formation is regulated after tooth size determination (i.e. macro-patterning) through the number and length of waves in a reaction-diffusion model.

Currently, it is anticipated that organ replacement therapy will represent the next generation of regenerative medical technology and successfully replace lost or damaged organs [22]. For such replacement tissues to function correctly, it will be necessary to reproduce the organ mass through the generation of a sufficient number of functional cells, and faithful replication of both the macro- and micro-morphologies [23]. As a concept for organ regeneration, an approach to developing a functional bioengineered organ from a bioengineered organ germ by reproducing the developmental process during organogenesis has now been investigated [4,24]. We have also previously reported from our laboratory that a bioengineered tooth germ reconstituted using an organ germ method can regenerate a fully functional bioengineered tooth through transplantation [14]. It is thought that the regulation of a bioengineered tooth morphology, such as the crown size and the number and location of the cusps, is important for occlusion to properly occur [11], which is an essential issue for future tooth regenerative therapy [4,24]. Previously, it has been reported that the morphology of a bioengineered tooth can be regulated by culturing tooth germ cells onto a tooth-shaped biodegradable scaffold [24]. In our present study, our cell manipulation technique to regulate the contact area between epithelial and mesenchymal cell layers will make a substantial contribution to the future clinical application of bioengineered teeth.

Our present data suggest that the spatiotemporal patterning of cell proliferation and Shh expression areas in the epithelium regulates the crown width and cusp formation of the tooth. Further studies of the molecular mechanisms underlying cell proliferation, differentiation and cell movement will contribute further to our understanding of tooth organogenesis. The development of future

Fig. 4. The spatiotemporal epithelial cell growth correlates with the Shh expression area and is involved the regulation of crown size. (A) Molar germs isolated from an ShhGFP mouse at ED14.5 after 1, 2, and 4 days of organ culture. (B) ShhGFP bioengineered tooth germs, which were generated from the molar germs of an ShhGFP mouse at ED14.5, were examined after 3 and 6 days of organ culture. Shh expression was visualized by the detection of GFP signals. Cell proliferation was immunohistochemically analyzed using a specific antibody against Ki67. Nuclei were detected by staining with Hoechst 33342. Dotted lines indicate the boundaries between the epithelium and mesenchyme. Scale bars, 100 μm.
technologies to more precisely regulate the morphology of bioengineered teeth will be required to realize tooth regenerative therapy.

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