

successful replacement of an entire and fully functioning organ could be achieved through the transplantation of bioengineered organ germ i.e. a successful organ replacement regenerative therapy [20].

Transplantation of a bioengineered mature organ will lead to immediately perform of the full functions *in vivo* and have a profound impact on the survival outcomes of many diseases [2,9]. Transplanted bioengineered organs are also expected to be viable over the long-term and achieve the continuous production of various functional cells and their progenitors from stem cells as efficiently as the natural organ *in vivo* [23,24]. It has also been proposed that mature organs can be developed from bioengineered organ germ by faithfully reproducing *in vivo* developmental processes. In the dental treatment, it has been expected to transplant of a bioengineered tooth unit comprising mature tooth, periodontal ligament (PDL) and alveolar bone into the tooth loss region through bone integration, which is connected between recipient bone and bioengineered alveolar bone in a bioengineered tooth unit [25]. Transplantation of a bioengineered tooth unit has also been proposed as a viable option to repair the large resorption defects in the alveolar bone after tooth loss [26]. However, there are currently no published reports describing successful transplantation or replacement using a bioengineered tooth [10,27].

In our current study, we have generated a bioengineered tooth unit, which was controlled for length and shape and report a successful tooth replacement by transplantation of a bioengineered tooth unit into the tooth loss region, followed by successful bone integration, and restoration of tooth physiological functions such as mastication, PDL function and an appropriate responsiveness to noxious stimulations. This transplantation of a bioengineered tooth unit could also regenerate alveolar bone formation in a vertical direction. Our results thus further demonstrate the potential for bioengineered tooth replacement as a future regenerative therapy.

## Results

### Generation of a Bioengineered Tooth Unit

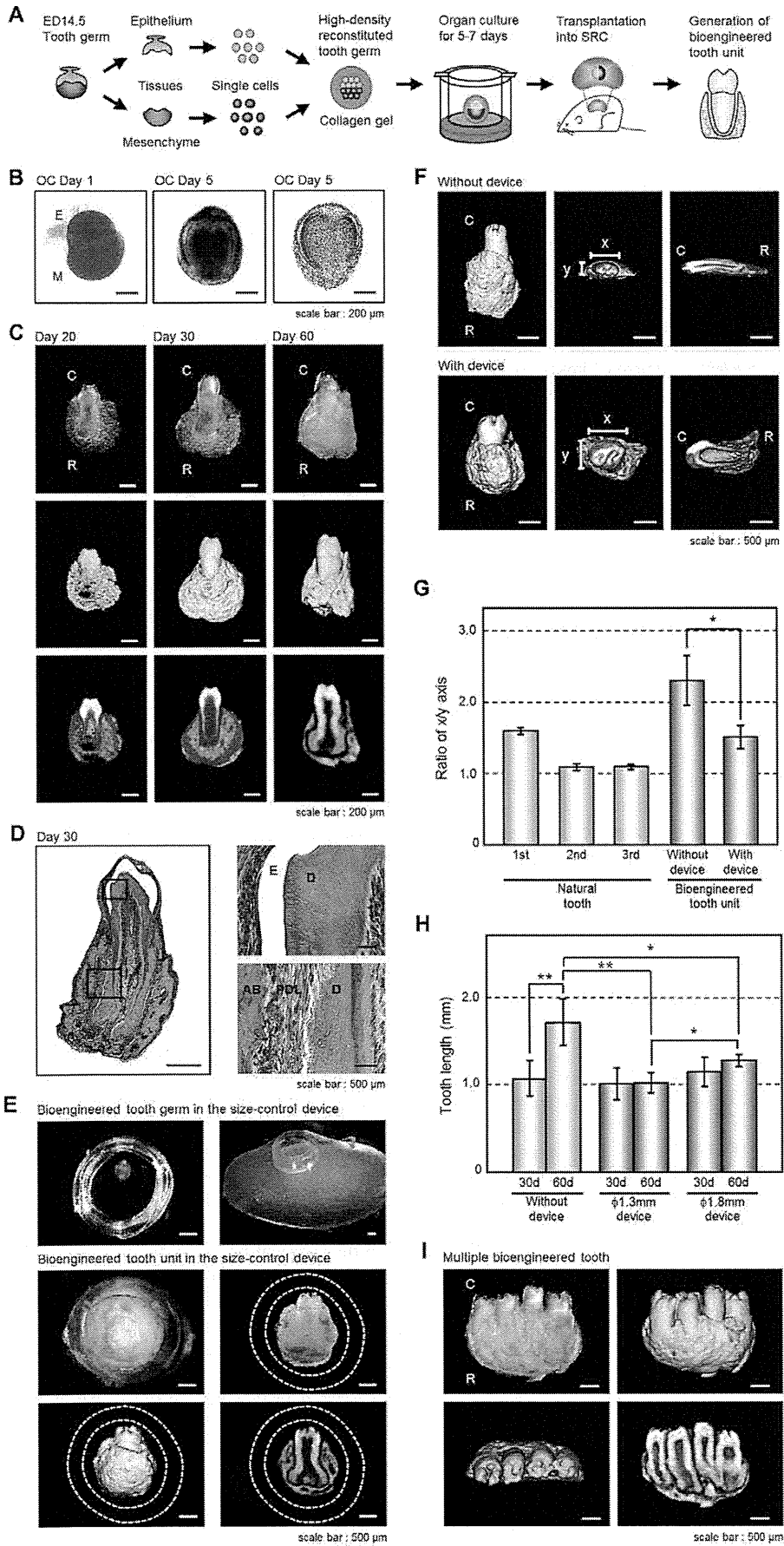
We have previously reported that bioengineered tooth germ can successfully develop a bioengineered tooth that by subrenal capsule transplantation can restore a mature tooth, including periodontal tissue and alveolar bone [22]. Because a three-dimensional *in vitro* organ culture has not yet been developed, we employed a strategy involving a bioengineered tooth unit, which has the necessary tissues to restore tooth functions, to investigation and advance the future potential of bioengineered tooth replacement (figure 1A). The bioengineered molar tooth germ was developed to a stage equivalent to the early bell stage of natural tooth germ for 5–7 days in an *in vitro* organ culture (figure 1B). Although we have previously reported that multiple bioengineered teeth have been formed from a bioengineered tooth germ reconstituted by our organ germ method [22], we recently developed a method to generate a single and width-controlled bioengineered tooth [28]. The bioengineered tooth germ gradually accumulated hard tissue, root extension, and an increased alveolar bone volume, depending on transplantation periods, and could successfully generate a tooth unit with the correct structure of a whole molar, and the proper formation of periodontal tissue and surrounding alveolar bone (figure 1C, D). However, the shape (x vs. y axis) of the bioengineered tooth unit was flattened by the pressure of the outer membrane of the subrenal capsule (figure 1F, G). The length of the tooth also showed continuous root elongation depending on the transplantation periods without occlusional mechanical stress (figure 1C, F, H).

To generate the shape- and length-controlled bioengineered tooth unit so that a suitable size was obtained for intraoral transplantation, the tooth germ was inserted into a ring-shaped size-control device and then transplanted into a subrenal capsule (figure 1E). The crown widths, calculated from the x/y axis ratios, of natural first, second and third molars of 9-week-old adult mice were  $1.61 \pm 0.05$  mm,  $1.09 \pm 0.04$  mm,  $1.12 \pm 0.04$  mm, respectively (each  $n = 5$ , figure 1G). The crown width of the bioengineered tooth units grown in the size-control device, which had a 1.8 mm inside diameter and 1.3 mm thickness, was  $1.46 \pm 0.16$  mm whereas when grown outside of the device the size was  $2.30 \pm 0.35$  mm (each  $n = 5$ , figure 1G). The device thus successfully generated a size-controlled bioengineered tooth so that it was similar to a natural tooth (figure 1F, G). This device could avoid the pressure by the subrenal capsule membrane, and reserve the three-dimensional space for developing a bioengineered tooth germ normally. We next evaluated the length of a bioengineered tooth unit generated in the size-control device (figure 1E). After 30 or 60 days, the lengths of the teeth transplanted without the devices were  $1.07 \pm 0.20$  mm and  $1.70 \pm 0.26$  mm, respectively, which was significantly associated with the transplantation period (each  $n = 5$ , figure 1H, figure S1A). Although the length of the bioengineered tooth transplanted without the devices was  $1.70 \pm 0.26$  mm after 60 days transplantation, bioengineered teeth transplanted in devices of 1.3 or 1.8 mm in diameter, was significantly regulated at  $1.02 \pm 0.11$  or  $1.27 \pm 0.06$  mm, respectively (each  $n = 5$ , figure 1H). The shape and length of the bioengineered tooth unit can therefore be controlled in three-dimensions using a specialized device.

Multiple bioengineered tooth units surrounded by alveolar bone could be also generated by the transplantation of several tooth germs into a single size-control device (figure 1I, figure S1B). Each resulting tooth had the correct structure including pulp cavities and partitioned periodontal spaces (figure 1I, figure S1C). Hence, multiple tooth replacements can be achieved with this regenerative transplantation method.

### Transplantation of a Bioengineered Tooth Unit into a Tooth Loss Region *in Vivo*

We next investigated whether a bioengineered tooth unit could be engrafted via the integration between the alveolar bone of this unit and that of the host recipient and then function appropriately by occlusion with an opposing tooth (figure 2A). The bioengineered tooth unit, which was generated by transplantation in a device of a 2.5 mm inside diameter for 50–60 days and labeled by the administration of calcein reagent into recipient mouse (figure 2B), was transplanted with the correct orientation into a properly-sized bony hole in the lower first molar region of the alveolar bone in a 4-week-old mouse (figure 2C). Briefly, in this mouse model, the lower first molar had been extracted, and the resulting gingival wounds had been allowed to heal for 4–6 days (figure S2A). When the bioengineered tooth unit was transplanted, it was located at a position reaching the occlusal plane with the opposing upper first molar (figure 2C, figure S2A). Partial bone integration was observed at 14 days after transplantation, and full bone integration around a bioengineered tooth root was seen at 30 days after transplantation (figure 2C). In the calcein-labeled alveolar bone of bioengineered tooth unit, resorption was partially observed at the surface at 30 days post-transplantation (figure 2D, figure S2B). The calcein-labeled bone finally disappeared and the recipient bone around the bioengineered tooth root replaced it completely at 40 days after transplantation at a frequency of 66/83 (79.5%; figure 2C, D, figure S2B). There have been many previously reported clinical cases of multiple tooth loss, the most serious condition being edentulism [29]. It is possible that a



**Figure 1. Generation of a bioengineered tooth unit.** (A) Schematic representation of the generative technology of bioengineered tooth unit. (B) Phase construct imagery of a bioengineered tooth germ on day 1 (left) and 5 (center) and HE staining (right) of an organ culture on day 5. Scale bar, 200  $\mu\text{m}$ . E, epithelium; M, mesenchyme. (C) Photographs (upper) and micro-CT images of the external surface area (middle) and cross section (lower) of a bioengineered tooth unit. Images were captured at 20 days (left), 30 days (center) and 60 days (right) after subrenal capsule transplantation (SRC). Scale bar, 200  $\mu\text{m}$ . C, tooth crown side; R, tooth root side. (D) Histological analysis of the bioengineered tooth unit on day 30 after SRC transplantation (left). (Scale bar, 500  $\mu\text{m}$ ). Higher magnification images of crown area (upper right) and the periodontal tissue area (lower right) are also shown. Scale bar, 50  $\mu\text{m}$ . E, enamel; D, dentin; AB, alveolar bone; PDL, periodontal ligament. (E) Photographs of the developmental processes occurring in bioengineered tooth germ in a subrenal capsule (SRC) using a size-control device. Images were captured of bioengineered tooth germ orientated in the device (top left), transplantation into the SRC (top right), and the bioengineered tooth at 50–60 days after transplantation in the SRC (middle). Micro-CT images of the external surface area (bottom left) and cross section (bottom right) are also shown. The dotted lines indicate the outlines of the device. Scale bar, 500  $\mu\text{m}$ . (F) Micro-CT images of a bioengineered tooth unit transplanted into the SRC for 30 days with (lower column) or without (upper column) the size-control device at an external (left), axial (center) or cross section (right) view. Scale bar, 500  $\mu\text{m}$ . x, x-axis of the crown; y, y-axis of the crown. (G) X-axis versus y-axis ratios (x/y) of the crowns of bioengineered tooth units at 30 days post transplantation into an SRC, and also of natural first, second and third molars from 9-week-old mice. Transplantations were performed with or without the 1.3 mm thickness size-control device. Error bars show the standard deviation ( $n=5$ ).  $*P<0.001$  (t-test). (H) The lengths of the bioengineered tooth units generated using size-control devices, which were of a 1.3 mm ( $\phi 1.3$  mm) or 1.8 mm ( $\phi 1.8$  mm) inner diameter, at 30 and 60 days post transplantation into an SRC were compared with or without the devices. Error bars show the standard deviation ( $n=5$ ).  $*P<0.01$  and  $**P<0.001$  (t-test). (I) Photograph (first figure from the left) and micro-CT images showing external (second figure), axial (third figure) and cross section (fourth figure) views of a multiple bioengineered tooth units, in which four teeth were contained in one alveolar bone, after 60 days transplantation into the SRC. Scale bar, 500  $\mu\text{m}$ .  
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bioengineered teeth unit could be transplanted into an edentulous jaw (figure S2E, F). Our current findings suggest that bioengineered teeth can be engrafted into regions of tooth loss through bone integration, which involves resorption of the alveolar bone of the bioengineered tooth unit through natural bone remodeling in the recipient.

The engrafted bioengineered tooth was found to be aligned appropriately and occlude with the opposing upper first molar (figure 2E, figure S2C). Micro-CT analysis also revealed that no root elongation was evident for the bioengineered tooth and that the apical foramen of the engrafted bioengineered tooth root significantly narrowed at 40 days after transplantation (each  $n=9$ , figure S2D). These results suggest that the bioengineered tooth in the tooth unit isolated from subrenal capsule transplantation is immature tooth, which has the potential to narrow of the apical foramen after the oral transplantation and would have the physiological ability to recapitulate mechanical stress by occlusion.

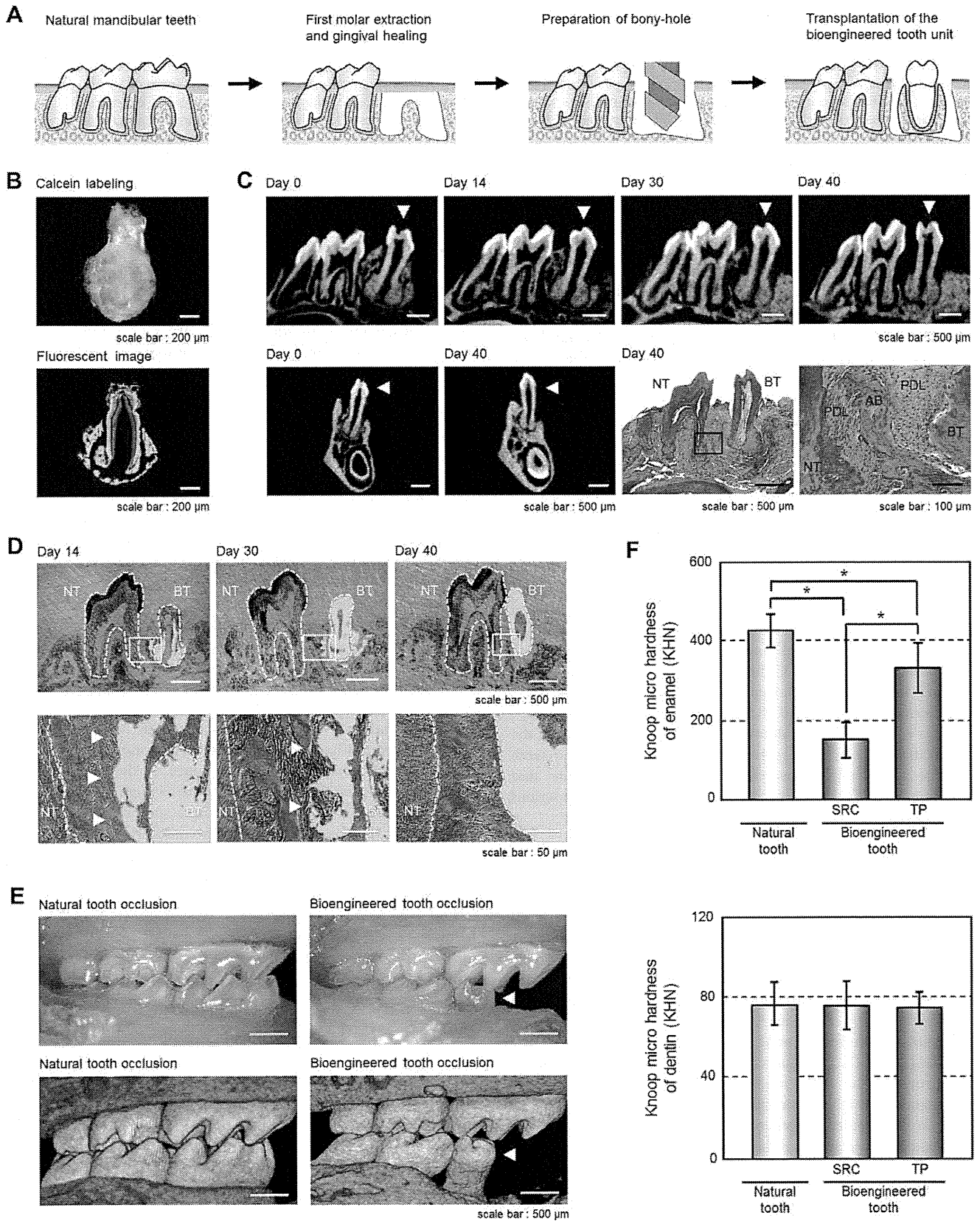
Masticatory potential is essential for proper tooth function and we next performed a Knoop hardness test, an important measure of masticatory functions, on bioengineered teeth including both the dentin and the enamel components. The Knoop hardness numbers (KHN) of the enamel and dentin in the natural teeth of 11-week-old adult mice were measured at  $404.2\pm 78.2$  and  $81.0\pm 11.5$ , respectively (each  $n=5$ , figure 2F). The bioengineered teeth generated in a subrenal capsule (SRC) and in jaw bone (TP) showed similar KHN values at  $179.6\pm 49.2$  and  $319.6\pm 78.3$  in the enamel, and  $80.7\pm 11.5$  and  $76.8\pm 13.6$  KHN in the dentin, respectively (each  $n=5$ , figure 2F). The value of enamel Knoop hardness of natural tooth increase in according to postnatal period [20]. Although the enamel hardness of the bioengineered tooth generated in a SRC showed low KHN values, the enamel hardness of the engrafted bioengineered teeth (TP) increased to the high KHN value in according to the period after the transplantation into jaw bone. Therefore, the hardness of the dentin in the engrafted bioengineered teeth was in the normal range. These findings indicate that the hardness of the enamel and dentin in the engrafted bioengineered teeth were in the normal range.

### Functional Analysis of the Periodontal Ligament and Neurons of the Engrafted Bioengineered Teeth

Previously, it had been demonstrated that the bioengineered tooth germ can recapitulate physiological tooth function in the adult murine oral environment [20]. In our present study, we next

investigated whether an engrafted bioengineered mature tooth unit can also restore physiological tooth functions *in vivo* such as the response to mechanical stress and the perceptive potential for noxious stimulations. It is essential for tooth functions that the engrafted bioengineered tooth in recipient has the cooperation with the oral and maxillofacial regions through the PDL. The response of the PDL to mechanical stress, such as orthodontic movements, induces alveolar bone remodeling, which is indicated by the localization of tartrate-resistant acid phosphatase (TRAP)-osteoclasts and osteocalcin (*Ocn*) mRNA-positive osteoblasts [20]. During experimental tooth movement, TRAP-positive osteoclasts and *Ocn* mRNA-positive osteoblasts were observed on the compression and tension sides, respectively (figure 3A). This demonstrated that the PDL of the bioengineered tooth unit successfully mediates bone remodeling via the proper localization of osteoclasts and osteoblasts in response to mechanical stress.

The perceptive potential for noxious stimulation including mechanical stress and pain, are important for proper tooth function [30]. Trigeminal ganglionic neurons, which innervate the pulp and PDL, can respond to these stimulations and transduce the perceptions to the central nervous system. Blood vessels that are detected in the pulp and PDL, maintain dental tissues such as odontoblasts, pulp, the PDL and alveolar bone. In our current experiments, we evaluated the responsiveness of nerve fibers in the pulp and PDL of the engrafted bioengineered tooth to noxious stimulations. Although von Willebrand Factor (vWF)-positive blood vessels were observed in the pulp and PDL of the bioengineered tooth generated in a subrenal capsule, anti-neurofilament (NF)-immunoreactive nerve fibers could not be detected (figure 3B, figure S3A, B). However, NF-positive nerve fibers could be detected in the pulp and PDL of the engrafted bioengineered tooth in the recipient bone and the neurons merged with vWF-positive blood vessels (figure 3B). Neuropeptide Y (NPY) and calcitonin gene-related peptide (CGRP), which are synthesized in sympathetic and sensory nerves, respectively, were also detected in both the pulp and PDL neurons (figure 3B, figure S3C–F). We found in our current analyses that c-Fos immunoreactive neurons, which are detectable in the superficial layers of the medullary dorsal horn following noxious stimulations such as mechanical and chemical stimulation of the intraoral receptive fields, were present in both normal and bioengineered teeth and drastically increased in number at two hours after orthodontic treatment and pulp exposure (figure 3C). These results indicate that an engrafted bioengineered tooth unit can indeed restore the



**Figure 2. Engraftment and occlusion of a bioengineered tooth unit in a tooth loss model.** (A) Schematic representation of the protocol used to transplant a bioengineered tooth unit in a murine tooth loss model. (B) Photograph (*Upper*) and sectional image (*Lower*) of a calcein-labeled bioengineered tooth unit at 60 days post-transplantation in an SRC. Scale bar, 200  $\mu$ m. (C) Micro-CT images of a bioengineered tooth unit (arrowhead) in cross section (*upper*) and frontal section (*first and second figures from the lower left*) during the processes of bone remodeling and

connection between the recipient jaw bone and alveolar bone of the tooth unit. Histological analysis of the engrafted bioengineered tooth unit at 40 days post transplantation was also performed. (Scale bar, 500  $\mu\text{m}$  and 100  $\mu\text{m}$  in the lower and higher magnification figure; *third and fourth figure from the lower left*). NT, natural tooth; BT, bioengineered tooth; AB, alveolar bone; PDL, periodontal ligament. (D) Sectional images of a calcein-labeled bioengineered tooth unit at 14, 30 and 40 days post-transplantation. The calcein-labeled bone of the bioengineered tooth units (arrowhead) was found to gradually decrease from the outside and finally disappear at 40 days post-transplantation. Scale bar, 500  $\mu\text{m}$  (*upper*), 50  $\mu\text{m}$  (*lower*). NT, natural tooth; BT, bioengineered tooth. (E) Oral photographs (*upper*) and micro-CT (*lower*) images showing occlusion of natural (*left*) and bioengineered teeth (*right*). Scale bar, 500  $\mu\text{m}$ . (F) Assessment of the hardness of a bioengineered tooth. Knoop microhardness values of the enamel (*upper*) and dentin (*lower*) of a bioengineered tooth at 60 days post-transplantation in a subrenal capsule (SRC) and at 40 days post-transplantation in jawbone (TP) were compared with those of natural teeth in 11-week-old mice. Error bars show the standard deviation ( $n=5$ ).  $*P<0.01$  (t-test). doi:10.1371/journal.pone.0021531.g002

perceptive potential for noxious stimulations in cooperation with the maxillofacial region.

### Regeneration of an Extensive Bone Defect by Transplantation of a Bioengineered Tooth Unit

Tooth loss is well known to cause significant alveolar bone resorption at the region in question [26]. Although there have been many studies of bone regenerative therapies [31], more effective methods to restore extensive bone defects during treatments such as dental implants are required and anticipated [26]. We investigated whether the transplantation of a bioengineered tooth unit would regenerate not only the missing tooth but also the surrounding alveolar bone of the recipient. To analyze whether such restoration of the alveolar bone occurred after transplantation, we developed a murine extensive bone defect model, which was prepared by the extraction of the lower first molar and then removal of the surrounding alveolar bone to generate a critical bone defect in the lower first molar region (figure 4A, figure S4A). When we transplanted a bioengineered tooth unit into this bone defect, vertical bone formation was observed from the marginal bone of the recipient at 14 days after transplantation (figure 4B, C, figure S4B). The regenerative bone volume post-transplantation significantly increased compared with a no transplant control ( $0.38\pm 0.07\text{ mm}^3$  vs.  $0.12\pm 0.08\text{ mm}^3$ ; each  $n=4$ , figure 4C, D), although the height and volume of the regenerated alveolar bone surrounding the bioengineered teeth was not completely recovered. These findings indicate that transplantation of a bioengineered tooth unit can restore a serious bone defect.

### Discussion

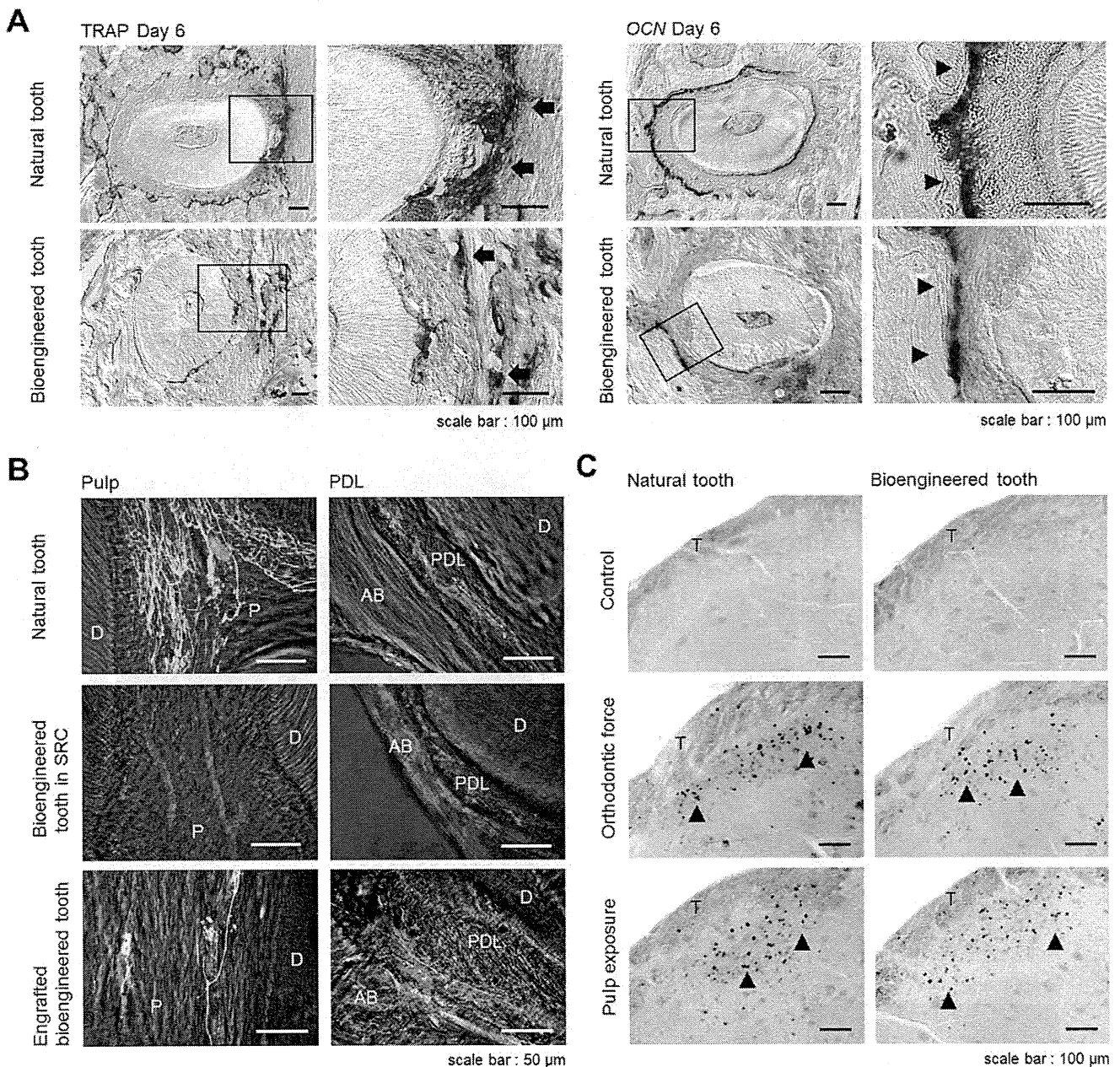
We here demonstrate the successful transplantation of a bioengineered tooth unit, which is a model for a bioengineered mature organ, into a missing tooth region *in vivo* and the subsequent restoration of tooth function by this graft. We also show that this transplantation can restore the bone volume in both the vertical and horizontal dimensions in a missing tooth mouse model with a serious extensive bone defect. These findings indicate that whole tooth regenerative therapy is feasible through the transplantation of a bioengineered mature tooth unit. This study also provides the first reported evidence of entire organ regeneration through the transplantation of a bioengineered tooth.

Organ replacement regenerative therapy, but not stem cell transplantation regenerative therapy for tissue repair, holds great promise for the future replacement of a dysfunctional organ with a bioengineered organ reconstructed using three-dimensional cell manipulation *in vitro* [11,19]. In previous reports, however, artificial organs, which were constructed with various cells and artificial materials could not restore functionality and thus are not a viable option for long-term organ replacement *in vivo* [15]. Previously, it has been shown that a bioengineered organ can be grown *in vivo* in amphibian models in which activin-treated cell

aggregates could form a secondary heart with pumping function and also regenerate eyes that were light responsive and connected with the host nervous system [32,33]. Recently, we have also regenerated bioengineered organ germs, including tooth germs and whisker follicles, and successfully achieved a fully functioning tooth replacement in an adult mouse through the transplantation of a bioengineered tooth germ in the lost tooth region [20,22]. It has been anticipated that replacement therapies will be developed in the future through the transplantation of a bioengineered mature organ with full functionality and long-term viability [2,19]. In our present experiments, we successfully generated a size-controlled bioengineered mature tooth unit, a strategy we adopted because the growth of functional organs *in vitro* is not yet possible [27]. Organs require a sufficient mass (cell number) and proper shape to function [34] and the tooth has unique morphological features, such as the tooth crown width and length (macro-morphology), and cusp and root shape (micro-morphology) [35]. However, the technology to regulate tooth morphogenesis for whole tooth regeneration remains unexplored [36]. We recently developed a novel organ germ method to regulate the crown width by regulating the contact area between epithelial and mesenchymal cell layers [28]. In our previous work, we demonstrated that the length of the bioengineered tooth is equivalent to that of natural tooth after the transplantation of the bioengineered tooth germ into oral environment [20]. In this study, the length of the bioengineered tooth unit could be controlled longitudinally, which would be provided by the limited space of the device. These findings provide the first evidence that the bioengineered tooth can be controlled in three-dimensions using a specialized device. It is also thought that bioengineered teeth could be generated with a controlled crown width through cell manipulation and tooth length by placement in a size-controlling device, which places a three-dimensional spatial limitation on size [20,28].

Loss of teeth and functional disorders in the PDL or temporomandibular joint, cause fundamental problems for oral functions, such as enunciation, mastication and occlusion, and associated health issues [21]. Although, missing teeth are traditionally restored by replacement with an artificial tooth, such as a bridge, denture or osseointegrated dental implant, it is thought that the proper restoration of tooth functions will require bone remodeling regulated by the PDL [20] and a proper responsiveness to noxious stimulations [30]. Previous reports of autologous tooth transplantations have indicated that natural periodontal tissue on the tooth could restore the physiological tooth function, including bone remodeling [37]. We recently showed that a fully functional bioengineered tooth can be achieved through the transplantation of a bioengineered organ germ [20]. In our current study, we demonstrate the successful replacement of an entire and fully functional tooth unit *in vivo*, which restored masticatory potential, the functional responsiveness, including bone remodeling, of the periodontal tissue to mechanical stress and proper responsiveness to noxious stimulations via both peripheral sensory and sympathetic nerves. This is a significant





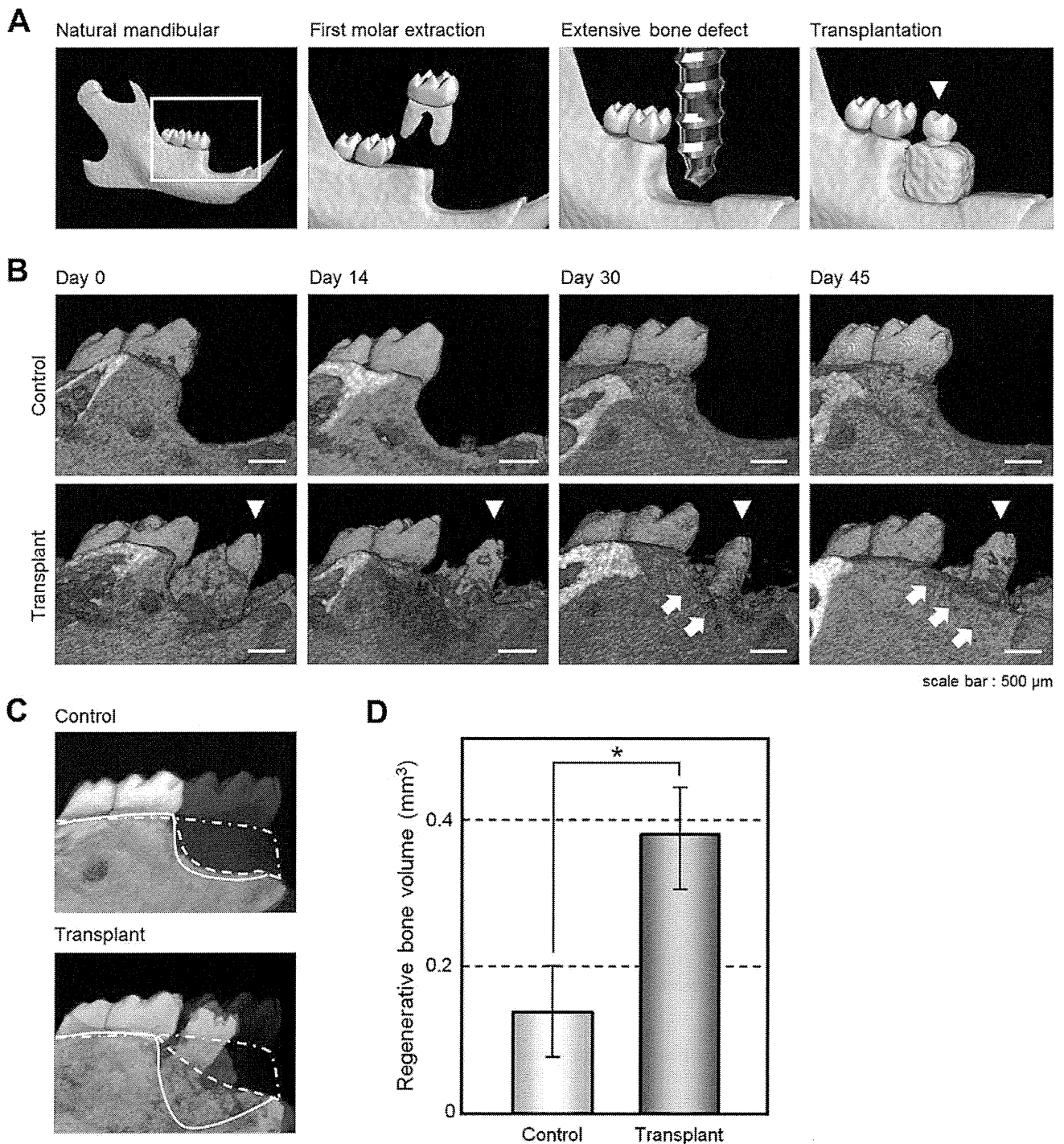
**Figure 3. Experimental tooth movement and pain response to mechanical stress.** (A) Sections of natural and bioengineered teeth were analyzed by TRAP-staining and *in situ* hybridization analysis of *Ocn* mRNA at day 6 of orthodontic treatment. TRAP-positive cells (arrow) and *Ocn* mRNA-positive cells (arrowhead) are indicated. Scale bar, 100  $\mu$ m. (B) Nerve fibers and blood vessels in the pulp and PDL of a natural tooth (top), a bioengineered tooth unit in an SRC (middle), and a bioengineered tooth at 40 days after transplantation (bottom) were analyzed immunohistochemically using specific antibodies for neurofilament (NF; green) and von Willebrand Factor (vWF; red). Scale bar, 50  $\mu$ m. D, dentin; P, pulp; AB, alveolar bone; PDL, periodontal ligament. (C) Analysis of c-Fos immunoreactive neurons in the medullary dorsal horns of mice after 0 hours (no stimulation, control; top), 2 hours of stimulation by orthodontic force (middle) and pulp exposure (bottom). C-Fos (arrowhead) was detectable after these stimulations in both natural (left) and bioengineered teeth at 40 days post-transplantation (right). Scale bar, 100  $\mu$ m. T, spinal trigeminal tract.

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advance for the concept of whole tooth regenerative therapy in which the transplantation of a bioengineered mature organ, and not organ germ, can replace an organ and restore its full function.

In order for a tooth to cooperate with the maxillofacial region, it is supported by the connection between the root cementum and alveolar bone through the PDL, which has essential roles in tooth support, resorption and repair of the root cementum, and the remodeling of alveolar bone [38]. Tooth loss causes a large

amount of alveolar bone resorption, which is mediated by the PDL, in the vertical and horizontal dimensions, and the loss of this bone, which leads to both functional and aesthetic problems, is difficult to rectify with standard dental therapies such as dental implant and autologous tooth transplantation [26]. Although bone regeneration has been attempted for many years through the use of tissue engineering technologies, guided bone regeneration methods, autologous bone or cell transplantation, and cytokine



**Figure 4. Alveolar bone regeneration following the transplantation of a bioengineered tooth unit.** (A) Schematic representation of a murine extensive bone defect model and the transplantation of a bioengineered tooth unit (arrowhead). (B) Micro-CT images of the vertical alveolar bone regeneration processes in a no transplantation control (*upper*) and following the transplantation of a bioengineered tooth unit (arrowhead, *lower*) in a murine extensive bone defect model. Vertical bone formation was observed from the marginal bone of the recipient (arrow). Scale bar, 500  $\mu\text{m}$ . (C) Three-dimensional superposition of micro-CT images of natural dentition (gray, double dotted line), a transplanted bioengineered tooth unit (*lower*) and a no transplantation control (*upper*) at day 0 in an extensive bone defect (red, straight line), and at 45 days after transplantation (green, dotted line). The superior edges of the recipient alveolar bone are indicated by each line. (D) Regenerative bone volume of the buccal area following the transplantation of a bioengineered tooth unit (transplant) and no transplantation (control) at day 45 in an extensive bone defect. Error bars show the standard deviation ( $n=4$ ).  $*P<0.01$  (*t*-test). doi:10.1371/journal.pone.0021531.g004

therapies with BMPs, FGFs or PDGF, no clinical protocol for bone regeneration in the vertical and horizontal dimensions has been established yet [31]. In our present study however, we demonstrate that a bioengineered tooth unit could be engrafted and integrate via recipient bone remodeling after transplantation into an extensive bone defect. The recipient alveolar bone of the vertical dimension was observed to maintain the height of the PDL in the bioengineered tooth unit. These findings indicate that the transplantation of a bioengineered tooth has great potential for not only future whole tooth regenerative therapy but also as a treatment in clinical cases where tooth loss is accompanied by a serious alveolar bone defect.

Further studies of three-dimensional organ culture technologies *in vitro*, which can generate a fully functional bioengineered organ, and the identification of available adult tissue stem cells for the reconstitution of a bioengineered tooth germ will be required in the future to realize whole tooth regenerative therapy in the clinic.

## Materials and Methods

### Ethics Statement

All animals and experimental protocols were approved by the Tokyo University of Science Animal Care and Use Committee (Permit Number: N10018). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

### Reconstitution of a bioengineered tooth germ from single cells

Molar tooth germs were dissected from the mandibles of ED14.5 mice. The isolation of tissues and single cell preparations from the epithelium and mesenchyme has been described previously [22]. Dissociated epithelial and mesenchymal cells were precipitated by centrifugation in a siliconized microtube and the supernatant was completely removed. The cell density of the precipitated epithelial and mesenchymal cells after the removal of the supernatants reached a concentration of  $5 \times 10^8$  cells/ml [22]. Bioengineered molar tooth germ was reconstituted using our previously described 3-dimensional cell manipulation technique, the organ germ method [22]. We used  $5 \times 10^4$  epithelial and mesenchymal cells each to generate single tooth structures. The bioengineered tooth germs were incubated for 10 min at 37°C, placed on a cell culture insert (0.4  $\mu$ m pore diameter; BD, Franklin Lakes, New Jersey, USA), and then further incubated at 37°C for five days in an *in vitro* organ culture as described previously [22].

### Generation of a bioengineered tooth unit

To control the length and shape of the bioengineered tooth unit, we manufactured a plastic ring-shaped structure, which was used as a size-control device, of a 1.3, 1.8 or 2.5 mm inside diameter and 1.3 mm thickness. After five days of cultivation, the reconstituted tooth germs were placed into this spacing device which was transplanted into a subrenal capsule for 60 days using 7-week-old female mice as the hosts. The bioengineered tooth unit was then isolated from the device.

### Fluorescent calcein labeling

Calcein (Wako, Osaka, Japan) was administered daily (1.6 mg/kg) via a subcutaneous dose to the transplanted bioengineered tooth germ in the subrenal capsule. These tooth units were then transplanted into the extracted regions of a lower first molar for 14, 30 or 40 days. Non-decalcified frozen sections were then prepared and observed using an Axiovert (Carl Zeiss, Oberkochen, Germany) with AxioCAM MRC5 (Carl Zeiss).

### Transplantation

The lower first molars of 4-week-old C57BL/6 (SLC, Shizuoka, Japan) mice were extracted under deep anesthesia and the resulting gingival wounds had been allowed to heal for 4–6 days. The transplantation of a bioengineered tooth unit was allowed the procedure as described previously [20]. To generate an extensive alveolar bone defect mouse model, the whole supporting alveolar bone (1.5 mm mesiodistally, 1.2 mm buccolingually and 0.6 mm vertically) was removed using a dental engine (NSK, Tochigi, Japan) under deep anesthesia. The bioengineered tooth units were transplanted into these defects using the same procedure described above.

### Microcomputed Tomography (Micro-CT)

The heads of the mice that had received a transplanted bioengineered tooth unit and normal mice were arranged in the centric occlusal position and radiographic imaging was then performed by x-ray using a Micro-CT device (R\_mCT; Rigaku, Tokyo, Japan) with exposure at 90 kV and 150 mA. Micro-CT images were captured using i-view R (Morita, Kyoto, Japan) and Imaris (Carl Zeiss).

### Histochemical analysis and immunohistochemistry

Histochemical and immunohistochemical tissue analyses were performed as described previously [20,22].

### Hardness measurements

Polished enamel and dentin samples from bioengineered tooth units extracted at 60 days after germ transplantation into the SRC or the mandible, and also a normal tooth (9-week postnatal) were embedded in acrylic resin ( $n=5$  for each group). The Knoop hardness test was then performed using a Miniloop Hardness Tester (HM-102; Mitutoyo, Kanagawa, Japan) equipped with a Knoop diamond tip (19BAA061; Mitutoyo). Five indentations were made on each specimen with a 10 g load for 10 sec.

### Experimental orthodontic treatments

Orthodontic treatment was performed as described previously [20]. Experimental tooth movements consisted of a horizontal orthodontic force of about 10–15 g applied continuously to the bioengineered tooth of the mice in the experimental group in a buccal direction using a dial tension gauge (Mitutoyo) for six days. In the control group, orthodontic force was applied in the buccal direction to the first molars of 7-week-old normal C57BL/6 mice in the same manner as the experimental group. Serial sections at day 6 were analyzed by TRAP staining and by *in situ* hybridization analysis for osteocalcin (*Ocn*) mRNA as previously described [20].

### Pulp exposure

A minimal pinpoint mechanical exposure of the pulp was made in the bioengineered tooth or control natural first molar of mice under anesthesia using a dental engine (NSK) supplied with dental diamond point (Shofu, Kyoto, Japan). For stimulation with cold water, ice was applied to the cavity of the tooth after pulp exposure.

### Measurement of the regenerative bone volume

To evaluate the extent of the alveolar bone recovery in our extensive bone defect mouse model, we used the Micro-CT device (Rigaku) to measure alveolar bone volume of the treated areas at 0 and 45 days after transplantation. We measured the volume of the alveolar bone in the operated region using TRI/3D-BON software (Ratoc, Osaka, Japan). The 3D region of interest (ROI)



was selected in the buccal alveolar bone area which was prescribed from the medial edge of lower second molar to the distal edge of the foramen mentale. We subtracted the alveolar bone volume of the area at day 0 from the volume at day 45, and calculated the regenerated bone volume.

### Statistical analysis

Statistical significance was determined with the unpaired Student's *t*-test, analyzed using the Common Gateway Interface Program (twk, Saint John's University).

### Supporting Information

**Figure S1 A method for controlling the size of a bioengineered tooth unit.** (A) Micro-CT images of the shapes of a bioengineered tooth unit, size controlled by devices of a 1.3 or 1.8 mm inner diameter, at 30 and 60 days after transplantation in an SRC. Scale bar, 500  $\mu$ m. (B) Photograph of plural bioengineered tooth germ arranged in a size controlled device. Scale bar, 500  $\mu$ m. (C) Micro-CT images (*left*) and histological analysis of the multiple bioengineered tooth units on day 60 after SRC transplantation (*middle and right*). The alveolar bone between the bioengineered teeth is indicated by arrowheads (*lower left*). Scale bar, 200  $\mu$ m. Higher magnification images of the periodontal tissue area (*lower middle and right*) are also shown. Scale bar, 50  $\mu$ m. D, dentin; AB, alveolar bone; PDL, periodontal ligament. (TIF)

**Figure S2 Engraftment and establishment of occlusion of a bioengineered tooth unit at the tooth loss region.** (A) Oral photographs and micro-CT images of bioengineered tooth unit transplantations into the adult mandible. Images were captured of lateral (*top*), occlusal (*middle*) and cross sections (*bottom*) views. The bioengineered tooth unit is indicated by an arrowhead. Scale bar, 500  $\mu$ m. (B) Sectional images of a calcein-labeled bioengineered tooth unit at 14, 30 and 40 days after transplantation into a murine model. Fluorescent and DIC images are merged. The alveolar bone of the bioengineered tooth unit is indicated by arrowheads. Scale bar, 500  $\mu$ m, *upper*; 100  $\mu$ m, *lower*. NT, natural tooth; BT, bioengineered tooth. (C) Oral photographs of an engrafted bioengineered tooth in a lateral view (upper left), a 45-degree view (*lower left*), an occlusal view (*upper right*) and a fluorescent image (*lower right*). Scale bar, 500  $\mu$ m. (D) Measurements of the tooth length (*left*) and apical foramen width (*right*) of a bioengineered tooth at day 0 and day 40 after transplantation. Error bars show the standard deviation ( $n = 9$ ). \* $P < 0.05$  (*t*-test). (E) Schematic representation of the protocol for transplanting multiple bioengineered tooth units in a murine edentulous model. (F) Micro-CT images of transplanted multiple bioengineered tooth

units in a murine edentulous model. Images were captured of the external surface area (*left*), sagittal section (*center*) and cross section (*right*). The bioengineered teeth are indicated by the arrowheads in the *left figure*. Scale bar, 500  $\mu$ m.

(TIF)

**Figure S3 Regeneration of nerve fibers and blood vessels in the engrafted bioengineered tooth unit.** (A, B) Nerve fibers and blood vessels in the pulp (A) and PDL (B) of a natural tooth (*top*), bioengineered tooth unit in an SRC (*middle*) and bioengineered tooth at 40 days after transplantation into an oral tooth loss region (*bottom*) were analyzed immunohistochemically using specific antibodies for NF and vWF. DIC (*first columns from the left*), NF images (*second columns*), vWF images (*third columns*), and merged images (*fourth columns*) are shown. Scale bar, 50  $\mu$ m. (C, D) Nerve fibers in the pulp (C) and PDL (D) of a natural tooth (*top*), bioengineered tooth unit in an SRC (*middle*) and bioengineered tooth at 40 days after transplantation (*bottom*) were analyzed immunohistochemically using specific antibodies for NF and neuropeptide Y (NPY). DIC (*first columns from the left*), NF images (*second columns*), NPY images (*third columns*), and merged images (*fourth columns*) are shown. Scale bar, 50  $\mu$ m. (E, F) Nerve fibers in the pulp (E) and PDL (F) of a natural tooth (*top*), bioengineered tooth unit in an SRC (*middle*) and bioengineered tooth at 40 days after transplantation (*bottom*) were analyzed immunohistochemically using specific antibodies for NF and calcitonin gene-related peptide (CGRP). DIC (*first columns from the left*), NF images (*second columns*), CGRP images (*third columns*), and merged images (*fourth columns*) are shown. Scale bar, 50  $\mu$ m.

(TIF)

**Figure S4 Alveolar bone regenerative potential of a bioengineered tooth unit.** (A) Photographs of a lateral (*left*) and occlusal (*right*) view of a natural mandibular dentition and an extensive bone defect (arrowhead). Scale bar, 500  $\mu$ m. (B) Micro-CT images of the frontal section of a no transplantation control (*upper*) and a transplanted bioengineered tooth unit at day 45 in a murine extensive bone defect model (*lower*). Significant vertical bone regeneration was observed following the transplantation of a bioengineered tooth unit when compared with the no transplantation control. The regenerated alveolar bone is indicated by an arrow. Scale bar, 500  $\mu$ m.

(TIF)

### Author Contributions

Performed the experiments: M. Oshima MM MY KN. Analyzed the data: AI M. Ogawa HY. Wrote the paper: M. Oshima TT. Designed the research plan: TT M. Oshima. Developed new assay systems and the discussion of the results: M. Ogawa RM EI TT-Y SK MS.

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## Review Article

**Dental Regenerative Therapy using Oral Tissues**

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**Abstract**

Anti-Aging Medicine is a theoretical and practical science which aims to ensure the achievement of a long and healthy life. Dental medicine plays an important role in its practice. Given the substantial influence of dental/oral diseases on general health, the maintenance and improvement of oral function promotes not only dental/oral Anti-Aging but also systemic Anti-Aging as well.

The current target of Anti-Aging dental medicine is the prevention or slowing down of the age-related decline in oral function by evaluating indicators of oral function, such as dental age, periodontal age, occlusion age, swallowing age, and salivary age. In this symposium, Dr. Kenji Mishima (Department of Dentistry, Tsurumi University), speaking on "Application of Cell Transplantation Therapy to Salivary Gland Dysfunction", Dr. Masahiro Saito (Research Institute for Science and Technology, Tokyo University of Science), speaking on "Role of Tooth Regeneration in Anti-Aging Medicine" and myself, Dr. Narisato Kanamura (Dental Medicine, Graduate School of Medical Science, Kyoto Prefectural University of Medicine), speaking on "Development of a New Periodontal Tissue Regeneration Method Aimed at Anti-Aging Use", delivered presentations about the current status and future prospects of regenerative dentistry, which aims not only to prevent a decrease in oral function but also to restore it when function is lost, and introduced the latest in regenerative dentistry involving the salivary glands, teeth, oral mucosal epithelia, and periodontal ligaments. In addition, to describe collaboration between dental medicine and ophthalmology, Dr. Takahiro Nakamura (Faculty of Life and Medical Sciences, Doshisha University), speaking on "Current Status and Future Prospects of Corneal Regenerative Therapy using Oral Tissue", introduced the current status and future prospects of corneal regenerative therapy using periodontal mucosal epithelium. Summaries of these lectures are presented here. In the "Dental Regenerative Therapy using Oral Tissues" symposium at the 2011 11th Scientific Meeting of the Japanese Society of Anti-Aging Medicine, the experts were invited to report recent findings on maintenance.

**KEY WORDS:** oral tissue, regenerative therapy, saliva, tooth regenerative therapy, periodontal ligament

**1. Application of Cell Transplantation Therapy to Salivary Gland Dysfunction**

The causes of salivary gland dysfunction include refractory diseases such as Sjogren's syndrome and Stevens-Johnson syndrome, radiation therapy against head and neck cancer, and a variety of drugs <sup>1)</sup>. Current treatments include the use of artificial saliva and oral therapy with muscarinic acetylcholine receptor agonists, which stimulate salivary secretion from residual acinar cells. Severe cases may be resistant to these treatments, and patients may develop oral cavity lesions such as mucositis, caries, or periodontal disease. In addition, as salivary gland dysfunction is a pathogenic factor in aspiration pneumonia in the elderly, serious concerns have been expressed about infection treatment methods. The

possibilities of regenerative medicine have therefore been investigated, specifically the reconstruction of lost gland tissues using transplantation of exogenous salivary gland stem cells. However, cell surface markers specific to salivary gland stem cells are difficult to isolate and thus remain unknown. We have therefore focused on a cell population called "side population (SP)" cells, which can be isolated without using a cell surface marker. Since their first isolation from bone marrow as a fraction containing a high frequency of stem cells, SP cells have been analyzed in a variety of organs <sup>2-6)</sup>. In the present study, we investigated the effects of experimental treatment with SP cells using a mouse model of irradiation-induced salivary gland dysfunction, and the possibility of establishing a treatment approach with a specific factor expressed in SP cells.

### *Investigation of effects of treatment with SP cells*

Salivary gland tissue collected from green fluorescent protein (GFP) transgenic mice was digested with collagenase and hyaluronidase to remove interstitial tissue, and epithelial clusters were isolated using a filter mesh. The isolated epithelial clusters were then treated with trypsin to disperse the cells, stained with Hoechst 33342, and subjected to FACS with UV laser irradiation and measurement at two wavelengths (450 nm and 675 nm). As a result, SP cells were detected as a characteristic cell population with low fluorescence intensity which accounted for about 0.5% to 1.0% of salivary gland cells. Next, Hoechst 33342-negative SP cells and Hoechst 33342-positive non-SP (main population, MP) cells were collected using FACS<sup>®</sup>. The collected cells were then transplanted into salivary glands of mice with irradiation-induced salivary gland dysfunction (15 Gy local irradiation). Saliva production associated with treatment with pilocarpine, a muscarinic acetylcholine receptor agonist, was then measured serially to determine the effects of SP cell transplantation. The results showed the restoration of secretion volume at 1 month after transplantation. In addition, examination of the removed tissues by fluorescence microscopy indicated the sparse distribution of GFP-positive cells. However, because the observed number of GFP-positive cells was low, the transplanted SP cells were unlikely to have directly contributed to the restoration of secretory ability, suggesting that soluble factor(s) secreted from SP cells may be involved in the secretory mechanism of residual acinar cells.

### *Functional analysis of SP cell-specific expression gene*

Hoechst 33342-negative SP cells and Hoechst 33342-positive non-SP (main population, MP) cells were collected using FACS, and RNAs were extracted from the collected SP and MP cells using a PicoPureRNA isolation kit (Arcturus). In addition, RNA amplification was performed by a T7 polymerase-based method using a RiboAmp RNA amplification kit (Arcturus). The amplified RNAs derived from SP and MP cells were then used to synthesize cDNAs, which were fluorescence-labeled with Cy3 or Cy5 and subjected to competitive hybridization on NIA 15K mouse cDNA array (Version 2) to compare their gene expression profiles based on the detected signals. This method identified multiple genes specifically expressed in SP cells, among which we selected clusterin for functional analysis. Specifically, clusterin gene was introduced into STO cells, a mouse embryonic fibroblast cell line, by lipofection to prepare a cell line that stably expresses clusterin following drug selection. We next investigated the possible function of clusterin in reducing damage caused by reactive oxygen species (ROS), on the basis that irradiation-induced cell damage is mediated by ROS. Specifically, we counted viable cells stained using trypan blue 24 hours after stimulation of clusterin-expressing and control cells with different concentrations of hydrogen peroxide solution. The results showed significantly higher cell viability among clusterin-expressing cells than control cells, and a decrease in ROS production in the cells.

We then investigated the effects of treatment with SP cells collected from clusterin gene knockout mice to verify the involvement of clusterin in the treatment effects of SP cells. Although autoimmune myocarditis has been reported in clusterin knockout mice, we saw no histological change in 12-week-old mice at least, and no difference in SP cell

fraction compared to control mice<sup>8)</sup>. However, pilocarpine-stimulated salivation was not restored in mice with irradiation-induced salivary gland dysfunction even after transplantation of SP cells of the above-mentioned knockout mice. These findings indicated that clusterin makes a critical contribution to treatment effects in SP cell transplantation.

### *Verification of treatment effects of clusterin using a mouse model with salivary gland dysfunction*

To determine whether clusterin directly contributes to the reversal of cellular dysfunction of the salivary gland, clusterin-expressing recombinant lentivirus (Lenti-Clu,  $5 \times 10^6$  TU) was injected into one submandibular gland of mice with irradiation-induced salivary gland dysfunction 4 days after irradiation, and GFP-expressing lentivirus (Lenti-GFP,  $5 \times 10^6$  TU) was injected into the other<sup>9)</sup>. Gene transfection efficiency and time-dependent change in saliva volume were then measured to assess restoration of secretory ability. The results indicated that Lenti-GFP transfection led to GFP positivity in approximately 16% of cells. In contrast, Lenti-Clu-injected mice showed an improvement in pilocarpine-stimulated salivation at 4, 8, and 16 weeks after virus injection compared to Lenti-GFP-injected mice. These results suggested that clusterin, which is expressed in SP cells, is involved in the functional restoration of glandular secretion.

These results revealed that SP cells or clusterin, a specific factor expressed in SP cells, is effective in the treatment of irradiation-induced salivary gland dysfunction. We are planning to examine the possible clinical application of these factors in the future.

## **2. Role of Tooth Regeneration in Anti-Aging Medicine**

### *Introduction*

Teeth possess not only a masticatory function (*i.e.*, "chewing") but also act as sensory receptors, sending masticatory stimulation to centers in the brain. Caries and tooth loss secondary to periodontal disease, the incidence of which are increasing in the elderly, are known to cause significant problems with masticatory function and to affect systemic condition. Thus, the development of dental regenerative medicine that can essentially restore the physiology of natural teeth will be useful in preventing a decline in oral function and in promoting Anti-Aging. Here, we discuss the current status of R&D in dental regenerative therapy against tooth loss, as well as its potential role in Anti-Aging Medicine.

### *Tooth regeneration by a bioengineered organ germ method*

Previously, functional complementary therapies with artificial devices such as dentures, dental bridges, and dental implants have been used as dental support for tooth loss. Although these complementary therapies are effective in the restoration of masticatory function, they cannot restore the innate physiological aspects of teeth, such as tooth movement associated with aging and response to masticatory stimulation.

Thus, a more biological treatment approach to tooth regeneration has been sought.

Teeth develop through continuous interaction between odontogenic epithelial cells and odontogenic mesenchymal cells which together constitute the embryonic tooth germ<sup>10)</sup>. For this reason, technologies that enable the regeneration of tooth germ from epithelial and mesenchymal cells through three-dimensional cell manipulation techniques have been developed with the goal of regenerating third teeth, in addition to primary and permanent teeth. To date, however, the ability to produce highly effective and normal tooth development has not been reached<sup>11)</sup>. In 2007, we developed the "bioengineered organ germ method," in which epithelial and mesenchymal cells derived from the tooth germ are compartmentally arranged at high cellular density (Fig. 1, top)<sup>12)</sup>. When ectopically transplanted *in vivo*, the bioengineered tooth bud cells develop with structurally normal regenerated teeth as well as periodontal tissue (periodontal ligament, cementum, alveolar bone), indicating the potential use of bioengineered tooth bud cells in tooth regenerative therapy<sup>12)</sup>.

### Regeneration of functional teeth

Successful tooth regenerative therapy requires not only the histological normality of the regenerated tooth but also its eruption/occlusion within the recipient's intraoral environment, and the full spectrum of normal tooth physiology, such as functional regeneration of the periodontal ligament and response to external noxious stimuli. When bioengineered tooth bud cells were transplanted into sites of tooth loss in adult mice, the bioteeth erupted and grew, and occlusion of the regenerated teeth was established with hardness comparable to that of natural teeth (Fig. 1, top). Further, the tissue structure of the regenerated teeth was similar to that of natural teeth, and a fully matured periodontal ligament structure was also observed, including alveolar bone. The periodontal ligament of the regenerated teeth was shown to retain the physiological

capacity to remodel surrounding alveolar bone in response to experimental orthodontic force and to move teeth in a similar manner to natural teeth. In addition, similarly to natural teeth, the dental pulp and periodontal ligament of the regenerated teeth had multiple peripheral nerves, including sympathetic and sensory nerves. Upon application of mechanical stress through dental pulp exposure or dental makeover, upregulation of c-Fos expression in response to intraoral noxious stimuli in some neurons in the spinal trigeminal nucleus was observed for both regenerated and natural teeth, revealing restoration of physiological response to external noxious stimuli in the regenerated teeth. These results demonstrated that not only masticatory function but also the full spectrum of normal tooth physiology could be restored in a tooth regenerated by means of transplantation of bioengineered tooth bud cells, and clearly indicate the clinical applicability of the approach to regenerative therapy against tooth loss<sup>13)</sup>.

### Tooth regeneration using bioengineered tooth units

Elderly people often have severe progressive periodontal disease presenting with extensive destruction of periodontal tissue essential for mastication, and tooth loss has been known to lead to the absorption of surrounding alveolar bone resulting in severe bone defects<sup>14)</sup>. For tooth regeneration in the elderly, an approach based on the regeneration of teeth with finished components (*e.g.*, dental prostheses) together with periodontal tissues for immediate functional recovery after implantation would be more appropriate than transplantation of bioengineered tooth germ. On this basis, we developed the "bioengineered tooth unit," which includes tooth and periodontal tissue (*i.e.*, functional unit of tooth), with the aim of developing tooth regeneration technology that allows for immediate functioning.

Because the culture of three-dimensional organs *ex vivo* is not currently possible, we demonstrated our concept by constructing bioengineered tooth units suitable for transplantation by transplanting bioengineered tooth bud

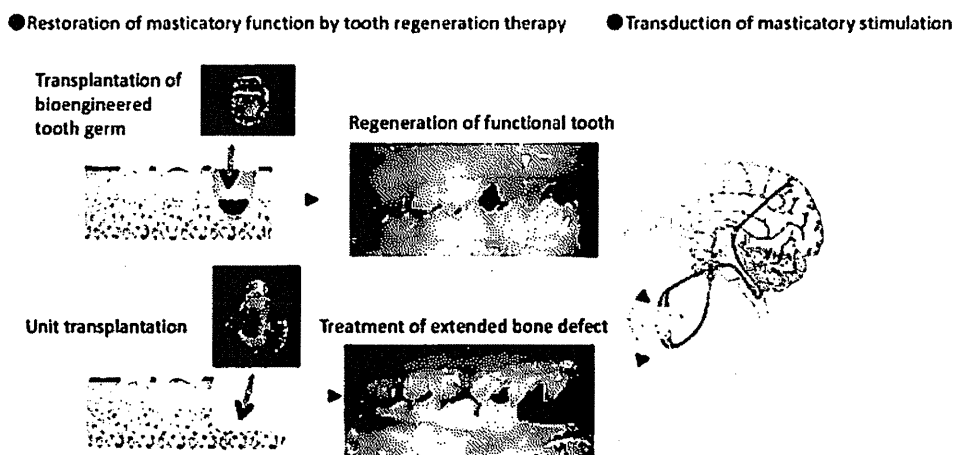


Fig. 1. Development of tooth regenerative therapy aimed at Anti-Aging. Regeneration of functional teeth using bioengineered tooth bud cells and treatment of extended bone defects by bioengineered tooth unit transplantation are expected to aid progress in anti-aging regenerative medicine technologies which enable the transduction of masticatory stimulation to be restored. (Scale bar: 200  $\mu$ m)



cells into kidney capsules. When the bioengineered tooth unit was implanted into sites of tooth loss to secure normal occlusion, osseointegration was seen between the alveolar bone of the bioengineered tooth unit and that of the recipient, as was the restoration of a functional periodontal ligament and responsiveness to external noxious stimuli (Fig. 1, bottom). In addition, when the bioengineered tooth unit was implanted into an extensive bone defect model in mice, osseointegration was seen between the alveolar bone of the bioengineered tooth unit and jaw bone of the recipient, indicating the ability of alveolar bone to regenerate vertically. These results indicate the potential of transplantation of bioengineered tooth units in regenerative therapy to generate an immediately functioning tooth and in patients who experience tooth loss with major bone defect<sup>15</sup>.

### Summary

Against the background of the aging society, dental therapy requires the development of dental therapeutic techniques that allow for the promotion of Anti-Aging. We consider regenerative therapy aimed at achieving a functional tooth is a desirable option. Our previous studies demonstrated that transplantation therapy against tooth loss using either bioengineered tooth bud cells or a bioengineered tooth unit can regenerate teeth that have similar physiological functions to natural teeth (Fig. 1). Realization of these therapies requires the identification of patient-derived stem cell seeds for use in regeneration of tooth germ<sup>16,17</sup>, development of technique to prepare bioengineered tooth bud cells using iPS cells<sup>18</sup>, and development of size control techniques to achieve suitable tooth size for the transplantation site<sup>19</sup>. Solving these problems would result in the realization of tooth regenerative therapy as an Anti-Aging dental therapy.

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## 3. Development of a New Periodontal Tissue Regeneration Method Aimed at Anti-Aging Use

### Introduction

Periodontal tissue, which consists of the gingiva, periodontal ligament (PDL), cementum, and alveolar bone, is a collective term for the tissues that surround the tooth and play a role in supporting its function. Periodontal disease (periodontal disorder), which destroys these periodontal tissues, is a chronic inflammatory disorder which ultimately leads to tooth loss. Periodontal disease affects approximately 80% of adults and is the most common cause of tooth loss in the aged. Thus, the regeneration of periodontal tissue that has been lost due to causes such as periodontal disease is a major goal of dental care. Recently, associations between periodontal disease and diabetes (a lifestyle-related disease), cardiovascular disease (*i.e.*, heart disease, arteriosclerosis), and systemic disorders (*e.g.*, aspiration pneumonia) have been suggested<sup>20</sup>, indicating that periodontal disease represents an aging factor that is associated not only with oral health but also general health. With the rapid transition into the era of an aging society, the regeneration of periodontal tissue destroyed by periodontal disease is a critically important research area in Anti-Aging Medicine, because of its systemic Anti-Aging effects, rather than simply the restoration of oral function, such as chewing.

The PDL, a periodontal tissue, is a fibrous connective tissue with a thickness of approximately 200  $\mu\text{m}$  that surrounds the tooth root and connects the tooth with the supporting alveolar bone. It lies between the alveolar bone and cementum, and fixes the tooth to the jaw bone<sup>21</sup>. The whole periodontal ligament is made up of a mixture of various types of cells, including fibroblasts, osteoblasts, cementoblasts, osteoclasts, undifferentiated mesenchymal cells, and epithelial cells derived from epithelial cell rests of Malassez. Of these, PDL-derived fibroblasts play an important part in the remodeling of PDL fibers<sup>20</sup>. However, when periodontal disease occurs, periodontal pockets are formed and the lysis/disappearance of PDL fibers occurs, leading to the destruction of periodontal tissue and finally to tooth loss.

### Regeneration of periodontal tissue using periodontal ligament-derived cells

Thanks to recent progress in dental medicine, the pathology and mechanism of periodontal disease have become increasingly clear. Previous studies have strongly suggested that the presence of newly formed PDL is important for the regeneration of periodontal tissue<sup>21</sup>. As such, researchers have investigated the regeneration of periodontal tissue using autologous transplantation of fibroblasts derived from PDL and grown *in vitro*<sup>22</sup>. Van Dijk *et al.* reported that the regeneration of periodontium-like hard tissues (newly formed cementum-like hard tissues) was possible using the autologous transplantation of PDL-derived cells into experimentally-created periodontal tissue defects in an experimental animal (beagle dog)<sup>23</sup>. In addition, Dogan *et al.* reported that periodontal tissues (newly formed cementum and new bone) could be regenerated using blood clots as carriers of cultivated PDL-derived cells<sup>24</sup>, and Nakahara *et al.* reported that differentiation of newly formed cementum was enhanced using collagen sponge as a cell culture substrate to facilitate close contact between cultivated cells and the tooth root surface<sup>25</sup>. These reports indicate that

## Dental Regenerative Therapy using Oral Tissues

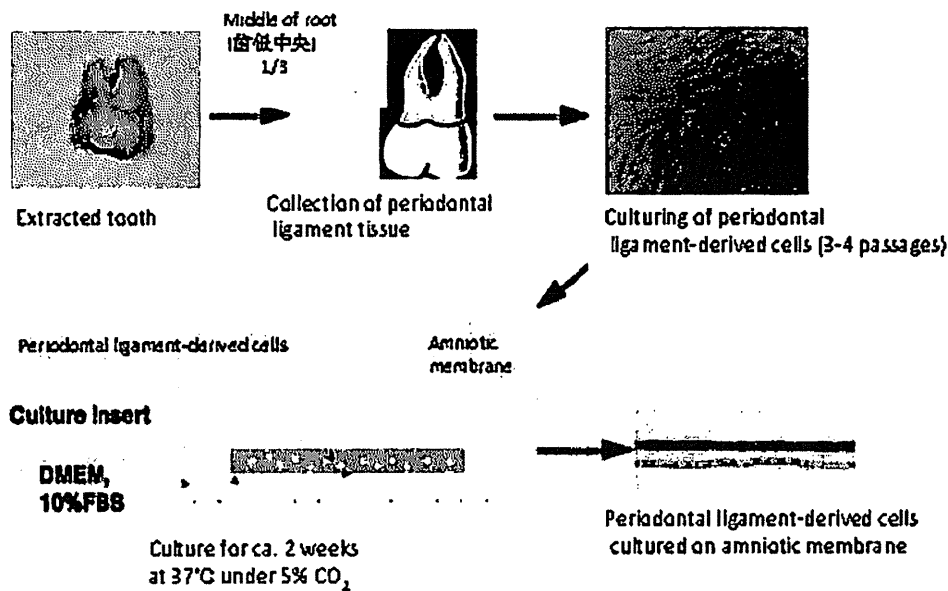


Fig. 2. Examples of reconstruction of oral mucosal defects in dental oral surgery using oral mucosal epithelial sheet cultured on amniotic membrane.

periodontal tissue can be regenerated using PDL-derived cells, and that a carrier (substrate) is important for the transplantation of cultivated cells. We have performed studies using amniotic membrane (AM), a biomaterial that has attracted interest as cell culture substrate in various medical fields<sup>26,32</sup>, and from these developed the idea of using this as a substrate for PDL-derived cells.

### Development of a new periodontal tissue regeneration method using amniotic membrane

The AM is a thin membrane that covers the outermost surface of the placenta and consists of parenchymal tissue with a specific thickness. The tissue is normally discarded after parturition, can be collected from the placenta almost aseptically, and can be obtained without ethical or technical problems. It has unique characteristics, including anti-inflammatory and infection-reducing effects<sup>33,34</sup>, and has been utilized as a biomaterial in various surgical therapies for purposes such as the prevention of adhesion/scarring in skin transplantation/abdominal surgery, healing acceleration as a skin burn wound dressing, and ocular surface reconstruction in ophthalmology<sup>35,39</sup>. In addition to its use as a transplantation material, it has also attracted attention for its high usefulness and effectiveness as a culture substrate<sup>39</sup>. We have previously shown the effectiveness of new amniotic membrane-based regenerative therapy to oral healthcare through successful preparation of a cultivated oral mucosal epithelial cell sheet on AM and the establishment and clinical application of an autotransplantation technique for various types of oral mucosal defects in dental oral surgery (Fig. 2)<sup>28,30,31</sup>. Recently, we applied this AM-based cell-culture system to culture PDL-derived cells for regenerative therapy for periodontal tissue. Below, we present progress to date and future prospects of our investigation for the development of cell sheets aimed at regeneration of periodontal tissue<sup>29,32</sup>.

### Preparation of periodontal ligament-derived cell sheets cultured on amniotic membrane

We have previously confirmed that PDL-derived cells can be successfully cultured to form a sheet using an AM-based cell-culture system<sup>26,27</sup>. In addition, we have reported that PDL-derived cell sheets cultured on AM could potentially regenerate periodontal tissue, based on the observation that periodontal tissues (*i.e.*, newly formed cementum and new bone) were regenerated by autologous transplantation of these sheets into periodontal tissue defects in an experimental animal (beagle dog)<sup>29</sup>. Growth factor, cell type, and substrate are important aspects of transplantation and regenerative therapies<sup>40</sup> which are expected to act in combination in the regeneration of tissues, including in periodontal tissue defects. Among them, a variety of culture substrates have been investigated for PDL-derived cells<sup>41</sup>, but an ideal substrate for periodontal tissue regeneration has not yet been developed. In addition, PDL-derived cells on substrate have not been evaluated, and a wide review of the literature reveals that the proliferation and differentiation abilities of PDL-derived cells on AM is poorly understood. Considering that the preparation of cultured human PDL-derived cell sheets will have clinical applications, we also performed an immunohistochemical study of the cell kinetics of PDL-derived cells on AM.

Human AM collected from placenta obtained during cesarean section was used. PDL tissues were collected from tooth roots after tooth extraction, etc., as appropriate. The collected PDL tissues were subjected to primary culture, and cells derived from them were used after 3-4 passages. The cells were seeded onto AM and cultured for approximately 2 weeks (Fig. 3), then subject to immunostaining for Ki-67 (cell proliferation marker), vimentin (mesenchymal marker), desmoplakin (desmosomal marker), and ZO-1 (tight junction marker). In addition, to investigate adhesion between the AM and these cultured cells (*i.e.*, cell-substrate adhesion), immunostaining for laminin 5/10 and collagen IV/VII (components of basement membrane) and scanning electron microscopic (SEM) observation were performed.

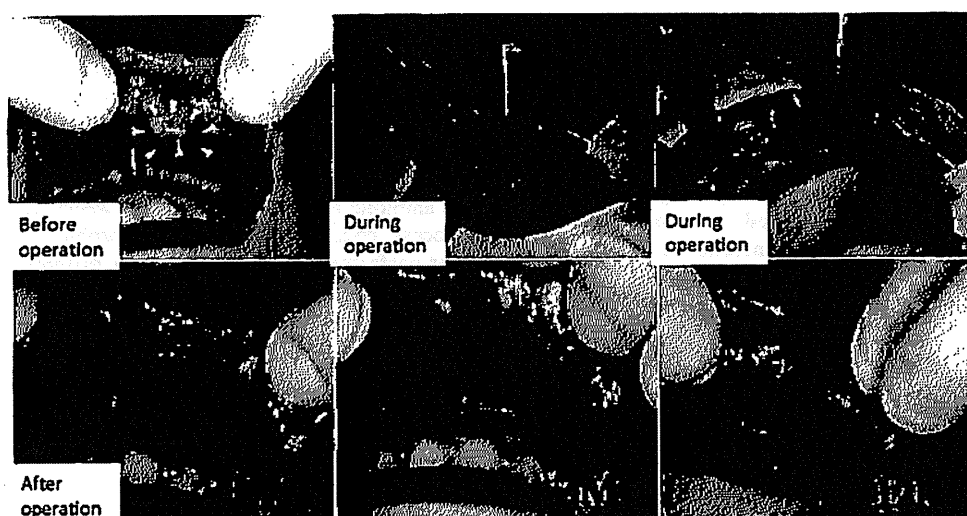


Fig. 3. Culturing of periodontal ligament-derived cells on amniotic membrane.

Tissue collection from extracted teeth and the use of AM for experimental purposes were conducted after obtaining informed consent from patients following sufficient explanation. Experimental use of PDL tissues, PDL-derived cells, and AM was approved by the Medical Ethical Review Board of Kyoto Prefectural University of Medicine (RBMR-R-21).

Results showed that the PDL-derived cells formed a monolayer on the AM after approximately 2 weeks of culture. Immunofluorescence showed the localization of Ki-67- and vimentin-positive cells and expression of desmoplakin and ZO-1. These cells were considered capable of proliferation and potentially maintaining their PDL-like properties even on AM. In addition, strong cell-cell adhesion structures, namely desmosomes and tight junctions, were shown to be present between cells<sup>32)</sup>. Laminin 5/10 and collagen IV/VII were expressed at the basal region of the PDL-derived cells (*i.e.*, cell-AM boundary), and SEM images showed that the cells had differentiated and proliferated on AM with lateral conjugation and adhesion to the AM, indicating strong adhesion between PDL-derived cells and AM.

#### Summary

These results confirm the proliferation of PDL-derived cells on AM and the presence of strong cell-cell adhesion structures and basal membranes. AM was shown to be a potentially suitable culture substrate, and PDL-derived cells were considered to form a sheet on AM, and not to be in the form of disparate individual cells. PDL-derived cell sheets cultured on AM can be considered to represent a novel material for a new periodontal tissue regeneration method, provided its ability to regenerate periodontal tissue is confirmed and some AM-specific effects are demonstrated.

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#### 4. Current Status and Future Prospects of Corneal Regenerative Therapy using Oral Tissue

##### Introduction

Human ocular surfaces consist of corneal epithelium and conjunctival epithelium. These are uniquely differentiated surface ectoderm-derived mucosal membranes which maintain homeostasis of the ocular surface in cooperation with lacrimal fluid. The tissue structure can be divided into three cellular layers: an outermost corneal epithelium layer, a corneal stroma layer, and an inner corneal endothelium layer. The corneal epithelium consists of stratified squamous epithelia with a thickness of approximately 50  $\mu\text{m}$  which provides physical/biological protection from the external environment to the ophthalmus. Thanks to progress in a variety of fundamental research programs, corneal epithelial stem cells have been found to exist in the basal layer of the corneal limbus, which is positioned at the periphery of the cornea<sup>42,43)</sup>. When the corneal limbus (*i.e.*, corneal epithelial stem cell) is lost for various reasons, biological reactions occur in which the surrounding conjunctival epithelia cover the corneal surface with accompanying inflammation or vascularization, etc., thereby resulting in significant visual disorder. Diseases associated with abnormalities of the corneal epithelial stem cell like the example above are called "refractory ocular surface disease," and have been extensively investigated in both fundamental and clinical studies to elucidate the condition and develop treatment methods.

**Development of ocular surface reconstruction**

To date, surgical reconstruction after refractory ocular surface disease has usually consisted of corneal epithelial cell transplantation (keratoepithelioplasty, corneal limbal transplantation) using donor tissue<sup>44,45</sup> and cultivated corneal epithelial cell transplantation<sup>46-49</sup>. However, because these involve allotransplantation, heavy long-term use of immunosuppressive agents is required after operation. The problems of postoperative rejection, infection, and decreased quality of life in these patients indicate the need for a safer and more effective transplantation technique. Because many refractory ocular surface diseases are binocular diseases, autologous corneal epithelium cannot be used, making it important to select a cell source that has no risk of postoperative rejection. We investigated the possibility of ocular surface reconstruction using autologous oral mucosal epithelium, with the aim of developing a novel surgical technique that uses mucosal epithelium other than ocular surface mucosal epithelium (Fig. 4).

**Development of a cultivated oral mucosal epithelial sheet using amniotic membrane**

**Cultivated oral mucosal epithelial sheet**

Regeneration of a living tissue *in vitro* requires the establishment of an extracellular environment that facilitates the differentiation and proliferation of cells (*i.e.*, scaffold for cells). Particularly in the case of refractory ocular surface diseases, normalization of the substrate, including the extracellular matrix, is considered essential, in addition to reconstruction of the epithelium. Amniotic membrane, a biomaterial, is a thin membrane over a thick basal membrane devoid of vasculature that covers the fetus and placenta within the uterus. It has been reported to have a variety of biological effects, including the suppression of scarring and inflammation, suppression of neovascularization, and acceleration of wound healing<sup>51-53</sup>.

First, we initiated the development of a cultivated oral mucosal epithelial sheet using amniotic membrane. Our research team first investigated the suitability of amniotic membrane with the epithelium scraped off as a culture substrate for oral mucosal epithelium in an animal study in rabbits<sup>39</sup>. An oral mucosal cell suspension was prepared from oral mucosa collected from white rabbits, and cultured on amniotic membrane for about 3 weeks. During the culture process, cocultivation with 3T3 fibroblasts by culture insert was performed using air-lifting for differentiation induction of epithelial cells. As a result, oral mucosal epithelial cells cultured on amniotic membrane adhered to and grew on the amniotic membrane substrate and reached confluence after 1 week. On culture for 2-3 weeks, they were found to stratify, forming 5-6 layers of cells, and to have a morphology comparable to that of the basal cells, wing cells, and superficial cells of normal corneal epithelium. Morphological investigation by electron microscopy revealed that the cultivated oral mucosal epithelial sheet has desmosomes, hemidesmosomes, and tight junctions, all of which are involved in cell adhesion between epithelial cells. Numerous microvilli were observed on the cell surface, showing properties of mucosal epithelium. Immunostaining for keratin, an epithelial cytoskeletal protein, showed the expression of keratin 4/13, a mucosal-specific keratin, but not keratin 1/10, which are epidermis-specific keratinizing type keratins. In addition, among keratinizing-type keratins 3/12, immunostaining was observed only for keratin 3. While normal oral mucosal epithelium is a unique mucosal membrane in the body that expresses keratin 3, our cultivated oral mucosal epithelial sheet was found to have the cytoskeleton characteristics of non-keratinized mucosa and cornea.

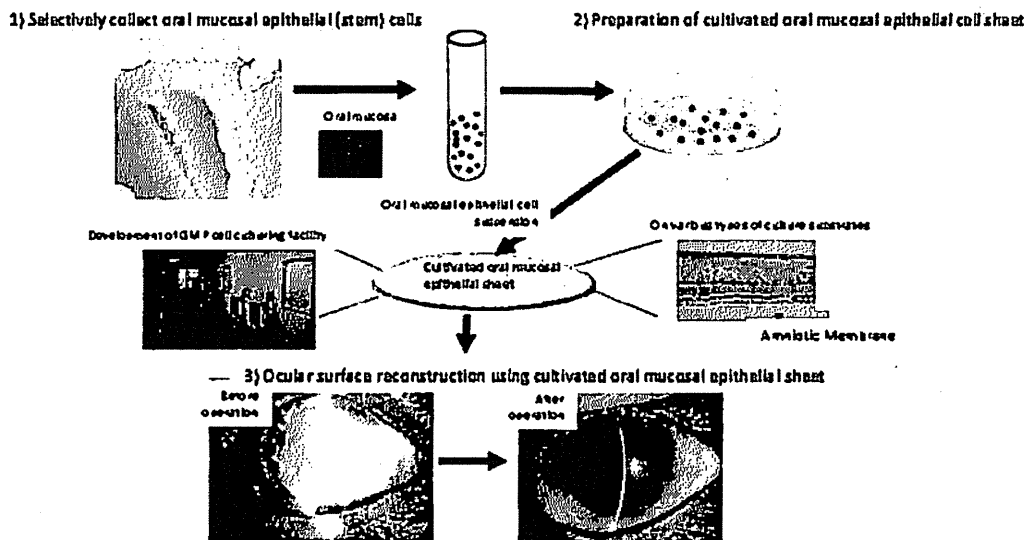


Fig. 4. Diagram showing the concept of cultivated oral mucosal epithelial transplantation for refractory ocular surface disease.

### *Ocular surface reconstruction using autologous cultivated oral mucosal epithelial sheet*

After examining the biological characteristics of the resulting cultivated oral mucosal epithelial sheet, it was autologously transplanted into the ocular surface of rabbits<sup>39)</sup>. A rabbit ocular surface disease model was created by superficial keratectomy. At 48 hours after transplantation, the mucosal epithelial sheet was confirmed by fluorescein staining to have remained transparent and on the ocular surface without defects. At 10 days after transplantation, it was observed to have remained on the ocular surface and to have extended outward compared to its position at 48 hours. In addition, histological examination of all corneal layers at this time point showed that the cultivated oral mucosal epithelial sheet had engrafted onto the ocular surface without stromal edema or cell infiltration, and with excellent biocompatibility with the ocular surface. These results indicate that our cultivated oral mucosal epithelial sheet has characteristics of corneal epithelium-like differentiation and stratified non-keratinized mucosal epithelium in terms of its histological and cell biology characteristics. In addition, oral mucosal epithelial sheet cultured on amniotic membrane was shown to engraft and survive even on the ocular surface, a unique environment in the body, suggesting its possible use as an alternative to corneal epithelium which maintains transparency after operation.

### *Clinical study of cultivated oral mucosal epithelial sheet transplantation*

Based on the above basic data obtained from animal studies, a clinical study of autologous cultivated oral mucosal epithelial transplantation against refractory ocular surface disease was initiated in 2002 after approval by the Institutional Review Board for Human Studies of Kyoto Prefectural University of Medicine<sup>54,55)</sup>. Of 17 eyes of 19 patients who underwent transplantation for corneal reconstruction at the Department of Ophthalmology, Kyoto Prefectural University of Medicine, up to January 2007 with long-term follow up for 3 years or more, approximately 53% showed a visual improvement of 1 grade or more at 3 years after operation. Postoperative complications included prolonged corneal epithelium disorder observed in approximately 37% during the follow-up period. During long-term follow-up, some patients showed ongoing reconstruction of the ocular surface with the transplanted cultivated oral mucosal epithelial sheet at 71 months after operation, revealing that oral mucosal epithelial cells, representing ectopic mucosal epithelial cells, can engraft and function on the ocular surface when applied using this surgical procedure. Considering that refractory ocular surface diseases have not been approved as an indication for corneal transplantation, the efficacy of cultivated oral mucosal epithelial sheet transplantation using autologous tissue was clinically adequate.

### *Future prospects*

In the history of corneal transplantation, recent progress in regenerative medicine/regenerative therapy research has produced significant innovation. Cell transplantation therapy from the *in vitro* to *in vivo* environments has produced a paradigm shift to corneal transplantation techniques in which replacement is limited to the defect site. The major challenges at present are the conduct of a comprehensive clinical examination of the long-term results of previous cultivated epithelium transplantation procedures, and ensuring the safety

and improving the quality of the cultivated epithelial sheets. Research tasks required to meet these challenges include the identification of stem cells in the cultivated epithelial sheet and the establishment of a culture environment, including niche. In addition, problems such as serum and feeder cells, which are used in the preparation process of the cultivated epithelial sheet, have to be resolved. Furthermore, in 2006, the Ministry of Health, Labour and Welfare (MHLW) implemented its "Guidelines for clinical research using human stem cells", which mandates ethics committee review when clinical research using tissue stem cells is conducted. Further development of culture techniques will need to conform with these guidelines. In any case, our responsibility is the development of safer and more evidence-based regenerative therapy.

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### *Conflict of interest statement:*

The authors declare no financial or other conflicts of interest in the writing of this paper.



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