Dentin-pulp complex tissue regeneration via three-dimensional cell sheet layering

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ABSTRACT

The dentin-pulp complex is a unique structure in teeth that contains both hard and soft tissues. Generally, deep caries and trauma cause damage to the dentin-pulp complex, and if left untreated, this damage will progress to irreversible pulpitis. The aim of this study was to fabricate a layered cell sheet composed of rat dental pulp (DP) cells and odontogenic differentiation of pulp (OD) cells and to investigate the ability to regenerate the dentin-pulp complex in a scaffold tooth. We fabricated two single cell sheets composed of DP cells (DP cell sheet) or OD cells (OD cell sheet) and a layered cell sheet made by layering both cells. The characteristics of the fabricated cell sheets were analyzed using light microscopy, scanning electron microscopy (SEM), hematoxylin-eosin (HE) staining, and immunohistochemistry (IHC). Furthermore, the cell sheets were transplanted into the subrenal capsule of immunocompromised mice for 8 weeks. Following this, the regenerative capacity to form dentin-like tissue was evaluated using micro-computed tomography (Micro-CT), HE staining, and IHC. The findings of SEM and IHC confirmed that layered cell sheets fabricated by stacking OD cells and DP cells maintained their cytological characteristics. Micro-CT of layered cell sheet transplants

revealed a mineralized capping of the access cavity in the crown area, similar to that of natural dentin. In contrast, the OD cell sheet group demonstrated the formation of irregular fragments of mineralized tissue in the pulp cavity, and the DP cell sheet did not develop any hard tissue. Moreover, bone volume/tissue volume (BV/TV) showed a significant increase in hard tissue formation in the layered cell sheet group compared to that in the single cell sheet group ($p<0.05$). HE staining also showed a combination of soft and hard tissue formation in the layered cell sheet group. Furthermore, IHC confirmed that the dentin-like tissue generated from the layered cell sheet expressed characteristic markers of dentin but not bone equivalent to that of a natural tooth. In conclusion, this study demonstrates the feasibility of regenerating dentin-pulp complex using a bioengineered tissue designed to simulate the anatomical structure.

Impact Statement

The dentin-pulp complex can be destroyed by deep caries and trauma, which may cause pulpitis and progress to irreversible pulpitis, apical periodontitis, and even tooth loss. Current treatments cannot maintain pulp health, and teeth can become brittle. We developed a three-dimensional layered cell sheet using dental pulp cells and odontogenic differentiation of pulp cells for dentin-pulp complex regeneration. Our layered cell sheet

enables the regeneration of an organized three-dimensional dentin-pulp-like structure comparable to that of natural teeth. This layered cell sheet technology may contribute to dentin-pulp complex regeneration and provide a novel method for complex tissue engineering.

Introduction

The dentin-pulp complex, which is composed of characteristic hard tissue formed by odontoblasts and soft connective tissue including pulp cells, not only possesses the ability to regenerate dentin but also serve functions in sensation and defense to maintain tooth homeostasis.[1,2] Dental caries and trauma damage the dentin-pulp complex and cause irreversible pulpitis by exposing pulp to the external environment.[3,4] To preserve the dentin-pulp tissue and effectively activate its functions, conventional treatments have been made by covering or cutting the pulp using dental materials[5,6] However, these techniques have various limitations, including a low degree of calcification of the dentinal bridge, incomplete formation in case of larger dentin defects, and reactive inflammation caused by capping materials.[7-9] Meanwhile, root canal treatment (RCT) is conventionally the only available option for irreversible pulpitis that remove the infected dentin and pulp tissue completely, and the pulp cavity or dentin fossa are filled with artificial materials.[10]

However, the low survival rate of teeth after RCT, the risk of residual infection, inflammation, and root fracture associated with pulp removal, has been a problem in dental procedure for many years.[11]

To overcome these problems, researchers have focused on regenerating dentin-pulp complex tissue to restore and protect the physiological functions of pulp tissue using stem/progenitor cells and candidate cytokines.[12] Recent stem cell biology studies in this field have identified tooth tissue-derived stem cells, including dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHED), and stem cells derived from apical papilla (SCAP), which can differentiate into odontoblast lineages.[13-15] These dental stem cells are considered a good resource for stem cell-mediated dentin-pulp complex regeneration.[16-20] These stem cells could form dentin and pulp in *in vivo* transplantation; however, they have not achieved an organized dentin-pulp complex formation because simple stem cell transplantation methods regenerated partially and randomly in the transplant site[21-23]. Cytokines and low-molecular-weight compounds are critical for recruiting stem/progenitor cells from the host and promoting their differentiation to regenerate dentin-like tissue,[24] and various studies have demonstrated that dental stem cells cultured with growth factors such as SDF-1, bFGF, and PDGF can form a dentin-pulp-like complex in small and large animal models. However, the formed

dentin-pulp tissue does not have an organized structure like natural teeth, and also secondary dentin formation for the purpose of overlying pulp tissue results in only a small scale of dentin regeneration.[3,25-27] To provide dentin-pulp complex regeneration for devitalized tooth with extensive dentin defects, where the infected dentin and pulp tissue has been completely removed, an effective transplantable tissue regenerative technology is needed. [14-16,28]

Cell sheet engineering using a temperature-responsive culture dish has various advantages to maintain the cellular attachment proteins and extracellular matrix.[29] Scaffold-free cell sheet engineering has been applied in the regeneration of various tissues such as the cornea, heart, and periodontal tissue.[30] However, a substantial problem in cell sheet technology is that it remains difficult to construct a three-dimensional complex tissue structure composed of multiple cell types.[31,33] The goal of tissue engineering technologies is to create a well-designed bioengineered tissue without artificial materials that reproduce anatomical tissue structures capable of enabling complete three-dimensional tissue regeneration equivalent to that of natural complex tissue.[32] In our previous study, we fabricated a complex cell sheet that reproduces the bone-ligament structure equivalent to natural periodontal tissue.[33] We also demonstrated a three-dimensional regeneration of periodontal tissue and represented the feasibility of a three-

dimensional tissue engineering from an anatomically designed bioengineered tissue. [33] Dentin-pulp complex tissue contains characteristic hard and soft tissue, and it is assumed that conventional tissue regeneration transplanting a single type of cell or applying single cell sheet is not sufficient to regenerate the natural tissue architecture.

In this study, we demonstrated the three-dimensional regeneration of dentin-pulp complex tissue using a layered cell sheet composed of dental pulp (DP) cells and odontogenic differentiated DP (OD) cells. Our layered cell sheet anatomically reproduced dentin-pulp complex structures similar to natural teeth in ectopic transplantation. This study indicates a newly technology for successful three-dimensional tissue regeneration using a bioengineered tissue designed to simulate anatomical structures.

Methods

Cell isolation and culture. The SD rats were sacrificed via isoflurane anesthetic inhalation (Mylan, Japan), and the maxilla and mandible were dissected to remove all incisors. The pulp was removed after cut both ends of the incisors, then minced it into small pieces. Further, the fragments were transferred to a tube containing 3 mg/mL collagenase type I solution (Gibco, Grand Island, NY 14072, USA), and enzymatic digestion was carried out for 45 min at 37 °C (gently tapped every 10 min). To terminate

enzymatic digestion in an equal volume of culture medium, Dulbecco's modified Eagle's medium (DMEM) (High Glucose) (Nacalai Tesque, Inc. Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS) (Biowest, Ireland) and 1% penicillin-streptomycin mixed solution (antibiotic solution) (Nacalai Tesque, Inc. Kyoto, Japan) were also added. The cell suspension was passed through a 70- μm cell strainer (Greiner Bio-One, Japan) and collected into a fresh 15 mL tube that was centrifuged at 1800 rpm for 3 min at 4 °C. Finally, DP cells were cultured in DMEM, 100 mm culture dish (Thermo Fisher Scientific, USA). Cells from 3rd passage were used for the subsequent experiments. DP cells were induced into odontogenic differentiation (OD) cells by culturing in α-minimum essential medium (α-MEM) (Nacalai Tesque, Inc. Kyoto, Japan), supplemented with 10% FBS, 1% antibiotic solution, 50 μg/ml ascorbic acid (Sigma Aldrich, Inc., USA), 10 nM dexamethasone (Sigma Aldrich, Inc., USA), and 5 mM β-glycerophosphate (Sigma-Aldrich, Tokyo, Japan) (OD medium).

Cell sheets Fabrication. All cell sheets were fabricated using a thermo-responsive culture dish (CellSeed, UpCell®, Tokyo, Japan). Three different cell sheets were prepared, of which two were single cell sheets with either DP cells (DP cell sheet) or OD cells (OD cell sheet), and the third was a layered cell sheet consisting of both cells. A single DP cell sheet was prepared by seeding 4×10^6 DP cells in an UpCell® dish and cultured in

DMEM and detached after 2 days. OD cell sheet was prepared by seeding 4×10^6 OD cells per dish and cultured in OD medium with 10 ng/ml BMP-2 (Peprotech, USA), which detached after 7 days. For layered cell sheet fabrication, firstly, made OD cell sheet with a density of 4×10^6 OD cells and cultured in OD medium along with 10 ng/ml BMP-2 for 7 days. Then washed with warm PBS to remove the remaining OD medium, and 4×10^6 DP cells were seeded onto the OD cell sheet. Finally, layered cell sheet was cultured in DMEM for 3 more days. All culture media were changed every 24 h. Cell sheets were detached using the respective 4 °C medium. The progress of cell sheet fabrication is shown in Fig. S1.

Subrenal Capsule transplantation. There were 4 groups in subrenal capsule transplantation which were scaffold tooth only (n=5), DP cell sheet (n=8), OD cell sheet (n=8), and layered cell sheet (n=8). To prepare the scaffold tooth, the mandibular first molar was securely positioned with a tweezer, and the occlusal cavity was prepared under a stereo microscope (Stemi DV4, Japan) using a hand drill (NSK Viva-mate Plus, Japan). We removed dentin in the pulp chamber to create an access cavity. The root canal was cleaned using H-file # 10 (Mani, Japan), and the remaining tissue of the scaffold tooth was further digested using collagenase type I solution in an incubator at 37 °C for 30 min. The tooth was then autoclaved to remove remaining live cells. In addition, the extracted

mandibular first molar was directly fixed in 4% paraformaldehyde phosphate buffer solution (PFA) (Nacalai Tesque, Kyoto, Japan) overnight. Subsequently, the natural tooth was used for Micro-CT analysis, and decalcified for HE staining or IHC staining. Regenerated tissue in the transplants was compared to the natural tooth (n=5) structure of mouse.

The detached cell sheets were cut into small pieces and placed covering the access cavity of the scaffold tooth. The DP cell side of the layered cell sheet was facing toward the pulp chamber, and the OD side was facing the outer surface. Single cell sheets were folded twice and inserted into the cavity of the scaffold tooth. We used 30μm collagen gel solution to embed each tooth-cell sheet constructs and incubated at 37 °C for 20 min to solidify into a gel. The collagen gel used for fixed the tooth-cell sheet structure in kidney. Additionally, scaffold tooth embedded into collagen gel without any cell sheets (negative control group) was also transplanted into subrenal capsule. Subrenal capsule transplantation was performed as previously reported.[31] The surgery was performed under a dental microsurgery microscope (Leica M 320 F 12) and transplants were collected after 8 weeks. Subrenal capsule transplantation procedures are shown in Fig. S2.

Experiment

Experiment design

Animals. All animal procedures were approved by the Animal Research Committee of Tokushima University (Approval No. T30-93 and T2021-6). All animals were obtained from CLEA Japan (Osaka, Japan). We used 4-week-old male Sprague-Dawley (SD) rats to harvest DP cells (totally 86 rats), and 4-week-old C57BL/6NJcl male mice were used to extract the mandibular first molar, which was subsequently used as natural tooth or used to prepare the scaffold tooth with an access cavity (totally 67mice). CB17/ICR-scid Jcl female mice were used for ectopic transplantation (totally 40 mice). All ectopic surgical techniques were performed under deep anesthesia using 75 mg/kg medetomidine (Nippon Zenyaku Kogyo Co., Ltd, Fukushima, Japan), 4 mg/kg midazolam (Sandoz K.K., Yamagata, Japan), and 5 mg/kg butorphanol (Meiji Seika Pharma Co., Ltd., Tokyo, Japan).

Alizarin red Staining. Mineralization of DP cells and OD cells was analyzed using alizarin red staining. $2.5x10^5$ DP cells were seeded per well in 6 well dish (Thermo Fisher Scientific, USA), which was cultured in DMEM or OD medium. The medium was changed once every two days. On days 7 and 28, the cells were fixed using 95% ethanol at 37 °C for 15 min and stained with 1% alizarin red water solution (MUTO PURE, Japan) at room temperature for 10 min. The culture dish was washed with running water three times and dried for further visualization. Images of the stained cells were captured using

a light microscope (Leica DMi 1, Wetzlar, Germany) and a digital camera (Canon 550D, Japan).

Quantitative real-time PCR. Cultured DP and OD cells were collected on day 14 for gene expression analysis. Total RNA was extracted from cells using TRIzol reagent (Life Technologies, CA, USA). Total RNA was dissolved in DEPC-treated water (Nacalai Tesque, Kyoto, Japan), and absorption, quantity, and purity were measured at 260 nm and 280 nm using a spectrophotometer (NanoDrop® ND-1000, Nanodrop Technologies, Inc., DE, USA). One microgram of total RNA was used to process the cDNA by reverse transcription. This was performed using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Thermo Fisher Scientific, USA). Quantitative RT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The relative gene expression levels were calculated using the ΔΔCt method. We used dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP), osteocalcin (OCN), and alkaline phosphatase (ALP), primers for the analysis based on previously reported methods.[34-35] For the endogenous control, the values were normalized to GAPDH (housekeeping gene). The primer sequences used are listed in Table 1.

Histological examination of cell sheets. The cell sheets were fixed overnight in 4%

PFA and dehydrated with graded concentrations of ethanol and Hemo-De (Falma, Japan). Next, they were embedded in paraffin for approximately 6 h and cut into 5 μm sections. Hematoxylin-eosin (HE) staining was performed to analyze the morphology.

For immunohistochemistry (IHC), the sections were dewaxed and dehydrated with graded concentrations of ethanol and Hemo-De. The sections were then treated with 3% H_2O_2 for 10 min. For antigen retrieval, the sections were autoclaved at 121 °C for 20 min in sodium citrate buffer solution (pH6.0) (Nacalai Tesque, Kyoto, Japan). To avoid nonspecific binding, the sections were initially blocked with Protein Block Serum-Free (Dako) for 30 min and incubated overnight with the primary anti-DSP antibody (1:75) (Aviva, USA). The sections were subsequently stained with the secondary antibody (rabbit anti-goat IgG, Vector, Japan) for 60 min, followed by the Vectastain ABC kit (rabbit IgG; Vector Laboratories, Japan) for 1 h. Furthermore, the sections were incubated with 3,3-diaminobenzidine (DAB; Sigma-Aldrich, Tokyo, Japan) chromogenic substrate and counterstained with hematoxylin to aid visualization. All digital images of HE staining and IHC analysis were captured using a light microscope (Olympus BX43, Cellsens Standard).

Scanning electron microscope (SEM) analysis of layered cell sheet. SEM analysis

was performed to identify the two-cell combinations in the layered cell sheet. We evaluated both sides of the layered cell sheet and paraffin sections. First, to observe the two sides, harvested cell sheets were fixed with 2.5% glutaraldehyde solution (Nacalai Tesque, Kyoto, Japan) at 4 °C overnight and dehydrated with graded concentrations of ethanol. The paraffin sections of the cell sheets were prepared using the same protocol as previously described in the histology section. Sections were dewaxed using Hemo-De and dehydrated with graded concentrations of ethanol. For electroconductive treatment, sections were stained with TI blue (3% platinum blue, Nissin EM, Tokyo, Japan) for 20 min at room temperature and then rinsed with distilled water. The cell sheets were observed using low-vacuum SEM, and images were acquired (TM-1000, Hitachi High-Tech Corporation, Tokyo, Japan).

Micro computed tomography (Micro-CT) analysis of transplants. The experimental animals were sacrificed after 8 weeks of isoflurane inhalation (Pfizer, Japan). Kidneys containing the transplants were dissected and fixed in 4% PFA overnight. Further Micro-CT was performed with SkyScan 1176 (SkyScan, Kontich, Belgium), the X-ray source at 50 kV and 500 μA using a 0.5 mm aluminum filter and at a reconstruction angular range of 360° under a voxel size of 9 μM. NRecon software (Bruker Micro-CT, Kontich, Belgium) was used to reconstruct the scanned images, and analysis of the three-

dimensional models was performed using CTVox software (Bruker Micro-CT, Kontich, Belgium). The regenerated dentin-like tissue volume at the tooth crown area was quantified and analyzed using CT analyzer software (CTAn, Bruker Micro-CT, Kontich, Belgium).

The region of interest was the area around the prepared access cavity in the scaffold tooth crown (Fig. S4 in blue). The regenerated dentin-like tissue volume at the tooth crown area of the scaffold tooth was quantified and analyzed using CT analyzer software (CTAn, Bruker Micro-CT, Kontich, Belgium).

Histological examination of transplants. Following the Micro-CT analysis, the transplants were immersed in decalcification solution (0.5)/L EDTA (pH 7.5) (Wako, Osaka, Japan) for 3–4 weeks and Plank Rychlo (Wako, Osaka, Japan) for 7 days each for HE staining and IHC analysis. After decalcification, the samples were further embedded in paraffin and cut into 5 μm sections for HE staining. In IHC staining, the samples were immersed in 0.3% H_2O_2 in methanol for 30 min and subjected to antigen retrieval in citrate buffer solution (pH 6.0) at 60 °C in a water bath overnight. The membranes were blocked with Protein Block Serum-Free for 30 min, followed by overnight incubation at 4 °C with the primary anti-DSP antibody (1:75) and anti-OCN antibody (1:50) (GeneTex, USA). The subsequent protocols were same as previous described.

Statistical analysis

SPSS 27 (IBM) was used for the statistical analysis. The experimental values are expressed as mean ± SEM. Statistical significance was evaluated by one-way analysis of variance followed by Tukey's HSD test. The t-test was used to examine between-group differences. The significance level was set at $p < 0.05$.

Experimental Results

Characteristics of cells & calcium deposition by OD of cells

The morphologies of DP and OD cells were observed under a light microscope. At 3rd passage DP cells showed a roughly star or polygonal morphology. (Fig. 1A) Whereas the OD cells after 7 days of differentiation showed a more homogenous pattern of polygonal shaped cells with higher cell density (Fig. 1B). Mineralization deposits were observed by alizarin red staining. On days 7 and 28, the OD cell culture showed increased mineral nodule formation. However, DP cell culture on days 7 or 28 did not indicate any mineral deposition (Fig. 1C, D).

OD cells upregulated dentin-pulp related genes

Quantitative real-time PCR was performed to compare the gene expression levels of OD cells after 14 days of differentiation and DP cells. There was a remarkable 5-fold

increase in the expression of DMP-1 in OD cells compared to DP cells (Fig. 2A) and significant augmentation of DSPP with a 3-fold difference in expression in OD cells compared to DP cells (Fig. 2B). Furthermore, OCN gene expression was also significantly increased in OD cells with a 2-fold difference (Fig. 2C). However, ALP expression did not differ between DP and OD cells (Fig. 2D).

Observations of cell sheets.

The single cell sheet consisted of either DP cells or OD cells, and the layered cell sheet was fabricated by layering the two cell types. Macroscopically, the detached single cell sheets were smaller, thinner, and had a slightly crumpled appearance compared to the layered cell sheet group (Fig. 3A-C). Light microscopic images revealed that cells in all three cell sheet groups maintained their cell morphology with a characteristic polygonal shape (Fig. 3D-F). Moreover, a higher cell density was observed in the OD cell sheet and layered cell sheet than in the DP cell sheet group (Fig. 3D-F).

HE and IHC staining of cell sheets

HE staining revealed that all cell sheets contained multiple layers of cells and detached as an intact continuous sheet along with the attachment matrix (Fig. 3G-I). A closely organized cell layer comprising four to five layers were observed in the DP cell sheet and

OD cell sheet (Fig. 3G, H). However, closely arranged cells were observed toward one side of OD cell sheet and the other side showed a lower cell density (Fig. 3H). Layered cell sheets appeared thicker compared to DP and OD cell sheets, which contained approximately 10 to 13 layers of cells, were observed along with two distinct cell layers on either side (Fig. 3I).

We performed IHC with anti-DSP to further confirm gene expression in the three types of cell sheets. IHC revealed that the OD cell sheet had a strong positive expression of DSP, whereas only weak staining was observed in the DP cell sheet. Strong DSP expression was localized to the OD layer in the layered cell sheet, whereas the side of the DP cell layer exhibited a lower expression.

SEM analysis of layered cell sheet

To further characterize the two distinct cell types in the layered cell sheet, we analyzed either side and the cross section of the layered cell sheets by SEM. Marked differences were observed between the two layers at both lower and higher magnifications (Fig. 4A-D). The DP cell side showed more fibrous and collagen-like structures, which are marked by white arrowheads in Fig. 4B. The OD side showed more mineralized structures, which are marked by white arrows in Fig. 4D. In addition, the cross-section of the layered cell

sheet consisted of two-layer cells that were demarcated by a low-density cell area (Fig. 4E, F), which is in concordance with the HE staining and IHC of layered cell sheets (Fig. S3).

Micro-CT analysis of hard tissue formation in scaffold tooth

We evaluated the hard tissue regeneration capability of cell sheets after 8 weeks of subrenal capsule transplantation. Groups were organized as follows for analysis: natural tooth (n=5), scaffold tooth only (n=5), DP cell sheet (n=8), OD cell sheet (n=8), and layered cell sheet (n=8). In the 3D horizontal view, no mineralized tissue formation was observed in the cavity of scaffold tooth only, DP cell sheet and OD cell sheet groups. The layered cell sheet group generated an almost complete hard tissue bridge capping in the access cavity of the scaffold tooth (Fig. 5A, S5, circled by red dotted line); the sagittal view indicated that mineralized tissue formation was not found in the root canal of the scaffold tooth only and DP cell sheet group (Fig. 5A). In contrast, the OD cell sheet group exhibited haphazardly arranged hard tissue formation in the root canal area of the scaffold tooth (Fig. 5A). The layered cell sheet group generated a uniform calcified cap-like structure at the access cavity of the scaffold tooth (Fig. 5A, S5, white arrowhead); the cross-sectional sagittal view further confirmed irregular hard tissue formation in the chamber of the OD cell sheet group and a cap-like hard tissue bridge formation in the

crown area of the scaffold tooth in the layered cell sheet group (Fig. 5A, S5, white arrow). Finally, a quantitative analysis of the newly formed hard tissue was conducted using Micro-CT. The bone volume/tissue volume (BV/TV) of the layered cell sheet group was significantly higher than that of the scaffold tooth, DP cell sheet, and OD cell sheet groups. However, there was no significant difference between the scaffold tooth only and DP cell sheet group (Fig. 5B).

HE and IHC staining of transplants

In the HE staining, only connective tissue-like formation was observed in the DP cell sheet-transplanted group (Fig. 6E, F). In contrast, OD cell sheet transplants showed irregularly regenerated mineralization in the root canal space of the scaffold tooth (Fig. 6G, H). The access cavity of scaffold tooth regenerated with a dentin-like bridge formation similar to that of natural dentin (black arrowhead in Fig. 6J) along with pulp-like connective tissue in the root canal space of layered cell sheet transplants (Fig. 6I, J). The scaffold tooth group without cell sheet transplants showed only the collagen gel structure embedded in the chamber (Fig. 6C, D).

To further evaluate the regenerated dentin-pulp–like tissue formation in our ectopic transplants, dentin mineralization marker DSP and bone formation gene OCN were used

in IHC analysis. Strong positive DSP expression was detected in the dentin-pulp–like tissue of layered cell sheet transplants (Fig. 7I, J). Similarly, DSP expression was also observed in OD cell sheet transplants (Fig. 7G, H). However, the DP cell sheet group only exhibited weak DSP expression (Fig. 7E, F). No DSP expression was observed in the scaffold tooth only group (Fig. 7C, D). In the natural tooth, DSP was expressed in the odontoblast area (Fig. 7A, B). The pulp-like connective tissue in the layered cell sheet group attached to the dentin wall of the scaffold tooth showed stronger DSP expression (Fig S6). However, no OCN expression was observed in the control groups of transplants and layered cell sheet transplants (Fig. 7K-T).

Discussion

Cell sheet technology is an available tissue engineering technology that can easily produce transplantable regenerative tissues for target site regeneration as a uniform tissue, and this approach is useful for treating various diseases and types of tissue damage.[36] Many attempts have been made to regenerate tissues using various types of stem cells and progenitor cells, and clinical applications are being improved for liver, cardiac muscle, esophagus, and cornea repair.[37-40] Advanced clinical trials of corneal regeneration using patient-derived induced pluripotent stem (iPS) cells have also been conducted as a transplantable scaffold-free bioengineered tissue.[41] Although cell sheet technology has

been shown to be effective in uniform tissue regeneration, it is still difficult to construct a 3D complex tissue composed of multiple cell linages.[31, 33] In this study, we focused on the regeneration of dentin-pulp complex tissue as an important functional unit of tooth that is frequently and irreversibly damaged by dental caries and trauma. We reconstructed the dentin-pulp complex tissue by stacking cell layers of odontoblasts and dental pulp cells using our bioengineered tissue engineering technology (Fig. 6I, J). The layered cell sheet was shown to be distinctly laminated to double layers while maintaining the cytological characteristics of each layer, which reflects the dentin-pulp complex tissue in natural teeth (Fig. 3I, L & 6I, J). Our layered cell sheet is a three-dimensional reconstruction using two types of cell populations induced to differentiate from the same cell origin; this approach can be regarded as a new three-dimensional cell manipulation technique for reconstructing bioengineered complex tissues.

Regenerative concepts have been attempted for the regeneration of dentin-pulp complex tissue using cytokines and low-molecular-weight compounds. They were limited to small-scale dentin and pulp regeneration; it has not yet been established any biological technology that can adequately regenerate for the extensive dentin-pulp tissue injury.[42-44] Furthermore, many studies reported dentin-pulp complex regeneration in *in vivo* transplantation using stem/progenitor cells, which can differentiate into odontoblast

lineages; however, they have not been established as a well-controlled transplantable technology because the dentin-pulp complex were partially and randomly regenerated on artificial scaffolds such as hydroxyapatite and beta-TCP.[27,45-46] In addition, stem cell transplantation into the pulp cavity results in a large amount of dentin formation onto the existing internal dentin-wall; however, a small amount of dentin regeneration to the dentin defect side (crown site) is not sufficient to serve as a seal for the pulp cavity.[21,27,47] In this study, we successfully carried out the regeneration of the dentin-pulp complex tissue with organized compartmentalized dentin and pulp tissue area into the scaffold tooth, and we achieved a biological near-capping of the pulp cavity in the crown site through the anatomically designed regeneration by transplantation of dentin-pulp layered cell sheet (Fig. 6I, J & 7I, J, S5). Our dentin-pulp layered cell sheet has a significant difference in the regenrated dentin volume compared to single DP cell sheet and OD cell sheet (Fig. 5B). For the functional regenration of complex tissue, several studies indicated the importance of a biological cross-talk in multiple constituent cells through the fabrication of a composite cell sheet by mixing both cells.[48,49] Additionally, another concept has been attempted to reconstruct the periodontal tissue structure by the transplantation with the combination of multiple types of uniform cell sheets; however, the complete complex structure of natural periodontal tissue was not established.[50] In our previous study, we

demonstrated the three-dimensional periodontal tissue regeneration using complex cell sheet composed of bone-ligament layering. The layered cell sheet method could contribute to constructing proper anatomical structures and regenerate three-dimensional complex tissues.[33]

The formed dentin tissue in our study expressed characteristic markers of dentin but not of bone; however, it did not seem to have a microstructure including dentin tubules similar to natural teeth (Fig. 6I, J & 7I, J, S, T). During tooth development, dentin formation progresses autonomously and is achieved with a characteristic structure that encloses peripheral nerve fibers within microstructures called dentinal tubules.[51,52] However, the secondary dentin, which is added due to age-related changes and external stimulations after the completion of the tooth root, is confirmed to exhibit heterogeneity of dentin calcification and reduction/loss of dentinal tubules.[53,54] In the present study, tissue-engineered layered cell sheets were applied to scaffold teeth and allowed to differentiate under subrenal capsule transplantation *in vivo*. Therefore, the formed-dentin tissue may resemble the tissue structure of secondary dentin without the microstructure of natural dentin.

In the present study, to demonstrate the concept of regeneration of dentin-pulp complex by layered cell sheet, we evaluated the tissue regeneration in the scaffold tooth with dentin

and pulp defect by using an ectopic transplantation model under the subrenal capsule (SRC). Since the SRC transplantation model has abundant blood flow and prevents the invasion of other tissues, it was employed to evaluate the ability for tissue formation of odontoblasts-pulp cells layered cell sheet.[55,56]. Whereas, the ultimate goal of this study is to apply the layered cell sheet into the oral transplantation. For clinical application of our technology, it is necessary to optimize the culture condition and handling of cell sheets and to integrate with conventional dental treatments. For devitalized teeth with infected dentin and pulp removed, *in vitro* layered cell sheets are transplanted into the pulp cavity while preserving the tissue structure both of odontoblast layer and pulp cell layer. To obtain pulp cells for the layered cell sheet construction, not only the patient's own third molars and deciduous teeth can be used, but also the tooth bank service for long-term preservation of extracted teeth can be assumed[57-59]. The formation of the dentin-pulp complex at the transplant site requires a maturation period, and the graft must be covered with sealing material like a crown restoration to prevent exposure and contamination of the transplanted layered cell sheet. Recent digitized crown restoration technology as a sealing for graft will contribute to the regenerative dental treatment of this dentin-pulp complex.[60]

Conclusion

In this study, we fabricated a layered cell sheet composed of DP cells and odontoblasts to reproduce a dentin-pulp complex tissue that reflects a natural tooth structure. Our study represents the feasibility of tooth complex tissue regeneration using a novel three-dimensional tissue engineering technology.

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Author Contributions

M. O., H. Y. and Y. M. were responsible for the study design and protocol. H. Y. and M. O. fabricated the cell sheet, scaffold tooth, and ectopic transplantation. M. I. and Miho, I. performed immunohistochemistry. O.B and T.M performed HE, and azan staining, R. R. and A. W. collected cells and performed micro-CT analysis, S.R performed RNA extraction and RT-PCR, K. S. performed part of SEM, and M. M. performed the data

statistics. H. Y. and M. O. wrote the manuscript, and all co-authors approved the final manuscript. Correspondence and requests for materials should be addressed to Y. M.

Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Table S1

Supplementary Figure S1

Supplementary Figure S2

Supplementary Figure S3

Supplementary Figure S4

Supplementary Figure S5

Supplementary Figure S6

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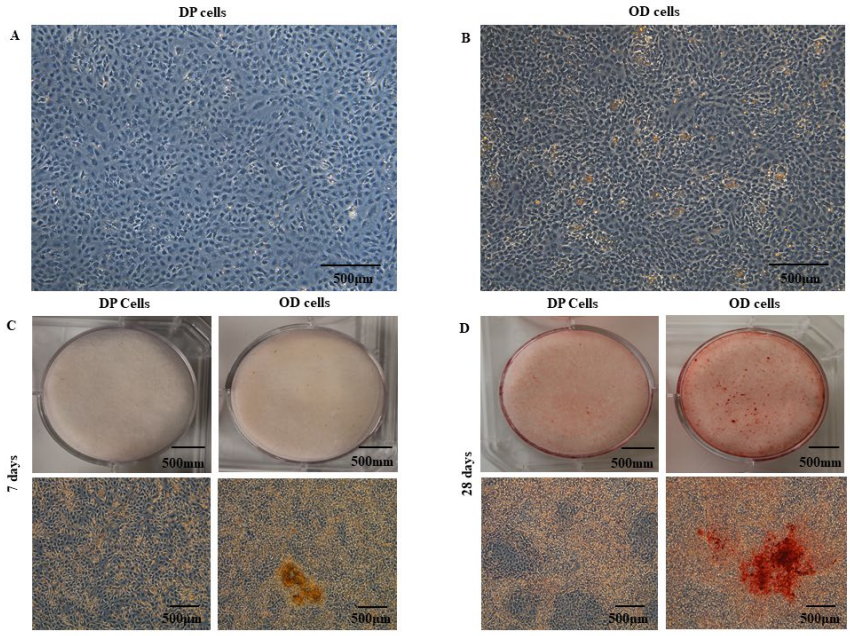

A
DP cells
500μm
B
OD cells
500μm
C
DP Cells
OD cells
7 days
500μm
500μm
500μm
500μm
D
DP Cells
OD cells
28 days
500μm
500μm
500μm
500μm

Fig.1

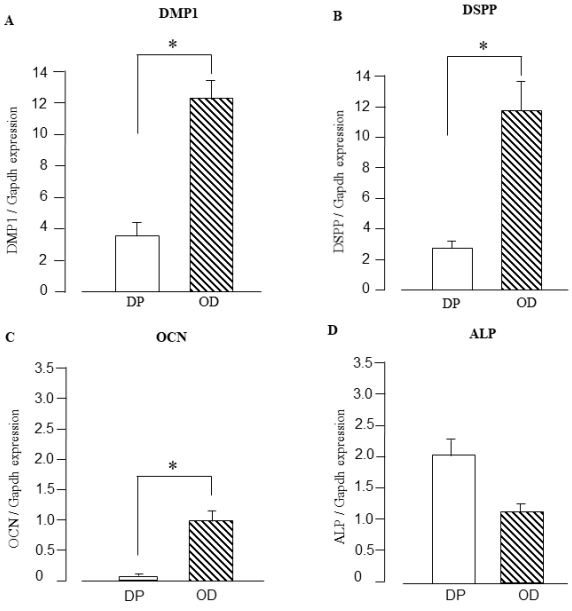

A
DMP1
DMP1 / Gapdh expression
B
DSPP
DSPP / Gapdh expression
C
OCN
OCN / Gapdh expression
D
ALP
ALP / Gapdh expression
DP
OD
*

Fig.2

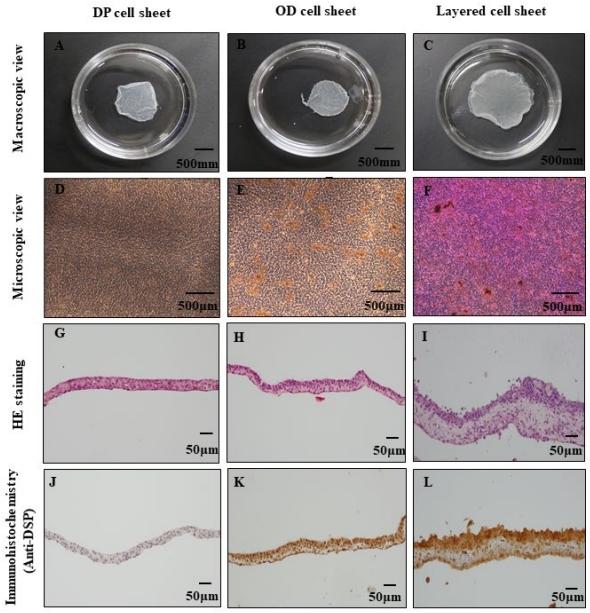

DP cell sheet
OD cell sheet
Layered cell sheet
Macroscopic view
Microscopic view
HE staining
Immunohistochemistry (Anti-DSP)
A
B
C
D
E
F
G
H
I
J
K
L
500mm
500μm
50μm

Fig.3

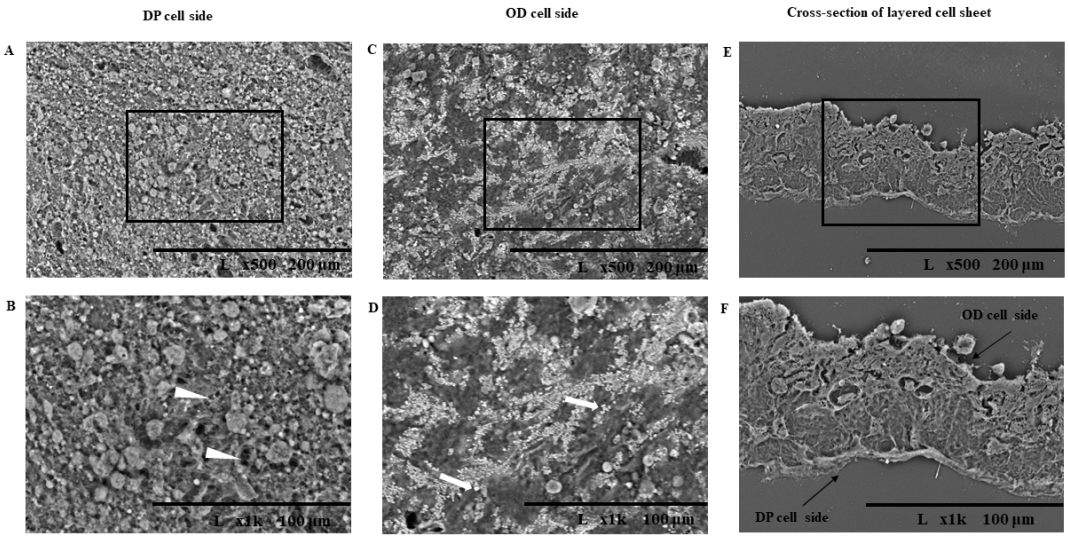

DP cell side
OD cell side
Cross-section of layered cell sheet
A
B
C
D
E
F
L x500 200 μm
L x1k 100 μm
OD cell side
DP cell side

Fig.4

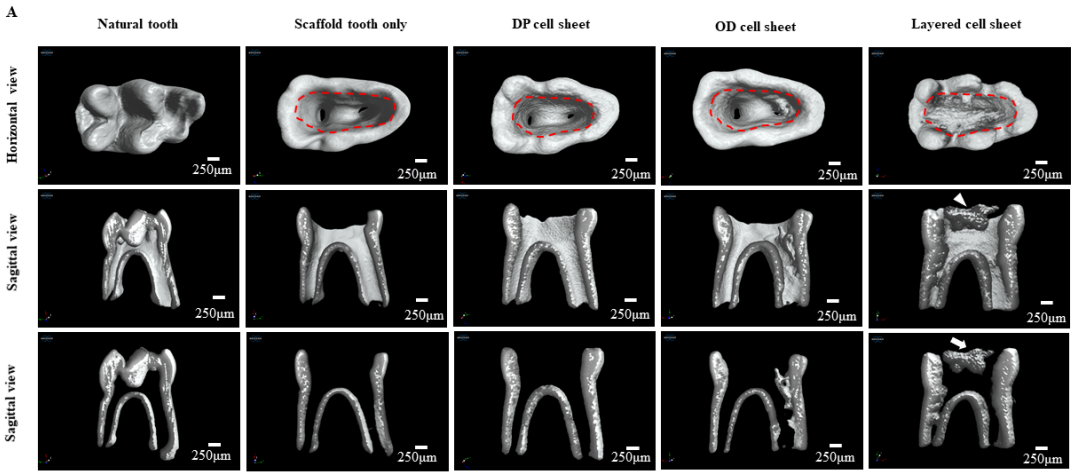

A
Natural tooth
Scaffold tooth only
DP cell sheet
OD cell sheet
Layered cell sheet
Horizontal view
Sagittal view
Sagittal view
250μm

Fig.5A

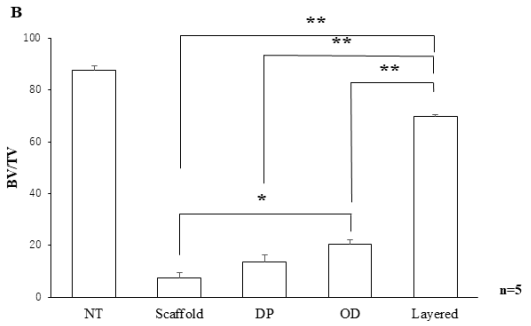

B
BV/TV
100
80
60
40
20
0
**
**
**
*
NT
Scaffold
DP
OD
Layered
n=5

Fig.5B

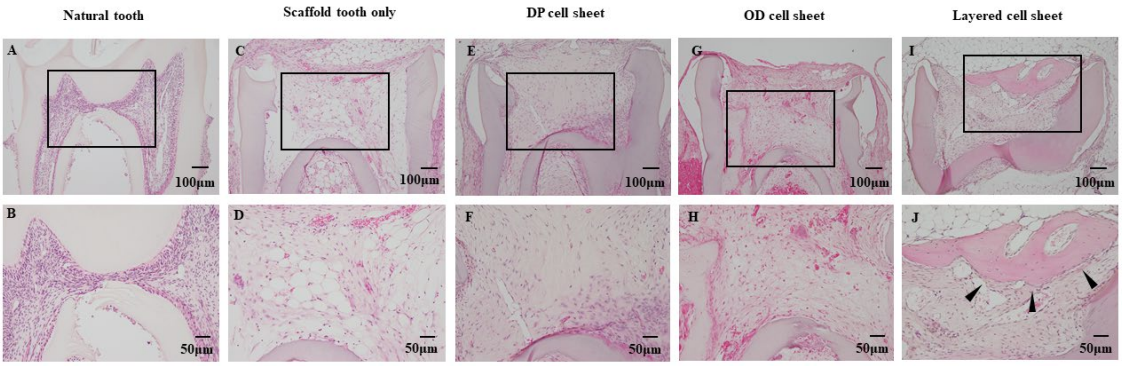

Natural tooth
Scaffold tooth only
DP cell sheet
OD cell sheet
Layered cell sheet
A
C
E
G
I
B
D
F
H
J
100μm
50μm

Fig.6

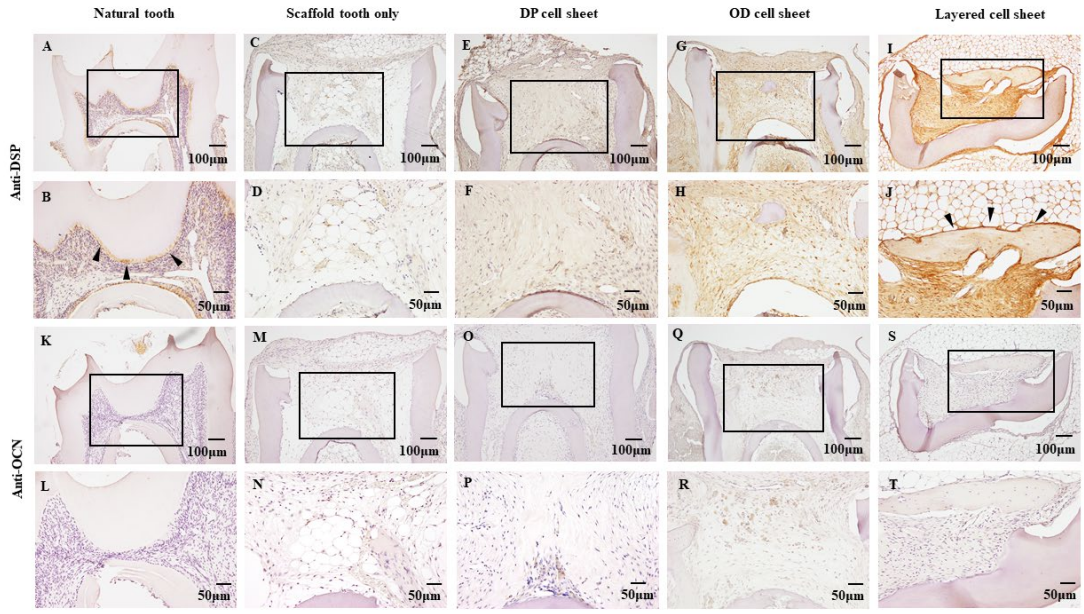
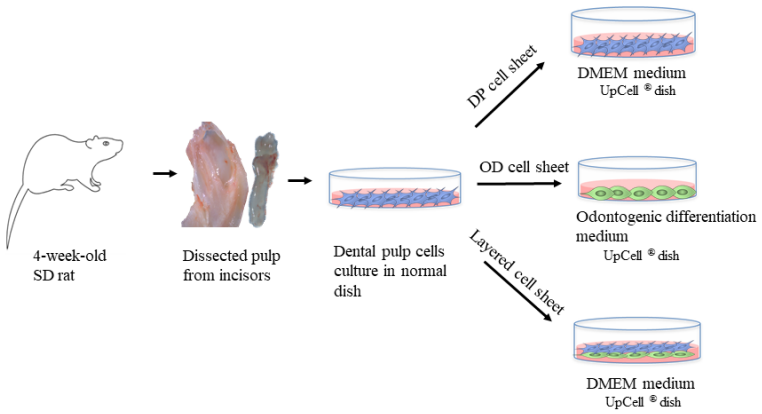
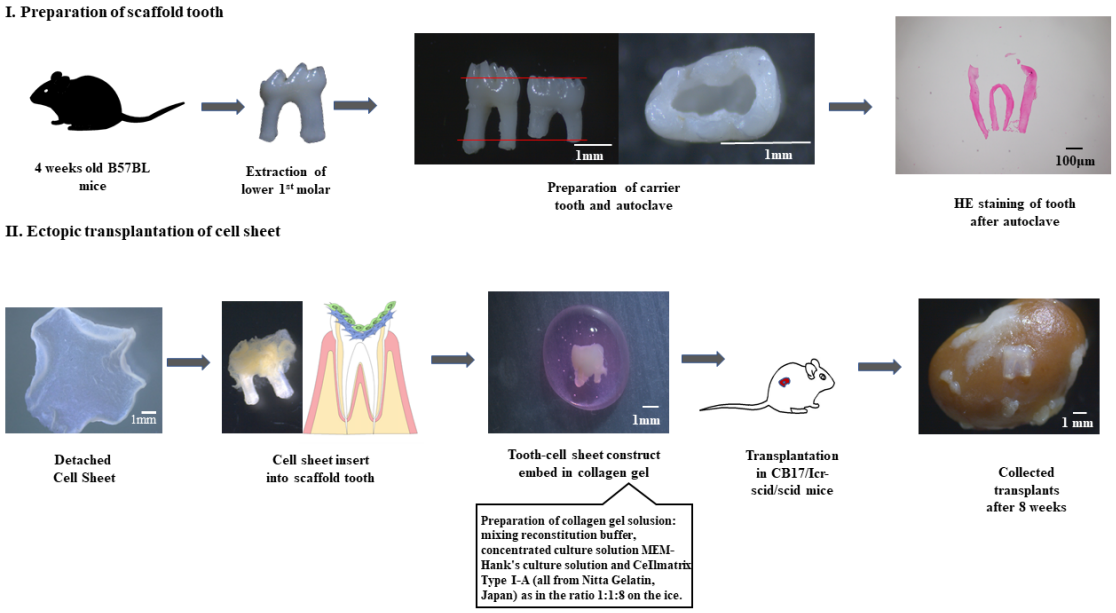

Natural tooth
Scaffold tooth only
DP cell sheet
OD cell sheet
Layered cell sheet
Anti-DSP
Anti-OCN
A
C
E
G
I
B
D
F
H
J
K
M
O
Q
S
L
N
P
R
T
100μm
50μm

Fig.7

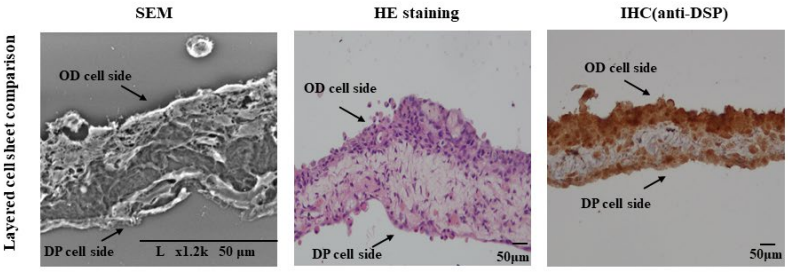
Supplementary Figure



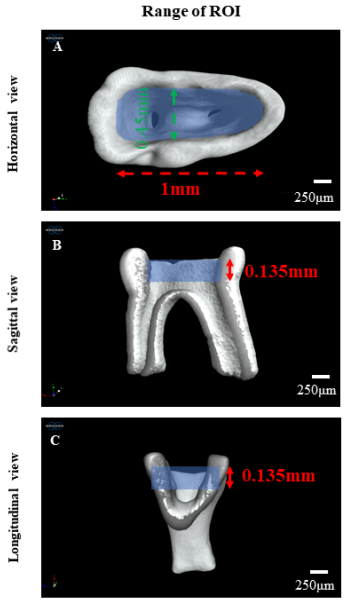
S1



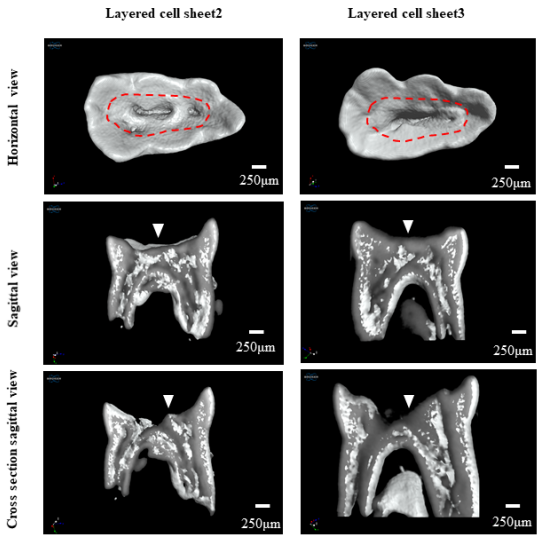
S2


SEM
HE staining
IHC(anti-DSP)
Layered cell sheet comparison
OD cell side
DP cell side
L x1.2k 50 µm
OD cell side
DP cell side
50µm
OD cell side
DP cell side
50µm

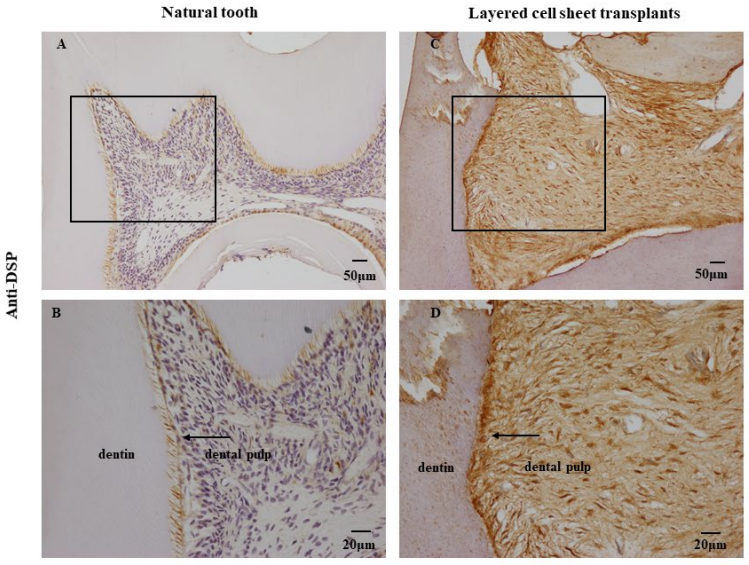
S3


Range of ROI
Horizontal view
Sagittal view
Longitudinal view
A
0.45mm
1mm
250µm
B
0.135mm
250µm
C
0.135mm
250µm

S4


Layered cell sheet2
Layered cell sheet3
Horizontal view
Sagittal view
Cross section sagittal view
250μm

S5


Natural tooth
Layered cell sheet transplants
Anti-DSP
A
B
C
D
50μm
20μm
dentin
dental pulp

S6