Original Article

Development of Multilayered Dental Pulp Cell Sheets and Consideration for Calcification Ability

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Abstract

Purpose: The purpose of this study was to analyze the calcification characteristics of three-dimensional (3D) culture of multi-layered dental pulp cell (DPC) sheets that have potential application in pulp regeneration and evaluation of dental materials and pharmaceuticals.

Methods: DPC were isolated from third molars extracted from three healthy patients aged 18–30 years old. DPC sheets were constructed using a temperature-responsive culture dish (UpCell) and a gelatin stamp (Cell Stamp for UpCell). Histological examination of three-layer and six-layer DPC sheets was performed using hematoxylin and eosin staining (HE). DPC sheets on mineral trioxide aggregates (MTA) were observed using a scanning electron microscope (SEM) to examine the microstructure. The LOX-1 probe was used to examine the effects of hypoxia. Calcification ability was analyzed by real-time PCR and Alizarin Red S staining (ARS). In this study, two-dimensional (2D) culture (monolayer) served as a control.

Results: Thick multilayered cell sheets were confirmed under the macroscopic view and with HE. The SEM images showed that the DPC sheets covered the MTA. Higher osteo/odontogenic differentiation was seen in the multi-layered cell sheets than in the monolayer cell sheets. The expression levels of alkaline phosphatase (*ALP*), type Ⅰ collagen (*COL1A1*), osteocalcin (*OCN*), and dentin sialoprotein (*DSPP*) genes were higher in the DPC sheets. With ARS, characteristic nodules of mineralization were observed in the cell sheets.

Conclusion: DPC sheets possessed higher mineralizing ability than the monolayer sheets. Cell sheets may be useful for comparative examination of pharmaceuticals, materials, and pulp regeneration in a 3D environment.

Key words: dental pulp cell (DPC), osteo/odontogenic differentiation, three-dimensional culture, cell sheets

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Introduction

In recent years, three-dimensional (3D) culture has been performed using various methods. It has been reported that 3D culture provides results that closely mimic natural tissues as compared to those of two-dimensional (2D) culture for accurate modeling of living organisms[1] and analysis of medicines[2,3]. In dentistry, 3D cultures can be used to assess biocompatibility of dental materials[4,5] and for regeneration of pulp[6] and periodontal ligament[7], using scaffolds[8]. However, hypoxia, promotion of calcification in the spheroid structure[9], and a decrease in viability due to a decrease in oxygen and nutrient supply can occur due to the scaling-up of the 3D structure[10]. Therefore, there may be underestimation or overestimation of the results when evaluating drugs and materials. In view of these facts, we wondered if it would be possible to perform 3D culture that maintains a specified thickness that does not cause hypoxia without using a scaffold and that focuses on cell sheet engineering. Cell sheet engineering refers to the use of a temperature-responsive culture dish (UpCell) to collect cell sheets that retain extracellular matrix without using enzymes such as trypsin from monolayer culture, and to construct 3D structures by layering these cell sheets[11]. It is possible to collect cell sheets that retain the binding and extracellular matrix and stack these cell sheets to construct a 3D structure[11]. In recent years, cell sheet technology has been clinically tested in humans[12,13]. However, there are few studies on the effects of calcification and hypoxia in cell sheets that retain the specified thickness by multi-layering of dental pulp cell (DPC) sheets using UpCell. Therefore, we used a gelatin stamp (CellStamp for UpCell) to stack cell sheets and analyze the characteristics of DPC sheets for application in pulp regeneration and evaluation of dental materials and pharmaceuticals.

Materials and Methods

1. Cell isolation and culture

In this study, DPC was collected from three third molars of three healthy patients (18–30 years old) who had undergone orthodontic treatment, following a protocol approved by the Institutional Review Board of The Nippon Dental University, School of Life Dentistry at Tokyo with informed consent. DPC was cultured according to the method described by Huang et al[14]. Pulp tissues were minced and digested in a solution containing 3 mg/m*l* collagenase type I (Sigma, St. Louis, MO, USA) and 4 mg/m*l* dispase (Gibco, Invitrogen, Carlsbad, CA, USA) at 37℃ for 60 minutes. Cell suspensions were obtained using a 70 μm strainer and then seeded in 60 mm culture dishes at 37℃ under 5% CO_2 and cells with passage numbers of 3–6 were used in all experiments. The growth medium used was as follows; minimum essential medium α (MEM-α, Gibco), 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 units/m*l* penicillin G, 100 μg/m*l* streptomycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 2 mmol/*l* L-glutamine (Wako), and 100 μmol/*l* L-ascorbicacid-2-phosphate (Sigma). The osteo/odontogenic differentiation medium (OM) used was as follows: 10 nmol/*l* dexamethasone (Sigma), 10 mmol/*l* β-glycerophosphate (Sigma), 50 μg/m*l* L-ascorbic acid phosphate (Sigma), and 10 nmol/*l* 1,25 dihydroxyvitamin D3 (Cayman Chemical, Ann Arbor, MI, USA).

2. Laminating procedure for the DPC sheets

DPC sheets were laminated by a modified procedure of the method described by Haraguchi et al[15] and the number of seeded cells was determined with reference to the manufacturer's protocol. DPC was seeded at a cell density of 1.5×10^5/well into a 24-well UpCell (CellSeed, Tokyo, Japan) and incubated for 5 days in GM, then the 7.5% gelatin stamp (CellStamp for UpCell; CellSeed, Tokyo, Japan) was placed in the well and left at 25℃ for 30 minutes. The cell sheets were transferred to a gelatin gel, which was then transferred to the next well. This process was repeated to create multiple layers. After the stacking was complete, the gelatin gel was heated at 37℃ for 30 min to dissolve the gelatin and was washed three times in the medium (Fig. 1).

3. Observation by hematoxylin and eosin (HE) staining

The monolayer, three-layer, and six-layer cell sheets in the GM were cultured on a polytetrafluoroethylene membrane for 3 days and fixed with 10% neutral formalin. Then, the cell sheets were embedded with optimal cutting temperature (OCT) frozen compound and a 7 μm section was cut from the OCT blocks and stained with HE. HE was observed using an optical microscope.

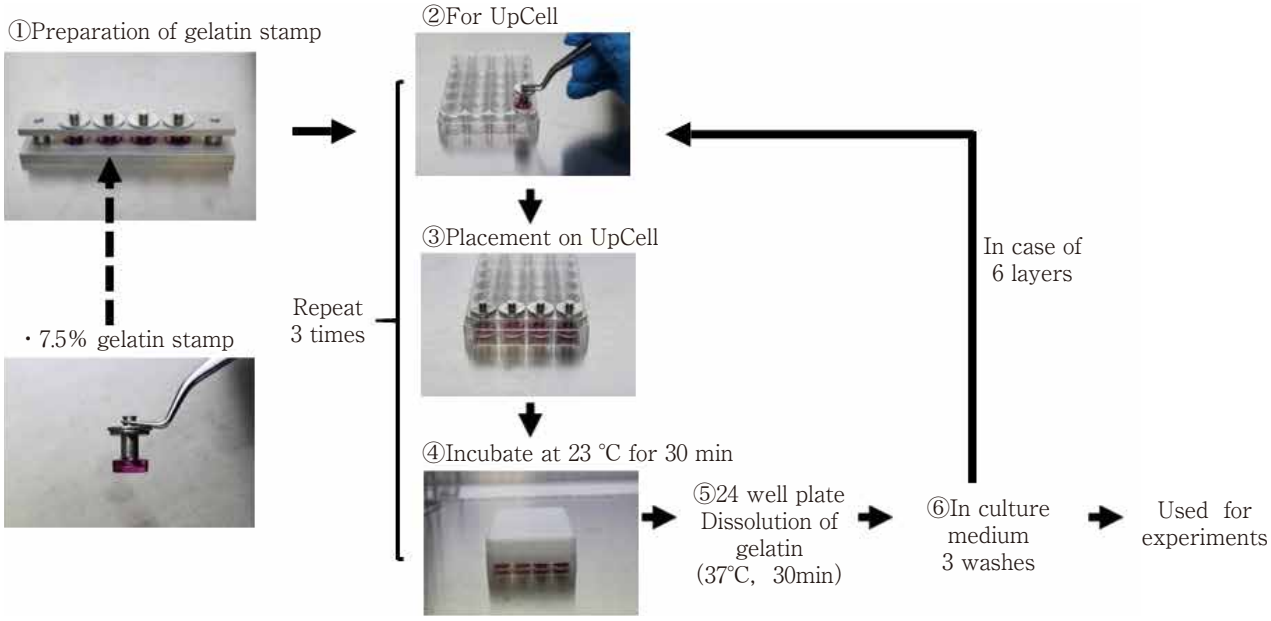

Fig. 1 Procedure for laminating cell sheets

①Gelatin gel (7.5%) was prepared by molding and placed on a 24-well UpCell plate. ②-④ : Cells were attached to the bottom of the gelatin stamp at 23℃ for 30 min, and the process was repeated three times to make three-layer sheets. Six-layer sheets were prepared by layering a three-layer sheet on top of another three-layer sheet after dissolving the gelatin. ⑤Gelatin was dissolved by heating at 37℃ for 30 min in the 24 well plate. ⑥Cell sheets were washed 3 times with GM.

4. Observation by scanning electron microscopy (SEM)

The monolayer, three-layer, and six-layer cell sheets in the GM were cultured on MTA (ProRoot MTA; Densply Tulsa Dental Specialties, Johnson City, TN, USA) with a diameter of 6 mm for 3 days. The samples were fixed with 2.5% glutaraldehyde solution for 30 min and post-fixed with 1% osmium tetroxide for 2 h at room temperature. The samples were then dehydrated with 50, 70, 80, 90, 95%, and 99.5% ethanol for 10 min, followed by replacement with t-butanol and freeze-drying. The samples were observed using SEM.

5. Alizarin Red S staining

The monolayer, three-layer, and six-layer cell sheets were cultured in the OM for 21 days, washed with phosphate-buffer solution, and fixed with 10% neutral formaldehyde at room temperature for 15 min. After washing twice with dH_2O, 300 m*l*/well of 40 mmol/*l* ARS (pH 4.1) was added, incubation at room temperature was performed for 20 min, then the wells were washed with 1 m*l* dH_2O 4 times for 5 min each. The samples were observed using an optical microscope and macroscopic views. Three random points on the well were measured by ImageJ software to determine the mean size of calcification (n=3).

6. Analysis of hypoxia

Monolayer, three-layer, and six-layer cell sheets were cultured in the OM for 3 days, and the hypoxia probe solution LOX-1 (Medical & Biological Laboratories, Aichi, Japan) was used in the medium at a final concentration of 2 μ/*l*. The cells were observed under a fluorescence microscope (Carl Zeiss LSM 700, Oberkochen, Germany).

7. Real-time polymerase chain reaction (PCR) analysis

Monolayer, three-layer, and six-layer sheets were cultured in the GM and collected at 3, 7, and 14 days. Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and Maxwell RSC simplyRNA Cells Kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Complementary DNA synthesis was performed from total RNA (1 μg) using SuperScript VILO Master Mix (Life Technologies, Foster City, CA, USA). Real-time PCR was performed with a THUNDERBIR Probe qPCR Mix (TOYOBO, Osaka, Japan) using a StepOnePlus real-time PCR system (Applied Biosystems Waltham, Massachusetts, USA) under the follow-

Table 1 Sequences of human primers used for real-time PCR

Genes	Upper primers	Lower primers
ALP	5′-TCCCTGATGTTATGCATGAGC-3′	5′-CGAGAGTGAACCATGCCA-3′
OCN	5′-CGCCTGGGTCTCTTCACT-3′	5′-CTCACACTCCTCGCCCTAT-3′
COL1A1	5′-TTCTGTACGCAGGTGATTGG-3′	5′-GACATGTTCAGCTTTGTGGAC-3′
DSPP	5′-TGACACATTTGATCTTGCTAGGA-3′	5′-TTTGGGCAGTAGCATGGG-3′

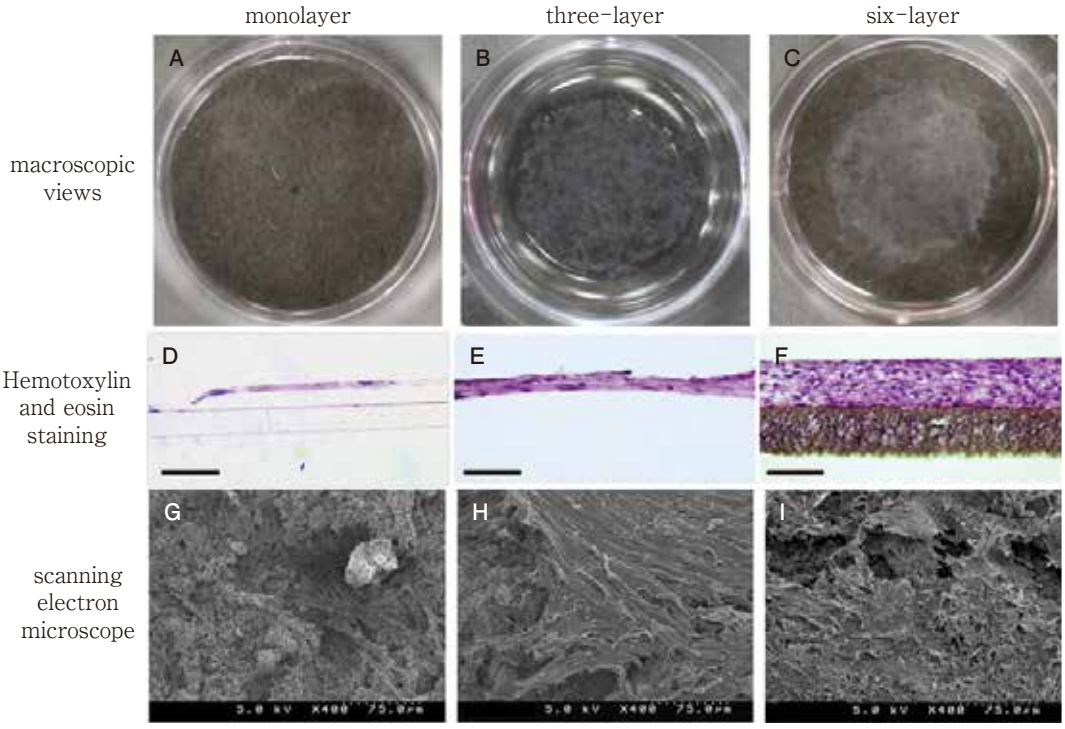

Fig. 2 Histological and SEM findings of cell sheets

Macroscopic views of the monolayer (A), three-layer (B), and six-layer (C) cell sheets. Hemotoxylin and eosin staining of the monolayer (D), three-layer (E), and six-layer (F) cell sheets. Scanning electron microscope view of the monolayer (G), three-layer (H), and six-layer (I) cell sheets.

Scale bar=50 μm

ing conditions: 95℃ for 3 min, followed by 40 cycles at 95℃ for 15 s. The primers for gene amplification were subjected to 40 cycles at 60℃ (45s) for ALP, COL1A1, and OCN and at 62℃ (45s) for DSPP. *β-actin* (NM_001101.2) was used as an endogenous control. The primer sequences used are shown in Table 1.

8. Statistical Analysis

Statistical significance was analyzed using one-way analysis of variance followed by Tukey's test. Data were expressed as mean±standard deviation, and $p<0.05$ was considered statistically significant.

Results

1. Histological analysis

The macroscopic view revealed membranous cell sheets with a thickness of three and six layers. The thickness of the monolayer, three-layer, and six-layer cell sheets, as seen with HE staining, was approximately 7 μm, 25 μm, and 45 μm, respectively. For SEM, when the cell suspension was seeded on the sample, the cells were scattered and attached to the MTA, but on the cell sheets, an image covering the sample was observed (Fig. 2).

2．Analysis of hypoxia

The monolayer cell sheets showed a few LOX-1 positive cells. More hypoxic cells were observed in the six-layer cell sheets than in the monolayer and three-layer cell sheets (Fig. 3).

3．Alizarin Red S staining

Several nodules of mineralization were observed in the three-layer and six-layer cell sheets but not in the monolayer sheets. In addition, the nodules of mineralization were larger in the six-layer cell sheets (1,573.4±669.1 μm^2) than in the three-layer sheets (895.4±269.7 μm^2) (Fig. 4).

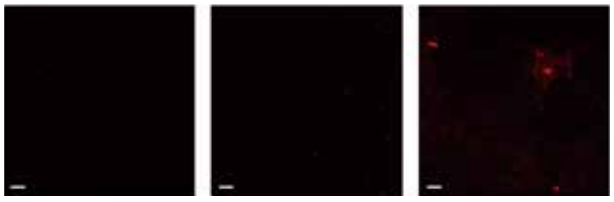

Fig. 3　Analysis of hypoxia

24-well Viewed from above hypoxia was detected with the LOX-1 probe in the monolayer, three-layer, and six-layer cell sheets.

Scale bar＝100 μm

4．Real-time PCR

The expression of *ALP* was upregulated in the monolayer, three-layer, and six-layer cell sheets at day 7 and downregulated at day 14, but tended to be significantly higher in the three-layer and six-layer cell sheets than in the monolayer sheets ($p<0.05$). The expression of *COL1A1* was upregulated in the monolayer, three-layer, and six-layer cell sheets on day 7 and was significantly higher in the six-layer cell sheets than in the monolayer sheets. The expression of *OCN* was upregulated in the monolayer, three-layer, and six-layer cell sheets at day 14 and was significantly higher in the three-layer ($p<0.05$) and six-layer ($p<0.05$) cell sheets than in the monolayer sheets. The expression of *DSPP* was upregulated in the monolayer, three-layer, and six-layer cell sheets at day 14 and was significantly higher in the six-layer cell sheets than in the monolayer sheets ($p<0.05$) (Fig. 5).

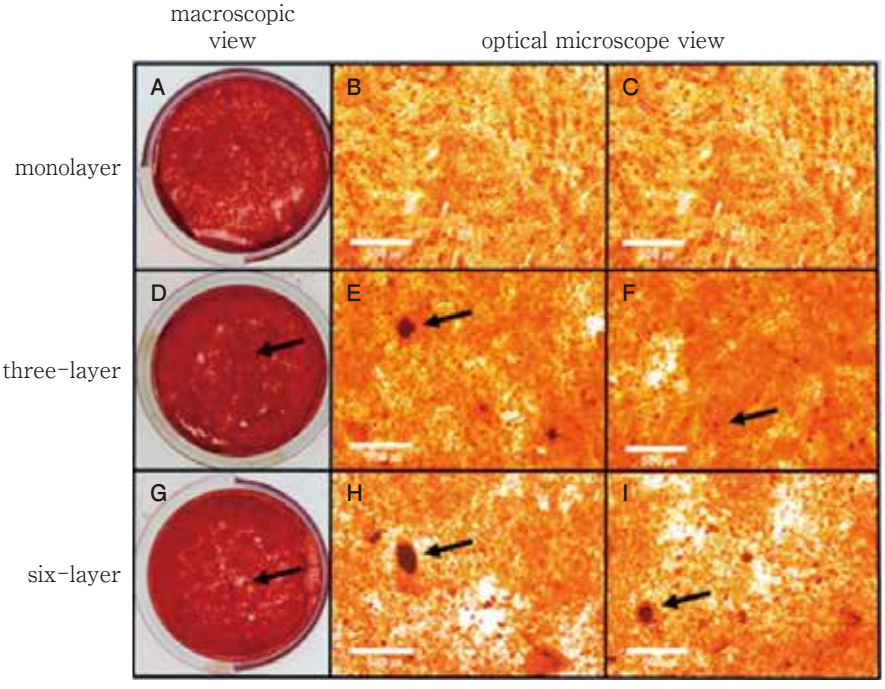

Fig. 4　Macroscopic and optical views

Macroscopic view of Alizarin Red S staining of the monolayer (A), three-layer (D), and six-layer (G) cell sheets in the 24-well plate. Optical microscope view of the monolayer (B, C), three-layer (E, F), and six-layer (H, I) cell sheets. Nodules of mineralization (indicated with black arrows) can be observed in relation to the three-layer and six-layer cell sheets.

Scale bar＝500 μm

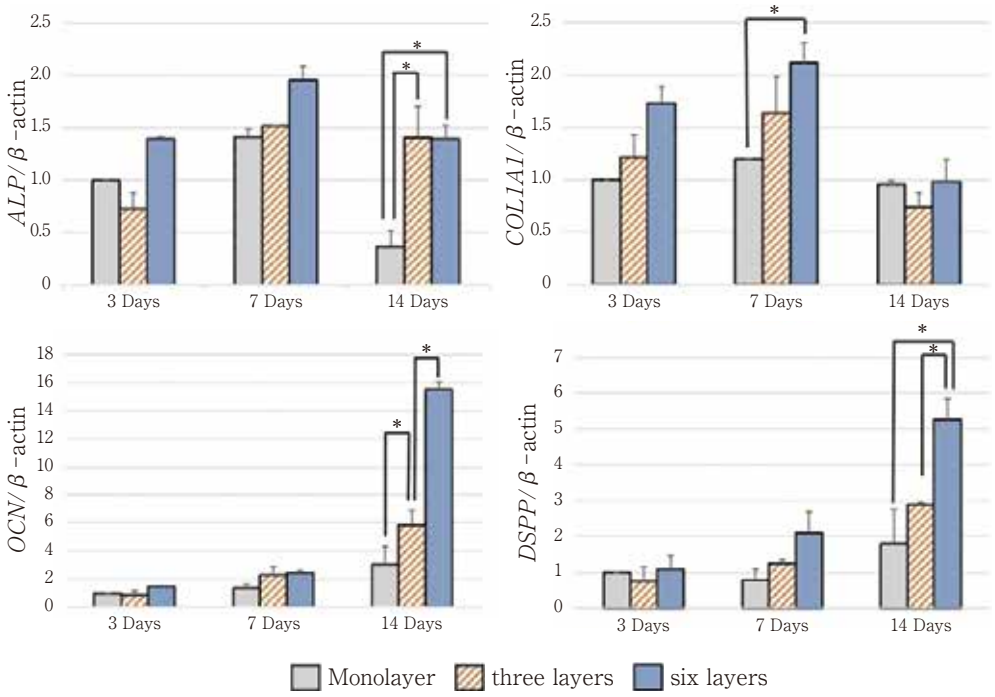

Fig. 5 Real-time PCR analysis of mineralization related marker genes

Real-time polymerase chain reaction was performed for *ALP*, *COL1A1*, *OCN*, and *DSPP* (n=3). The results of two independent experiments performed in triplicate are expressed as the mean (±SD) (＊：$p<0.05$).

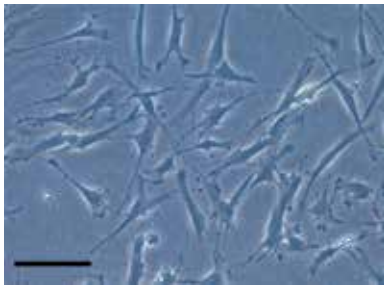

Fig. S1 Phase contrast image of DPC

Scale bar=100 μm

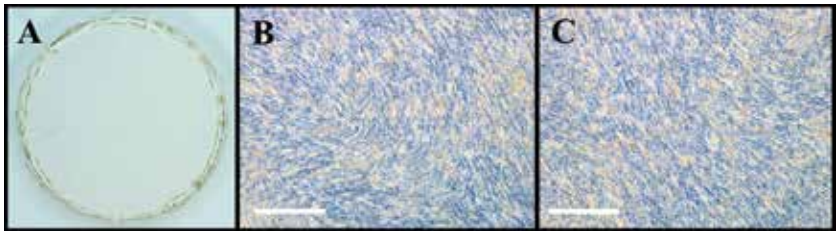

Fig. S2 Macroscopic and optical views

Macroscopic view of ARS of the monolayer (A) was cultured in the GM for 21 days. Optical microscope view of the monolayer (B, C). Calcification was not observed (Scale bar=500 μm).

Discussion

In the present study, it was possible to fabricate cell sheets to form in membranous by multilayering, as shown by Kikuchi et al[16]. In addition, some studies on MTA have reported the necessity of direct contact between cells and MTA for their differentiation into odontoblast-like cells[17]. Based on these studies, if it is possible to cover the MTA as shown by SEM, it may be possible to analyze the interaction with cells that occurs in the surface layer of the material in more detail and to study the material properties in an environment similar to that *in vivo*. Considering the mineralization-associated marker gene, after the initial expression of calcification and in *ALP*, which is an important factor for mineral deposition[18], downregulation was observed after 14 days in the monolayer sheets, while the levels were high in the three-layer and six-layer sheets, which may have been due to the occurrence of heterogeneous calcified areas within the sheets. *COL1A1*, a major collagen in dentin and bone matrix, also showed a similar trend. The expression of *OCN*, a non-collagenous protein and a marker of osteo/odontogenic differentiation, and *DSPP*, a representative marker of odontogenic differentiation, was also higher in the multilayered sheets. The expression trends of the markers were consistent with those seen in previous studies[19]. The above-described upregulation of the mineralization-related marker and increased nodular calcification seen with ARS were observed, especially in the six-layer sheets. This may have been due to the thickness of the cells (as seen with HE), causing the expansion of hypoxic areas and the upregulation of mineralization-associated marker expression[20] induced by localized hyper-calcified areas and hypoxic areas in ARS. However, when considered in terms of materialogical characterization, since many dental materials are often compared with cells for their odontogenic induction[21], a comparative study of calcification-related factors using six-layer sheets may result in an overestimation of calcification. Therefore, we hypothesized that a 3D culture with about three layers would not be affected by scaffolding or hypoxia and would allow us to perform comparative material studies in a 3D environment similar to that in vivo. In addition, there is a correlation between LOX-1 and hypoxia-inducible factor-1α (HIF-1α)[22]. The effect of hypoxia on cells may cause activation of angiogenesis[23] by inducing vascular endothelial growth factor (VEGF) expression related to HIF-1α, which may be advantageous for pulp regeneration by transplantation. The above results suggest that cell sheet technology may be further developed by changing the application depending on the thickness of the cell sheets.

Note that there were some limitations to this study. We were not able to produce stable cell sheets. Therefore, it is necessary to consider a more appropriate protocol.

In the future, we plan to conduct a more detailed study to investigate the material science of multilayered cell sheets with DPC for regeneration of dental pulp.

Conclusion

DPC sheets possessed higher mineralizing ability than monolayer sheets. Cell sheets may be useful for comparative examination of pharmaceuticals, materials, and pulp regeneration in a 3D environment.

The authors have no conflicts of interest directly relevant to the content of this article.

Supplemental Date

DPC image (Fig. S1) and ARS staining control (Fig. S2) were added as Supplemental Date.

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