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Original Article

Stable preparation of *in vivo* transplantable periodontal ligament-derived mesenchymal stem cell sheets in thermoresponsive culture dishes with tunable cell detachability

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A B S T R A C T

Tissue engineering plays a pivotal role in the advancement of regenerative medicine. Thermoresponsive culture dishes, coated with specialized polymers that control cell adhesion through temperature fluctuations, enable the processing of cells into sheets for medical applications while maintaining their intact state. Cell sheets prepared using these culture dishes have been incorporated into several commercial pharmaceutical products. However, controlling the detachability of cell sheets using conventional thermoresponsive culture dishes remains a challenge, and often leads to unexpected detachment during cultivation. In this study, we developed a thermoresponsive culture dish with tunable cell detachability using a thermoresponsive block copolymer, poly(butyl methacrylate)-*b*-poly(*N*-isopropylacrylamide) (PBMA-PIPAAm), which is a specialized polymer that allows precise control of the amount of surface-immobilized polymer and polymer layer thickness. Culturing periodontal ligament-derived mesenchymal stem cells on these dishes demonstrated fully tunable detachability without compromising cell properties compared to conventional thermoresponsive dishes (UpCell®). Thermoresponsive PBMA-PIPAAm-coated culture dishes enable the complete on-demand detachment of transplantable cell sheets, thereby avoiding unexpected detachment that may increase production costs and reduce technical hurdles in the manufacturing process. The PBMA-PIPAAm coating method has the potential to contribute to biomedical and clinical applications of mesenchymal stem cell sheets.

Abbreviations: ATR/FT-IR, attenuated total reflection-Fourier transform infrared spectrometer; DPBS, Dulbecco's phosphate-buffered saline; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FDR, false discovery rate; FITC, fluorescein isothiocyanate; HE, hematoxylin-eosin; $\log_2$ FC, $\log_2$ fold change; MSC, mesenchymal stem cell; PBMA-PIPAAm, poly(butyl methacrylate)-*b*-poly(*N*-isopropylacrylamide); PCA, principal component analysis; PDL-MSC, periodontal ligament derived mesenchymal stem cell; PE, phycoerythrin; PBMA, poly(butyl methacrylate); PIPAAm, poly(*N*-isopropylacrylamide); SCID, severe combined immunodeficient; TCPS, tissue culture-treated polystyrene.

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1. Introduction

Cell-based regenerative medicine enables the regeneration and functional recovery of lost tissues and organs, providing transformative effects that are difficult to achieve using conventional pharmaceuticals. Cell sheets have demonstrated remarkable therapeutic outcomes in fields such as ophthalmology [1], dermatology [2], and cardiovascular surgery [3] and have emerged as a pivotal dosage for regenerative medicine. Cell-based regenerative medicine products, particularly those utilizing cell sheets, have received regulatory approvals in the USA and Japan. In the USA, three out of 17 FDA-approved cellular therapy products employ cell sheet technology (https://www.fda.gov/). Similarly, in Japan, five out of ten products utilize this technology (https://www.pmda.go.jp/). In the field of periodontal regeneration, we have prepared human periodontal ligament-derived mesenchymal stem cell (hPDL-MSC) sheets using thermoresponsive culture dishes and demonstrated their safety and efficacy in regenerating alveolar bone defects caused by periodontal diseases [4]. These findings underscore the recognition of cell sheets as a viable and effective approach in regenerative medicine.

Tissue engineering plays a significant role in the creation of cell sheets and is essential for their preparation. The immobilization of poly(*N*-isopropylacrylamide) (PIPAAm), which exhibits reversible hydrophilic-hydrophobic switchable properties in response to temperature changes, has enabled the production of thermoresponsive culture dishes, allowing for the non-enzymatic production of cell sheets containing the extracellular matrix (ECM) [5,6]. However, the preparation of cell sheets remains challenging because of the sensitivity of the required culture techniques, which poses long-standing issues related to operability and production efficiency. In cell cultures on thermoresponsive culture dishes, the unexpected detachment of cell sheets occurs during manufacturing due to sharp changes in temperatures during medium changes, leading to unintended detachment upon minor impacts. Manufacturing failures arising from the unexpected detachment of cell sheets lead to increased production costs and, more critically, an inability to deliver planned medical treatments, posing a significant disadvantage in the development of regenerative medical products.

To resolve these issues, we developed a novel thermoresponsive culture dish called Smart Surface Cultureware (SSCW®) via nanocoating poly(butyl methacrylate)-*b*-poly(*N*-isopropylacrylamide) block copolymers (abbreviated as PBMA-PIPAAm) on various culture substrates [7]. The PBMA block strongly supports the water-stable surface adsorption of the thermoresponsive poly(*N*-isopropylacrylamide) (PIPAAm) block, regardless of temperature [7]. In addition, SSCW® technology demonstrates that the feature of cell detachability can be controlled by changing the amount, chain length, and thickness of PIPAAm-based layers on the culture surfaces [6,8]. However, the applicability of SSCW® technology to hPDL cells has not yet been thoroughly investigated.

In this study, we investigated thermoresponsive culture dishes with tunable cell detachability for the stable preparation and recovery of transplantable cell sheets. Herein, the controlled detachability and *in vivo* transplantation quality of hPDL cell sheets using SSCW® with PBMA-PIPAAm layer were investigated in comparison with a commercial thermoresponsive culture dish, UpCell®, utilized in our previous clinical trials [4].

2. Materials and methods

2.1. Ethics statement

This study was conducted in accordance with the principles outlined in the Declaration of Helsinki and approved by the Institutional Review Board of Tokyo Medical and Dental University Human Subjects Research (D2020-077). All patients and guardians were fully informed and provided written consent for the donation of their teeth and their subsequent use in this research project.

All experimental protocols involving animal use and euthanasia were approved (A2021-184C3 and A2023-036C2) by the Animal Care Committee of the Experimental Animal Center at Tokyo Medical and Dental University. Compliance with the Cartagena Act was confirmed by the Genetically Modified Organisms Safety Committee of Tokyo Medical and Dental University (approval no. 12076-001).

2.2. Preparation and characterization of SSCW® dishes

PBMA-PIPAAm block copolymer (the molecular weights of PBMA and PIPAAm were 15,000 and 40,000, respectively) was obtained using a previously reported method with slight modifications [6,8]. SSCW® dishes were prepared by spin-coating the PBMA-PIPAAm solution on a tissue culture-treated polystyrene (TCPS) dish (35 mm in diameter) (430165; Corning, NY, USA) at 5000 rpm for 10 s (Fig. 1A) [8]. The amount of surface-immobilized PIPAAm segments and the thickness of polymer layers were varied by changing the polymer concentration (T1: 0.25 wt/v%, T2: 0.3 wt/v%, T3: 0.4 wt/v%, and T4: 0.45 wt/v%).

The number of immobilized thermoresponsive PIPAAm segments was determined using an attenuated total reflection-Fourier transform infrared spectrometer (ATR/FT-IR) (NICOLET 6700 FT-IR; Thermo Fisher Scientific, NY, USA) equipped with a germanium ATR crystal (Harrick Scientific, NY, USA). The intensity ratio of the peak derived from the polystyrene base material (1602 cm^{-1}) and the peak of isopropylacrylamide (IPAAm) derived from the block copolymer (1648 cm^{-1}) was used to determine the amount of PIPAAm on the polystyrene surface using a calibration curve [6]. The thicknesses of the coated polymer layers in the dry state were estimated using OPTM-A1 (Otsuka Electronics, Osaka, Japan) at a wavelength of 230–800 nm.

2.3. Cell culture

hPDL cells were obtained and prepared as previously described [9] and exhibited the hMSC phenotype defined by Dominici et al. [10]. Briefly, periodontal ligament tissues were isolated from the mid-third of extracted human teeth and dispersed in alpha-minimum essential medium (α-MEM) with GlutaMAX (Thermo Fisher Scientific, MA, USA) containing 1 % sulbactam/ampicillin (UNASYN-S KIT; Pfizer, Tokyo, Japan), 1200 PU/mL dispase (Sanko Junyaku, Tokyo), and 0.8 PZ-U/mL collagenase type 1 (SERVA Electrophoresis, Heidelberg, Germany). The resulting single-cell suspension was plated in a T25 Primaria culture flask (Falcon, NY, USA; Passage 0), and cultured in complete medium, consisting of α-MEM supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin containing 10 % fetal bovine serum (FBS), at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. The medium was changed twice per week, and the cells were passaged using 0.25 % trypsin-EDTA (Thermo Fisher Scientific). hPDL cells at the seventh passage were used for experiments.

2.4. Cell proliferation assay

To evaluate cellular proliferation at 24, 48, and 96 h after seeding, hPDL cells were plated at a density of 3×10^4 cells per dish on eight different types of cell culture surfaces (35 mm UpCell® (Cell Seed, Tokyo), TCPS, T1, T2, T3, and T4). hPDL cells were cultured in complete medium throughout the experiment. Cell

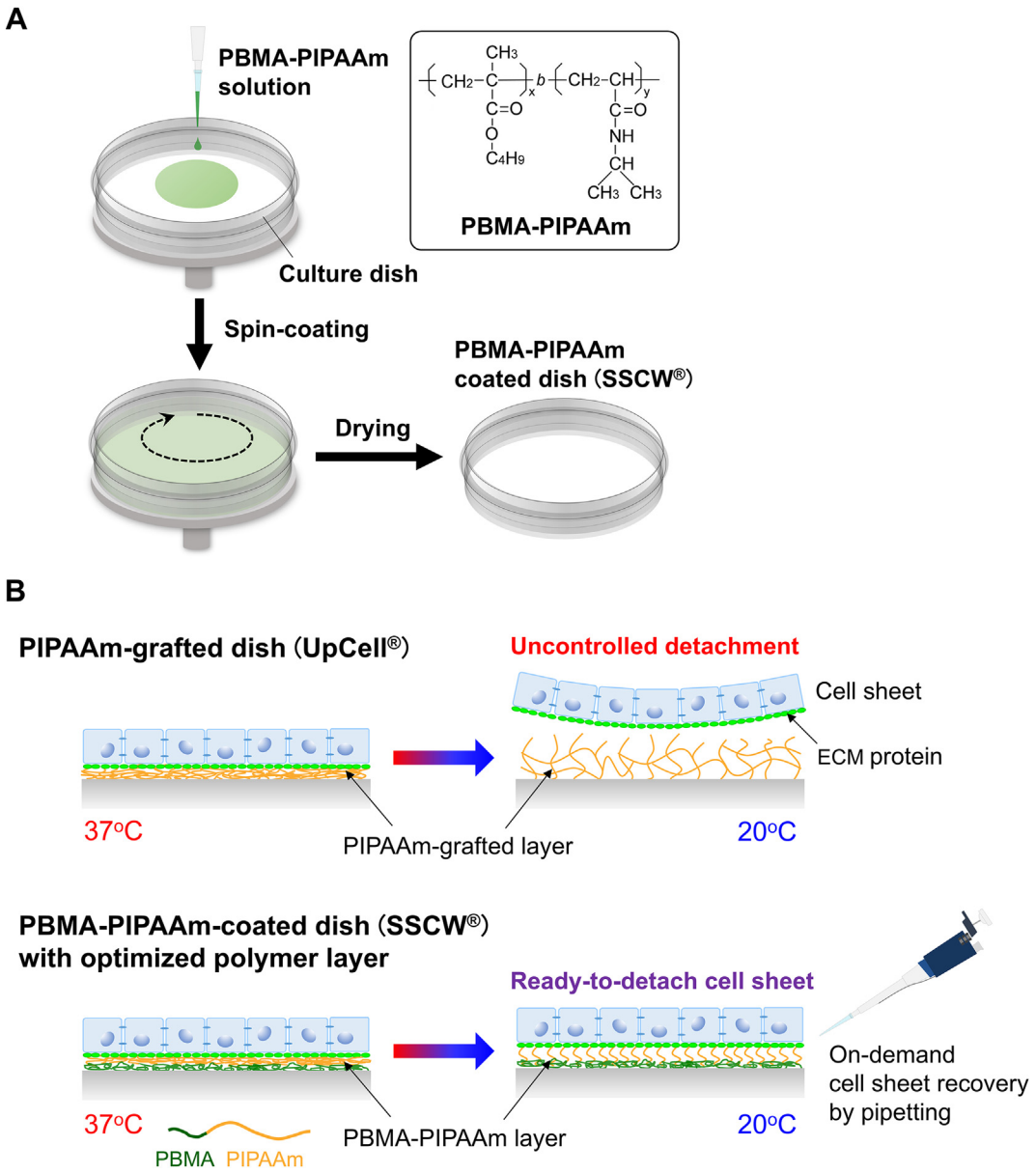

Fig. 1. Schematic representation of the mechanics behind thermoresponsive cell culture dishes. (A) The chemical structure of PBMA-PIPAAm and the process used to fabricate PBMA-PIPAAm coated dishes (SSCW®). (B) Schematic illustration of on-demand cell sheet manipulation using PBMA-PIPAAm-coated dish (SSCW®) compared with conventional PIPAAm-grafted dish (UpCell®).

proliferation was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) at 24, 48, and 96 h post-seeding, according to the manufacturer's protocol (reaction time: 30 min). The optical absorbance of the samples was measured at 450 nm using a microplate reader (SpectraMax ABSPlus; Molecular Devices, CA, USA). The relative cellular proliferation levels were expressed as the ratio of the proliferative activity of cells on each surface to that on TCPS at 24-h time point.

2.5. hPDL cell sheets

To obtain hPDL cell sheets, hPDL cells were seeded onto two different kinds of thermoresponsive dishes (UpCell® and SSCW®) at a density of 5×10^4 cells per dish and cultured with complete culture medium at 37 °C. After 2 days, the culture medium was changed to osteogenic induction medium, consisting of complete culture medium supplemented with 73.1 μg/mL L-ascorbic acid phosphate magnesium salt n-hydrate (FUJIFILM Wako, Osaka), 10 nM dexamethasone (DEXART; Fuji pharma, Toyama, Japan), and 10 mM β-glycerophosphate (Sigma-Aldrich, MO, USA). After an additional 12 days of culture, the hPDL cell sheets were harvested using enzyme-free methods. Two harvesting methods were employed: waiting for spontaneous detachment and applying water flow by pipetting. For spontaneous detachment, the edge of the dish culturing hPDL cells was cut with fine tweezers and left to stand at room temperature (20 °C) for several tens of minutes. For water flow detachment, hPDL cell sheets were physically detached using a P1000 micropipette. The detached hPDL cell sheets were fixed in 4 % paraformaldehyde (Muto Pure Chemicals, Tokyo) at 4 °C for 15 min, routinely processed into 6-μm thick paraffin-embedded sections, stained with hematoxylin–eosin (HE).

2.6. Cell count of PDL cell sheets

hPDL cell sheets are resistant to enzymatic dissolution because of their rich ECM. To accurately count the cells, a combined treatment method with collagenase and trypsin-EDTA was used with slight modifications, as previously described [11]. Briefly, an hPDL cell sheet was incubated with 440 μL of collagenase (NB4 standard grade; SERVA Electrophoresis) diluted in serum-free medium to a PZ activity of 0.9 IU/mL at 37 °C for 15 min. The suspension was then agitated by pipetting to completely dissolve the cell sheet, after which 400 μL of 0.25 % trypsin-EDTA (Thermo Fisher Scientific) was added. After treatment for 5 min at 37 °C and dispersion by pipetting, the cell suspension was washed with 2 mL of Dulbecco's phosphate-buffered saline without $CaCl_2$ and $MgCl_2$ (DPBS; FUJIFILM Wako) containing 10 % FBS and then centrifuged. The supernatant was discarded and the resulting hPDL cell pellet was resuspended in 1 mL of DPBS containing 10 % FBS. The number of cells was determined using the cell suspension, which was stained with 10 μL of trypan blue (Sigma-Aldrich), and viable (unstained) cells were counted.

2.7. Preparation of the total RNA library and sequencing

For these studies, hPDL cells were isolated from four different subjects and cultured using two different methods: (1) hPDL cells were cultured in complete medium for 4 days on TCPSs (undifferentiated condition); (2) hPDL cells were cultured in complete medium for 2 days, followed by a switch to osteogenic induction medium for 12 days on each type of culture surfaces (TCPS, UpCell®, and SSCW®). Total RNA was purified and concentrated using the RNeasy Mini Kit (Qiagen, CA, USA). The purified total RNA was used to construct a library with the NEBNext® Ultra™ II Directional RNA Library Prep Kit (Illumina, CA, USA), which was subsequently sequenced using the NovaSeq 6000 platform (Illumina). All procedures were performed according to the manufacturer's instructions.

2.8. Whole-transcriptome analysis

The NovaSeq 6000 platform generated approximately 100 million pair-ended 150 bp reads from 16 samples, comprising hPDL cells isolated from four different subjects, with RNA samples taken from four experimental groups. Low-quality adapter sequences were trimmed from paired-end reads using Trimmomatic v0.39. Trimmed reads were aligned to the human reference genome (GRCh38) using HISAT2 v2.2.1. Transcript assembly and abundance estimation based on GRCh38 gene annotations were performed using StringTie v2.2.1. Differential expression analysis was conducted using the “DESeq2” v1.34.0 package for R.

2.9. Differentiation assay

To evaluate the osteogenic differentiation potential of hPDL cells on different culture surfaces, 5×10^4 cells were seeded on each dish (TCPS, UpCell®, and SSCW®) and cultured in osteogenic induction medium for 21 days. The medium was changed every three days, and differentiation properties were assessed using Alizarin Red S staining (FUJIFILM Wako) following brief fixation with 4 % paraformaldehyde for 15 min at 4 °C.

To evaluate adipogenesis differentiation potential, 5×10^4 cells were seeded on each dish (TCPS, UpCell®, and SSCW®) and cultured in adipogenic medium, consisting of complete medium supplemented with 100 nM dexamethasone (Fuji Pharma), 0.5 mM isobutyl-1-methylxanthine (Sigma-Aldrich), and 50 mM indomethacin (FUJIFILM Wako) for 21 days. The medium was changed every three days, and differentiation properties were evaluated using Oil Red O staining (FUJIFILM Wako) following brief fixation by 4 % paraformaldehyde for 15 min at 4 °C.

2.10. Flow cytometry

The PDL cell sheets were dissociated into single cells using the method described above. After surface staining with fluorescent-conjugated antibodies against hALPL (phycoerythrin (PE)) (FAB1448P; R&D Systems, MN, USA) and CD105 (fluorescein isothiocyanate (FITC)) (152905; Ancell Corporation, MN, USA) diluted in staining buffer (2 % FBS/DPBS), at 4 °C for 30 min. The cells were then washed and resuspended in staining buffer. As an isotype control, PE-conjugated non-specific mouse IgG1κ (IC002; R&D Systems) and FITC-conjugated non-specific mouse IgG1κ (555748; Becton Dickinson, NJ, USA) were used in the place of the primary antibodies. Each sample was routinely stained with 7-Amino-Actinomysin D (7-AAD) (559925; Becton Dickinson) to discriminate dead cells and subsequently analyzed using Attune NxT (Thermo Fisher Scientific). Data were processed and analyzed using the FlowJo Software V10 (Becton Dickinson).

2.11. Cell sheet transplantation with dentin disk in immunodeficient rat models

To determine whether the cell sheets remained *in vivo*, hPDL cell sheets combined with dentin disks were implanted subcutaneously into the parietal bone of severe combined immunodeficient (SCID) rats (F344/Il2rg knockout, 7-week-old male; RIKEN BRC, Tokyo, Japan). After culturing for 2 weeks, the hPDL cell sheets were sectioned into quarters and harvested using a non-enzymatic method. The rats were anesthetized via intraperitoneal injection of ketamine and xylazine. Subcutaneous incisions were made on the head surface, and hPDL cell sheets and dentin disks (048-32171; FUJIFILM Wako) were placed under the subcutaneous tissue (Fig. 6A, Supplementary Fig. 2). Four weeks post-implantation, the rats were euthanized and the samples were excised. The samples were fixed in 4 % paraformaldehyde, decalcified in 10 % EDTA disodium salt solution (pH 7.0) (20251; Muto Pure Chemicals) for 6 weeks, processed into 6-μm thick paraffin-embedded sections, and stained with HE.

2.12. Immunostaining for human periostin

For immunostaining, 6-μm thick paraffine-embedded sections were deparaffinated using the routine process. After washing with distilled water, slides holding the tissue sections were pretreated with 10 mM citrate buffer (pH 6.0) at 90 °C for 15 min for antigen retrieval. Sections were washed with 0.1 % Tween-20 (P1379-500 ML; Sigma-Aldrich) with PBS (PBT) for 5 min. Endogenous peroxidase activity was quenched by treatment with BLOXALL Blocking Solution (PK-6101; Vector Laboratories, CA, USA). Next, the sections were incubated for 20 min at room temperature in 2.5 % normal horse serum/PBS (PK-6101; Vector Laboratories) to block non-specific antibody binding. Subsequently, the sections were incubated overnight at 4 °C with anti-human periostin rabbit monoclonal antibody (1:2000, ab219057; Abcam, Cambridge, UK). The VECTASTAIN Universal Elite ABC PLUS Kit (Vector Laboratories) was used for secondary antibody and ABC reactions according to the manufacturer's protocol. After washing, visualization was performed using ImmPACT™ DAB Eqv Regent (Vector Laboratories). Sections were counterstained with hematoxylin and mounted. Negative controls for immunostaining were prepared by omitting the primary antibody or using a sham control without hPDL cell sheets. (Supplementary Fig. 1B).

2.13. Statistical analysis

The values presented in the graphs are expressed as mean ± standard deviation of the mean (SDM) from at least three independent experiments. Statistical comparisons between samples were performed using the paired Student's t-test, with differences considered statistically significant at $P < 0.05$.

3. Results

3.1. Optimal amount of PBMA-PIPAAm for PDL cell sheet preparation

To obtain thermoresponsive culture dishes with controlled cell detachability, TCPS dishes coated with various PBMA-PIPAAm layers (SSCW® sample, T1–T4) were prepared by changing the concentration of the PBMA-PIPAAm solution. The number of PIPAAm segments and the thickness of the spin-coated PBMA-PIPAAm layers were well controlled depending on the polymer concentration (Fig. 2A and B). To determine the optimal coating condition for cultivating PDL cells, the cell proliferation profiles on four different types of SSCW® were compared with non-polymer-coated TCPS and UpCell®. After seeding the cells on each dish, CCK-8 assays were conducted at 24, 48, and 96 h to estimate total cell activity. The results showed no significant difference in cell proliferation rates between UpCell® and SSCW® sample T1 (PIPAAm amount: 1.74 μg/cm^2 and thickness of polymer layer: 8.5 nm). The TCPS dishes exhibited significantly higher cell proliferation rates than the other culture dishes, while no significant differences were observed between UpCell® and T1 (Fig. 2C). Representative phase-contrast images of each culture dish on day 14 are shown in Fig. 2D. Insufficient space for cell proliferation was observed in dishes T3 and T4 (Fig. 2C, yellow arrows). Consequently, dish T1 was determined as the most suitable for PDL cell proliferation. Subsequent experiments were conducted using T1 culture dishes, which were designated as SSCW®.

3.2. PBMA-PIPAAm coatings for controlled cell sheet detachment

Initially, hPDL cells were cultured on UpCell® and SSCW® surfaces for 14 days to prepare cell sheets. After the cell sheets were cut along the dish perimeter, they were maintained at room temperature (20 °C). Within 30 min, cell sheets on UpCell® began detaching from the edges and achieved full detachment after 60 min. Conversely, cell sheets on the SSCW® did not spontaneously detach, even after 60 min (Fig. 3A).

Subsequently, whether cell sheets could be detached using a physical method involving pipetting was evaluated. Through pipetting, hPDL cell sheets were fully detached from both UpCell® and SSCW®, leaving no residual cells on the culture (Fig. 3B). Sections of hPDL cell sheets were prepared and stained with HE. Both UpCell®- and SSCW®-prepared cell sheets demonstrated nuclei stained with hematoxylin and cytoplasm or extracellular matrix stained with eosin (Fig. 3C). This indicates that SSCW® is capable of preparing completely intact cell sheets, comparable in quality to those produced by UpCell®. Additionally, cell counts of hPDL cells cultured for 14 days on TCPS, UpCell®, and SSCW® surfaces showed no statistically significant differences. These results demonstrate the capacity of PBMA-PIPAAm coatings to enable the controlled detachment of cell sheets, thereby preventing spontaneous detachment.

3.3. Equivalence of hPDL cells on SSCW® to UpCell® in comprehensive gene expression analysis by RNA-Seq

To further confirm that the differences in culture dishes did not affect the cellular characteristics of hPDL cells, we collected the mRNA samples on day 14 from four distinct hPDL cell lines (subject 1-4) cultured on each dish, and conducted a transcriptome analysis. The principal component analysis (PCA) plot revealed that the data points did not disperse across the different culture dishes, but instead clustered according to the cell line (Fig. 4A). For a more detailed analysis of gene expression differences among cells on each dish, volcano plots were generated for the following comparisons: TCPS vs. UpCell®, TCPS vs. SSCW®, and UpCell® vs. SSCW® under the osteogenic induction conditions (Fig. 4B). The analysis indicated that the majority of the differentially expressed genes (DEGs) identified were read-through genes and pseudogenes (Tables 1–3).

3.4. Evaluation of the differentiation potential of hPDL cells on SSCW®

To determine whether the coated PBMA-PIPAAm block copolymers influenced the cellular characteristics and differentiation potential of hPDL cells, experiments were conducted under osteogenic and lipogenic induction conditions. The hPDL cells were cultured in each dish and subjected to Alizarin Red S staining for calcification and Oil Red O staining for lipogenesis. Satisfactory levels of calcification and lipogenesis were observed in both culture conditions (Fig. 5A). In contrast, undifferentiated hPDL cells did not stain with either Alizarin Red S or Oil Red O (Supplementary Fig. 1A). Moreover, the expression of surface markers, including CD105 (a marker of mesenchymal stem cells) and ALPL (a marker of calcification), was evaluated through flow cytometry for the hPDL cells cultured in each dish for 14 days under osteogenic induction conditions. The expression of surface antigens on the cells cultured in each dish showed minimal differences (Fig. 5B).

3.5. Evaluation of hPDL cell sheets prepared with SSCW® in vivo

To evaluate the *in vivo* performance of hPDL cell sheets prepared using SSCW®, a comparative experiment was conducted with cell sheets prepared using UpCell®. hPDL cell sheets from both types of culture dish were transplanted into the parietal bone of immunodeficient rats, along with a dentin disk (Fig. 6A). After four weeks, the rats were euthanized and paraffin-embedded tissue sections were prepared for anti-human periostin immunostaining. Both sets of transplanted cell sheets exhibited residual clumped structures stained with anti-human periostin antibody between the dentin disk space and the parietal bone, indicating preservation of the sheet structure. Notably, no cementum formation was observed in either set of hPDL cell sheets. These results demonstrate that hPDL cell sheets prepared using SSCW® were able to retain a stable sheet structure *in vivo*, comparable to that of those prepared with UpCell®.

4. Discussion

4.1. Characteristics of thermoresponsive PBMA-PIPAAm-coated cell culture dishes

The use of thermoresponsive polymers in culture dishes to generate cell sheets was first proposed by Okano et al. Cell sheet transplantation has advantages over traditional cell suspensions because it preserves the tissue structure and facilitates the transplantation of an intact ECM [12]. Our group has previously

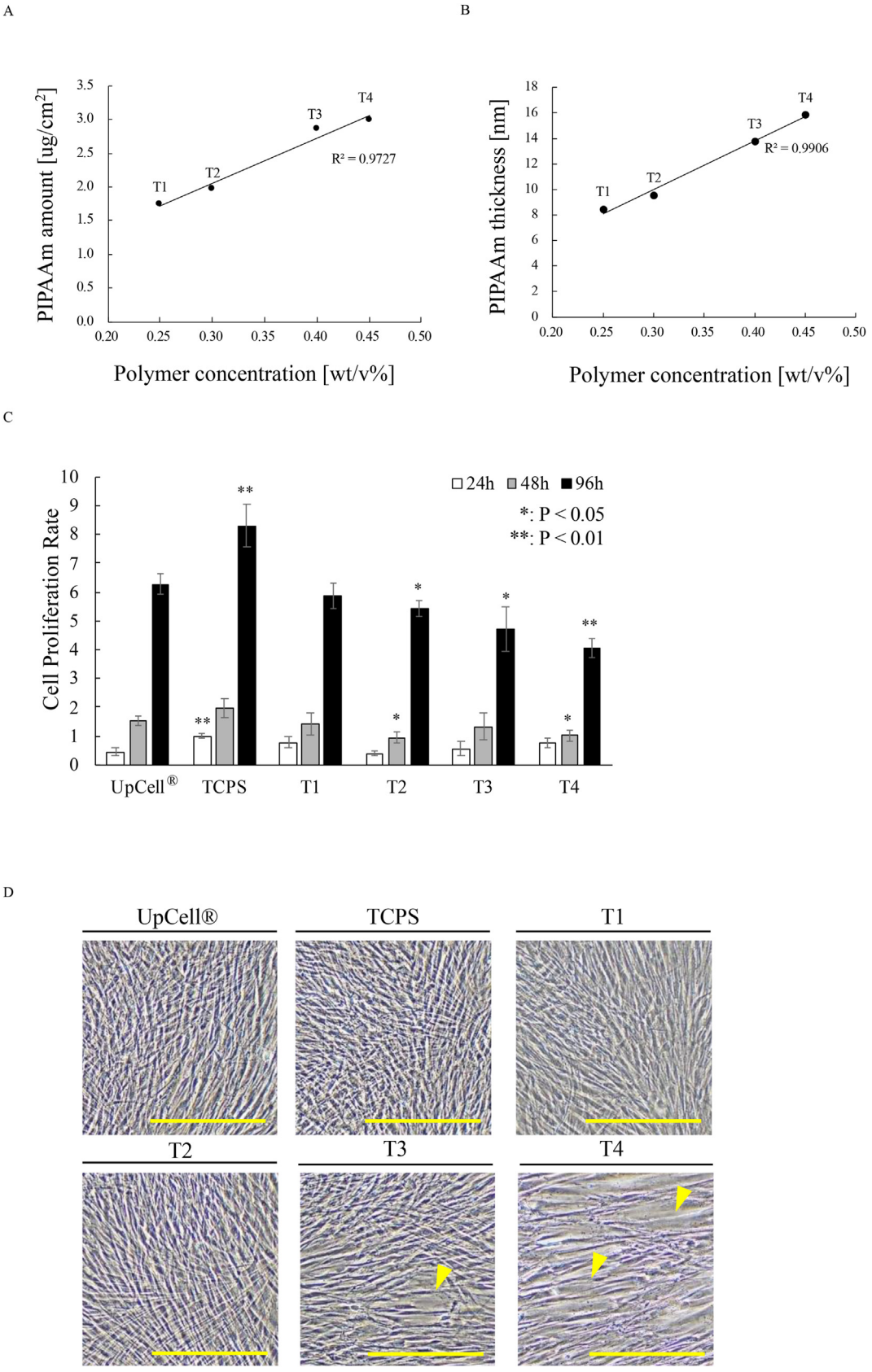

Fig. 2. Optimization of PBMA-PIPAAm polymer quantity for PDL cell culture. (A) Correlation between polymer concentration and PIPAAm amount [μg/cm^2] on each cell culture dish. (B) Correlation between polymer concentration and PBMA-PIPAAm thickness on each culture dish. (C) Relative proliferation of hPDL cells on different culture dishes at 24 h (white bars), 48 h (gray bars), and 96 h (black bars) using CCK-8 (mean ± SD). *P < 0.05, **P < 0.01 (paired Student's t-test). (D) Phase-contrast images of hPDL cells on each type of dish after 14 days of culture. Yellow arrows indicate gaps between cells. Scale bar: 500 μm.

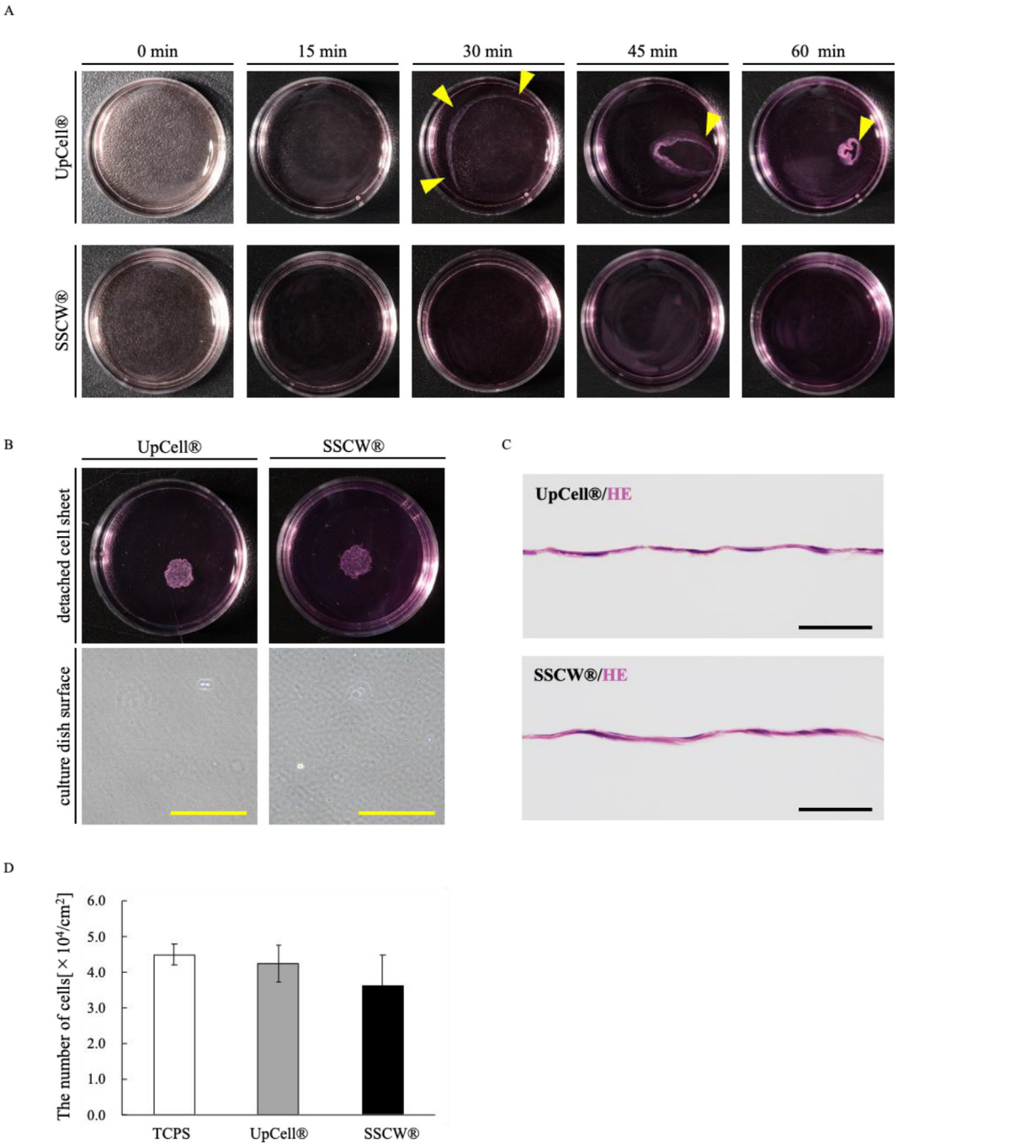

Fig. 3. Comparison of cell sheet detachment on SSCW® and UpCell®. (A) Demonstration of hPDL cell sheet detachment over a period of up to 60 min. Yellow arrows indicate the edges of the detached hPDL cell sheet on UpCell®. (B) Both types of cell culture dishes were capable of generating hPDL cell sheets through water flow using pipetting, leaving no residual cells attached to the surfaces after detachment. Yellow scale bars represent 500 μm. (C) Histological sections of hPDL cell sheets embedded in paraffin and stained with hematoxylin and eosin (HE). Black scale bars represent 50 μm. (D) There were no significant differences in the number of cells within the cell sheets between the different types of cell culture dishes.

conducted clinical trials involving the preparation of hPDL cell sheets using UpCell®, a technology that is already commercially available, and their administration to humans for treating periodontal disease. However, unexpected detachment of cell sheets during the preparation process has emerged as a significant challenge in manufacturing management. This requires customization of the culture dish detachment and adhesion properties tailored to specific cell types, accomplished by adjusting the amount and thickness of the thermoresponsive polymer-coated layers.

In recent years, PBMA-PIPAAm-coated culture dishes, SSCW®, have been developed as novel type of thermoresponsive substrates that can be easily customized according to the properties of various cell types [6–8]. In addition, the PBMA-PIPAAm layers on culture surfaces are significantly stable during and after cell culture and cell sheet harvest because hydrophobic PBMA block strongly enhances the water stability of physically adsorbed polymers [7]. For SSCW® technology, the amount of surface-immobilized PIPAAm segments and the thickness of spin-coated polymer layers can be readily controlled by changing the concentration of polymer solution

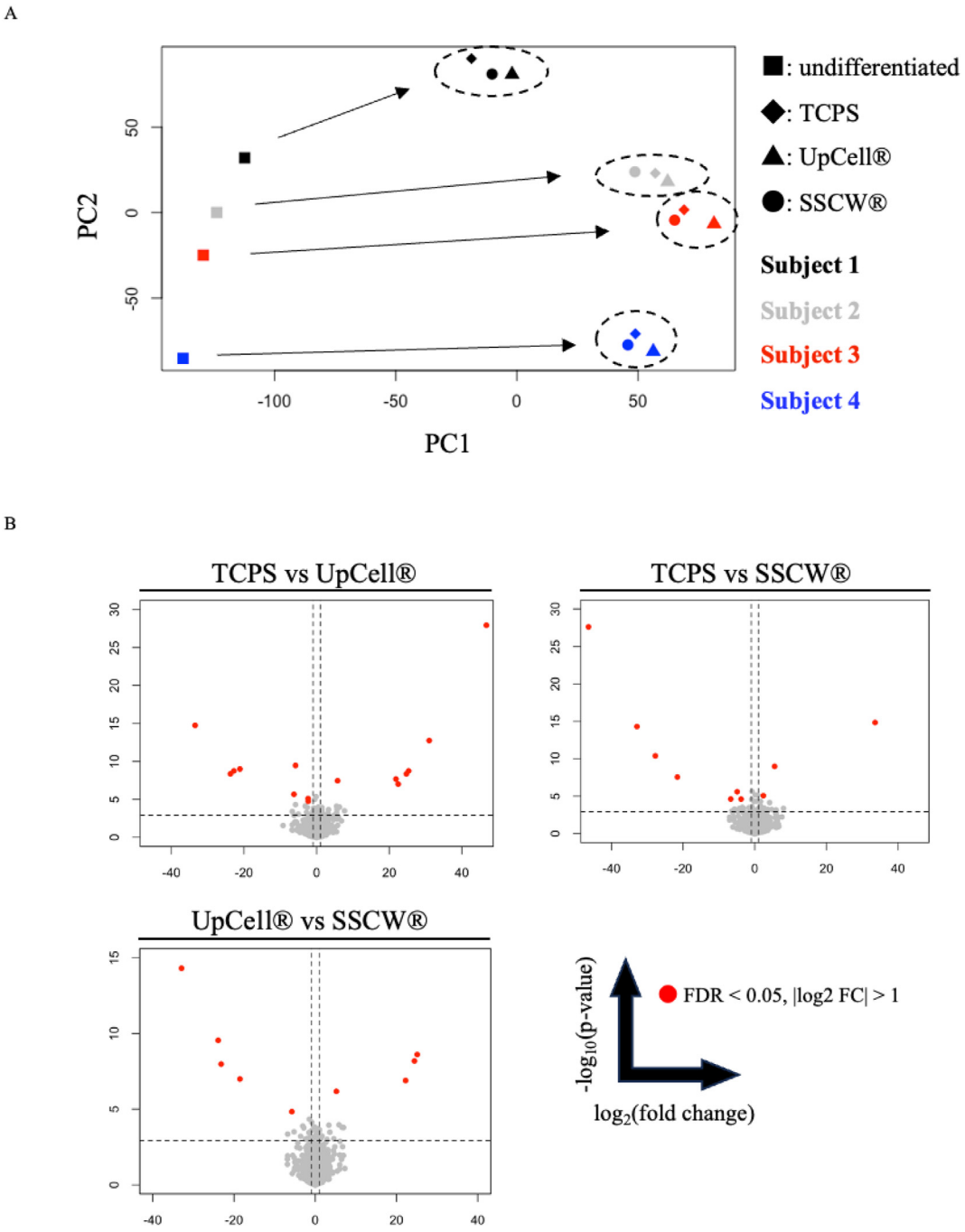

Fig. 4. RNA-seq analysis of PDL cells cultured on SSCW® and UpCell®. (A) PCA plot illustrating the RNA-seq results for hPDL cells cultured under each condition. Cells cultured in osteogenic induction medium cluster on the right side of the plot. Note that the PCA plot is primarily influenced by the donor source of the hPDL cells rather than the culture surface used. (B) Volcano plot displaying differentially expressed genes (DEGs); red dots represent genes with a false discovery rate (FDR) < 0.05 and $|\log_2$ fold change$| > 1$.

(Fig. 2A and B), modulating the thermal behavior of cell adhesion/proliferation and detachment. Our previous study demonstrated that decreases in the amount of PIPAAm segments and thickness of polymer layers promoted cell proliferation and reduced cell detachability on SSCW® surfaces [6]. These characteristics of SSCW® represent an advantage in the design of personalized thermoresponsive dishes compared with UpCell® fabricated by electron-beam-induced graft polymerization. In this study, through an extensive investigation of culture dishes coated with various PBMA-PIPAAm layers, those dishes with optimal detachment properties for preparing hPDL cell sheets were identified. The results demonstrate that hPDL cell sheets cultured on dishes with optimally tuned PBMA-PIPAAm coatings do not undergo unexpected detachment due to a temperature decrease and can be detached while retaining ECM integrity through non-enzymatic physical detachment methods.

When preparing cell sheets, it is necessary to detach the cells while preserving the ECM in response to temperature changes. However, cells that detach easily can lead to unexpected detachment of cell sheets, posing significant challenges in the manufacturing process. In general, the features of cell detachment from thermoresponsive culture dishes vary significantly,

Table 1
Differentially expressed genes (DEGs) between TCPS and UpCell®.

TCPS vs UpCell®					
Gene name	Description	baseMean	log_2 fold change	p-value	FDR
BLOC1S5-TXNDC5	ncRNA	8531.37791	46.7101006	1.18E-28	2.55E-24
C7orf55-LUC7L2	Protein cording	174.566819	−33.469983	1.76E-15	1.90E-11
AL645922.1	–	143.956355	30.9769425	1.78E-13	1.28E-09
MIR2277	ncRNA	43.8561246	−5.831714	3.47E-10	1.87E-06
AC010197.2	–	9.34504336	−21.144481	1.00E-09	4.32E-06
AP000295.1	–	50.675596	−22.792188	1.79E-09	5.55E-06
BORCS7-ASMT	ncRNA	160.706988	25.2994027	1.80E-09	5.55E-06
HSPE1-MOB4	Protein cording	100.123573	24.6826798	4.41E-09	1.06E-05
ZNF497	Protein cording	62.1207155	−23.758477	4.36E-09	1.06E-05
AC009133.6	–	11.2670679	21.8088297	2.13E-08	4.60E-05
POC1B-GALNT4	Protein cording	31.3762383	5.75857508	3.55E-08	6.95E-05
AC078927.1	–	18.509201	22.4356806	9.70E-08	0.000174295
SPART-AS1	ncRNA	9.16993063	−6.2610447	2.14E-06	0.003552118
JRK	Protein cording	838.877978	−2.375015	7.99E-06	0.011490139
SNRPGP10	Pseudogene	58.1611053	−2.330923	1.77E-05	0.022422387

*Gene descriptions were retrieved from the National Library of Medicine, NIH website (https://www.ncbi.nlm.nih.gov/).
Abbreviations: FDR, false discovery rate; ncRNA, non-coding RNA.

Table 2
Differentially expressed genes (DEGs) between TCPS and SSCW®.

TCPS vs SSCW®					
Gene name	Description	baseMean	log_2 fold change	p-value	FDR
BLOC1S5-TXNDC5	ncRNA	8531.377905	−46.42976276	2.49E-28	5.36E-24
C7orf55-LUC7L2	Protein cording	174.5668195	33.56768633	1.46E-15	1.57E-11
INO80B-WBP1	ncRNA	194.534776	−32.92210766	4.99E-15	3.59E-11
AL645922.1	–	143.9563547	−27.772988	4.08E-11	2.20E-07
MIR2277	ncRNA	43.85612457	5.535608003	1.05E-09	4.55E-06
AC009133.6	–	11.26706786	−21.62991434	2.79E-08	0.000100235
POC1B-GALNT4	Protein cording	31.37623832	−4.924678918	2.59E-06	0.006991519
JRK	Protein cording	838.877978	2.36658348	8.63E-06	0.016914519
AC026954.2	–	84.75831461	−6.726144025	2.44E-05	0.041311325
FAM209B	Protein cording	9.397609701	−3.840315842	2.50E-05	0.041311325

*Gene descriptions were retrieved from the National Library of Medicine, NIH website (https://www.ncbi.nlm.nih.gov/).
Abbreviations: FDR, false discovery rate; ncRNA, non-coding RNA.

Table 3
Differentially expressed genes (DEGs) between UpCell® and SSCW®.

UpCell® vs SSCW®					
Gene name	Description	baseMean	log_2 fold change	p-value	FDR
INO80B-WBP1	ncRNA	194.534776	−32.93074576	4.91E-15	1.06E-10
AP000295.1	–	50.67559602	−23.89333059	2.84E-10	3.06E-06
BORCS7-ASMT	ncRNA	160.706988	25.09205382	2.44E-09	1.75E-05
HSPE1-MOB4	Protein cording	100.123573	24.40718143	6.54E-09	3.52E-05
ZNF497	Protein cording	62.12071547	−23.16670726	1.05E-08	4.51E-05
AC010197.2	–	9.345043357	−18.54846845	1.02E-07	0.000366476
AC078927.1	–	18.50920099	22.22457239	1.28E-07	0.000393429
AMIGO3	Protein cording	286.6198261	5.181692134	6.68E-07	0.001801604
SPART-AS1	ncRNA	9.169930631	−5.759488289	1.46E-05	0.034988199

*Gene descriptions were retrieved from the National Library of Medicine, NIH website (https://www.ncbi.nlm.nih.gov/).
Abbreviations: FDR, false discovery rate; ncRNA, non-coding RNA.

depending on the cell type. Moreover, the cell sheet production process requires long-term cell culture involving multiple medium changes, which makes process management to prevent unwanted detachment a substantial burden on manufacturing. Thermoresponsive culture dishes, such as SSCW®, which do not cause cell sheet detachment solely due to temperature changes but instead enable retrieval of cell sheets at desired timings, minimizing the risk of unexpected detachment. This feature can reduce manufacturing risks and alleviate the stress on operators, aligning them well with their clinical needs.

From a clinical perspective, this feature has the potential to reduce risks associated with manufacturing failures and contribute to reducing medical care costs. The optimized SSCW® prevents the unexpected detachment of cell sheets, a major cost risk in manufacturing while simplifying inherently complex process management involved in cell sheet preparation. Additionally, SSCW® reduces the risk of manufacturing failures that could lead to the cancellation of scheduled surgeries, allowing physicians to deliver regenerative medicine on time. These benefits provided by tunable detachment control are expected to support the production

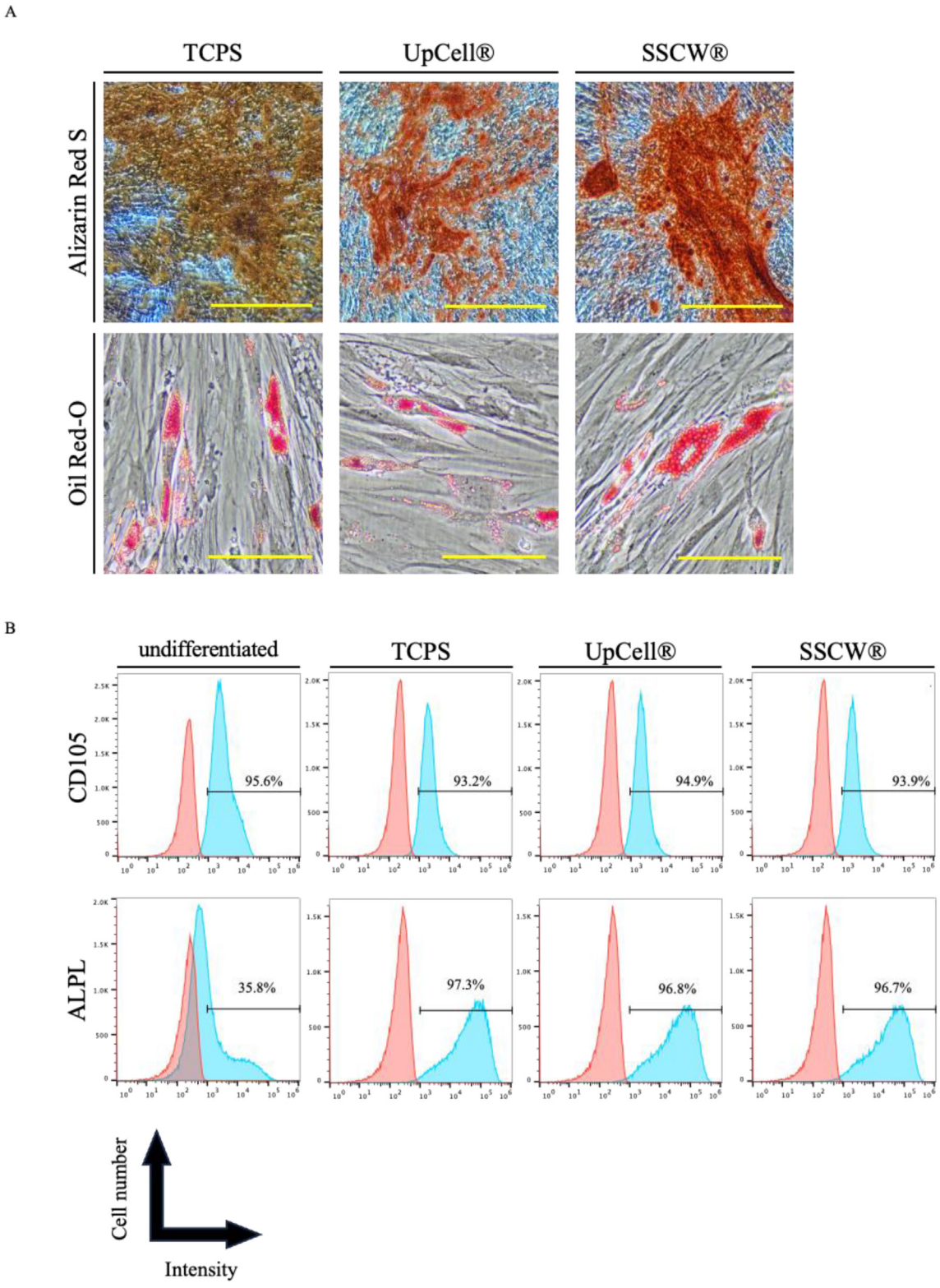

Fig. 5. Histological and flow cytometry analysis for hPDL cells cultured on SSCW® and UpCell®. (A) Alizarin Red S and Oil Red-O staining of hPDL cells cultured on each type of dish. Cells were cultured for 21 days in either osteogenic induction medium or adipogenic medium. Yellow scale bars represent 500 μm in the upper panels and 100 μm in the lower lane. (B) Flow cytometry analysis of representative markers for hPDL cells, showing CD105-positive cells as a mesenchymal stem cell marker and ALPL-positive cells as an osteogenic marker. Representative histogram plots on each cell culture surface are shown, with cells cultured for 14 days in either a complete medium (undifferentiated state) or osteogenic induction medium.

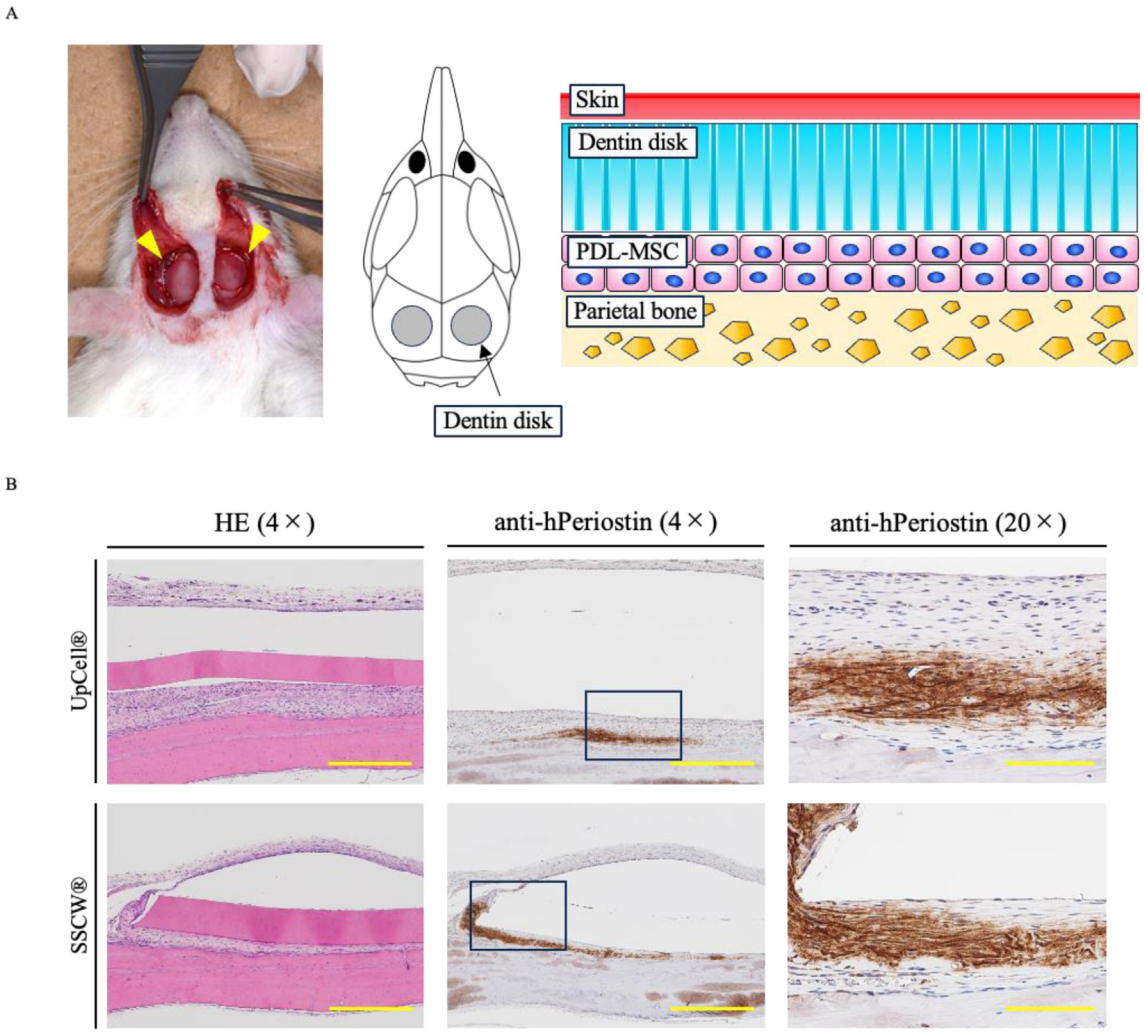

Fig. 6. Historical analysis of hPDL cell sheets *in vivo*. (A) Schematic illustration of hPDL cell sheet transplantation into the parietal bone of rats. (B) Immunohistochemical analysis of human periostin. Images were captured using a 4 × and 20 × objective lenses at magnification. Yellow scale bars represent 500 μm (4 ×) or 100 μm (20 ×).

of more stable and consistently reliable hPDL cell sheets at a lower cost.

4.2. Differences in culture dishes did not alter the characteristics of PDL cells

PDL cells are mesenchymal stem cells derived embryologically from the cranial neural crest with the potential to differentiate into dental tissues, such as the alveolar bone and periodontal ligament. The mesenchymal stem cell markers and multipotency of the PDL cells used in this study have been extensively characterized [9]. However, it has been well established that the surface characteristics of culture dishes can influence cell differentiation [13,14]. In our study, PDL cells cultured on TCPS, UpCell®, and SSCW® showed no differences in cell differentiation assays or in surface antigen evaluation by flow cytometry. Additionally, a detailed examination of differentially expressed genes (DEGs) through transcriptome analysis revealed that the influence of culture surfaces on PDL cells was minimal. Notably, PCA revealed that gene expression differences in PDL cells were significantly influenced more by the cell strains than by the culture dishes. However, one limitation of transcriptome analysis is that it only reflects trends in the overall cell population, potentially overlooking variations within subpopulations. Given that mesenchymal stem cells represent a heterogeneous cell population, single-cell analysis can provide a more detailed examination of the effect of culture dishes on PDL cells. Nevertheless, the results indicate that the influence of culture dishes on changes in cell characteristics was minimal and histologically undetectable.

4.3. Evaluation of hPDL cell sheets in vivo

The primary mechanisms of tissue regeneration through cell transplantation involve the differentiation and proliferation of the transplanted cells themselves, as well as the activation of surrounding cells via the trophic functions induced by factors secreted by the transplanted cells [15–17]. Typically, transplanted cells are eventually eliminated by recipient immune responses, and their contribution to long-term tissue regeneration is thought to be mediated primarily through trophic functions. However, MSCs, including PDL cells, possess immunomodulatory properties that allow them to evade recipient immune responses, suggesting that the transplanted cells may directly contribute to regeneration [18]. In our previous studies, the potential of PDL cells to differentiate into periodontal tissues, such as the cementum and periodontal ligament, *in vivo* and their contribution to periodontal tissue regeneration in animal models has been well-documented [19–21].

In this study, an *in vivo* model was developed by transplanting hPDL cell sheets and dentin disks into the parietal bone of SCID rats to replicate the complex structure of dentin, cementum, and alveolar bone found in natural teeth. By transplanting an onlay graft consisting of PDL cells followed by a dentin disk onto the parietal bone of rats, we simulated a native periodontal tissue structure that served as a niche for PDL cells. Our findings demonstrated the robust adhesion of hPDL cells, forming tissue structures between the dentin disk and the parietal bone even after a 4-week post-transplantation period. The lack of evident cementum formation, indicating an incomplete evaluation of PDL cell differentiation, could be attributed to the substantial mobility of subcutaneous tissues in rats, which may have caused displacement of the transplanted grafts. Nonetheless, these results indicate that cell sheets cultured using both SSCW® and UpCell® maintained strong adhesion to the host tissue and preserved robust tissue structure, thereby demonstrating the reliability of cell sheet transplantation.

5. Conclusion

SSCW® addresses the longstanding challenge of spontaneous detachment during prolonged culture, which has historically posed significant obstacles. Through histological analysis, transcriptome analysis, and *in vivo* cell sheet transplantation assays, we have demonstrated that cell sheets cultured on SSCW® exhibit comparable performance to those cultured on conventional counterparts. These findings highlight the potential of SSCW® to mitigate the risk of cell detachment during culture, improve the efficiency of cell sheet production, and facilitate the harvesting of cell sheets while preserving the ECM. Additionally, the versatility of the SSCW® extends beyond that of hPDL cells, as its detachability can be precisely controlled by tuning the coating polymers. This versatility makes SSCW® a promising candidate for diverse cell sheet preparations in regenerative medicine.

Author contributions

Conceptualization: KM, MN, TO, TI. Methodology: KM, MN, HS, TO, TI. Data collection: KM, JW. Animal experiments: KM, JW, YO, YT, HN. Statistical analysis and transcriptome analysis: KM, SO, MH. Interpretation of data: KM, MN, TO, TI. Project administration: TO, TI. Supervision: TO, TI. Writing—original draft: KM. Writing—review and editing: KM, AL, TI. All authors approved the final version of the manuscript.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this study, the authors used ChatGPT 4o to improve language and readability. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

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Declaration of competing interest

T.O. is the founder and representative director of the board of CSTERM. T.O. is a founder and stakeholder at Cellseed, Inc. H.S. was engaged by CSTERM as the project leader of SSCW® development (until March 2023). Tokyo Medical and Dental University received research funding from the CSTERM. No potential conflicts of interest relevant to this article have been reported.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2025.01.004.

References

[1] Soma T, Oie Y, Takayanagi H, Matsubara S, Yamada T, Nomura M, et al. Induced pluripotent stem-cell-derived corneal epithelium for transplant surgery: a single-arm, open-label, first-in-human interventional study in Japan. Lancet 2024;404(10466):1929–39.
[2] Matsumura H, Matsushima A, Ueyama M, Kumagai N. Application of the cultured epidermal autograft "JACE®" for treatment of severe burns: results of a 6-year multicenter surveillance in Japan. Burns 2016;42(4):769–76.
[3] Kawamura M, Miyagawa S, Miki K, Saito A, Fukushima S, Higuchi T, et al. Feasibility, safety, and therapeutic efficacy of human induced pluripotent stem cell-derived cardiomyocyte sheets in a porcine ischemic cardiomyopathy model. Circulation 2012;126 (11_suppl_1):S29–S37.
[4] Iwata T, Yamato M, Washio K, Yoshida T, Tsumanuma Y, Yamada A, et al. Periodontal regeneration with autologous periodontal ligament-derived cell sheets - a safety and efficacy study in ten patients. Regen Ther 2018;9:38–44.
[5] Kushida A, Yamato M, Konno C, Kikuchi A, Sakurai Y, Okano T. Decrease in culture temperature releases monolayer endothelial cell sheets together with deposited fibronectin matrix from temperature-responsive culture surfaces. J Biomed Mater Res 1999;45(4):355–62.
[6] Nakayama M, Yamada N, Kumashiro Y, Kanazawa H, Yamato M, Okano T. Thermoresponsive poly (N-isopropylacrylamide)-based block copolymer coating for optimizing cell sheet fabrication. Macromol Biosci 2012;12(6):751–60.
[7] Nakayama M, Toyoshima Y, Chinen H, Kikuchi A, Yamato M, Okano T. Water stable nanocoatings of poly (N-isopropylacrylamide)-based block copolymers on culture insert membranes for temperature-controlled cell adhesion. J Mater Chem B 2020;8(34):7812–21.
[8] Nakayama M, Toyoshima Y, Kikuchi A, Okano T. Micropatterned Smart culture surfaces via multi-step physical coating of functional block copolymers for harvesting cell sheets with controlled sizes and shapes. Macromol Biosci 2021;21(3):2000330.
[9] Iwata T, Yamato M, Zhang Z, Mukobata S, Washio K, Ando T, et al. Validation of human periodontal ligament-derived cells as a reliable source for cytotherapeutic use. J Clin Periodontol 2010;37(12):1088–99.
[10] Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Kause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8(4):315–7.
[11] Washio K, Kuroda H, Iwata T, Yoshida T, Yamato M, Okano T. Improved enzymatic treatment for accurate cell counting from extracellular matrix–rich periodontal ligament cell sheets. Oral Craniofac Tissue Eng 2012;2(3).
[12] Okano T, Yamada N, Sakai H, Sakurai Y. A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly (N-isopropylacrylamide). J Biomed Mater Res 1993;27(10):1243–51.
[13] Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, Cordenonsi M, et al. Role of YAP/TAZ in mechanotransduction. Nature 2011;474(7350):179–83.
[14] Mikami R, Arisaka Y, Hakariya M, Iwata T, Yui N. Improved epithelial cell–cell adhesion using molecular mobility of supramolecular surfaces. Biomater Sci 2021;9(21):7151–8.
[15] Forbes SJ, Rosenthal N. Preparing the ground for tissue regeneration: from mechanism to therapy. Nat Med 2014;20(8):857–69.

[16] Chimenti I, Smith R, Li T, Gerstenblith G, Messina E, Giacomello A, et al. Relative roles of direct regeneration versus paracrine effects of human cardiosphere-derived cells transplanted into infarcted mice. Circ Res 2010;106(5):971–80.
[17] Samsonraj RM, Raghunath M, Nurcombe V, Hui JH, van Wijnen AJ, Cool SM. Concise review: multifaceted characterization of human mesenchymal stem cells for use in regenerative medicine. Stem cells translational medicine 2017;6(12):2173–85.
[18] Song N, Scholtemeijer M, Shah K. Mesenchymal stem cell immunomodulation: mechanisms and therapeutic potential. Trends Pharmacol Sci 2020;41(9):653–64.
[19] Iwata T, Yamato M, Tsuchioka H, Takagi R, Mukobata S, Washio K, et al. Periodontal regeneration with multi-layered periodontal ligament-derived cell sheets in a canine model. Biomaterials 2009;30(14):2716–23.
[20] Washio K, Iwata T, Mizutani M, Ando T, Yamato M, Okano T, et al. Assessment of cell sheets derived from human periodontal ligament cells: a pre-clinical study. Cell Tissue Res 2010;341(3):397–404.
[21] Tsumanuma Y, Iwata T, Washio K, Yoshida T, Yamada A, Takagi R, et al. Comparison of different tissue-derived stem cell sheets for periodontal regeneration in a canine 1-wall defect model. Biomaterials 2011;32(25):5819–25.