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Original Article

Explant culture of oral mucosal epithelial cells for fabricating transplantable epithelial cell sheet

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ABSTRACT

Introduction: Carrier-free autologous mucosal epithelial cell sheets have been clinically utilized as a cell therapy for various epithelial disorders. Fabrication of a transplantable oral mucosal epithelial cell sheet without mouse feeder layers requires a higher seeding density than that of a sheet with mouse feeder layer culture; therefore, a large amount of donor mucosal tissue is needed. However, cell grafts co-cultured with mouse feeder layers are classified by the US Food and Drug Administration (FDA) as xenogeneic products. The goal of this study was to evaluate the utility of oral mucosal epithelial cells expanded by primary explant culture for the fabrication of an adequate number of transplantable epithelial cell sheets without mouse feeder layers.

Methods: Small fragments derived from minced oral mucosal tissue were placed into culture dishes for primary explant culture in keratinocyte culture medium. After primary explant culture, the outgrown cells were treated with trypsin-EDTA and were seeded on a temperature-responsive cell culture insert. After subculture, the cultured cells were harvested as a confluent cell sheet from the culture vessel by temperature reduction.

Results: Carrier-free human oral mucosal epithelial cell sheets were fabricated in all human cases, and autologous transplantation of the harvested cell sheets showed rapid epithelial regeneration to cover epithelial defects in a rabbit model. The explant culture method, involving the use of small fragments for primary culture, was sufficient for preparing a large number of mucosal epithelial cells without mouse feeder layers. Moreover, oral mucosal epithelial cells derived from the primary explant culture after cryopreservation allowed for the fabrication of cell sheets.

Conclusions: This method for fabricating transplantable oral mucosal epithelial cell sheets is an attractive technique for regenerative medicine. It offers a patient-friendly manufacturing method in which a small amount of biopsy material from the patient represents a sufficient epithelial cell source, and a manufacturing plan for preparing cell grafts can be easily tailored.

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1. Introduction

Regenerative medicine using cultured epithelial cell grafts is an attractive novel therapy for various epithelial disorders, such as

severe burns [1], giant congenital nevi [2], and corneal limbal stem cell deficiency [3]. For fabricating cultured human oral mucosal epithelial cell sheets, oral mucosal tissue is subjected to disaggregation by a standard enzymatic method to form a cell suspension, and the cells are seeded on culture vessels. The seeded oral mucosal epithelial cells proliferate and form epithelial tissue *in vitro*.

We previously developed a method for fabricating a carrier-free epithelial cell sheet using a temperature-responsive culture surface that does not require the use of proteinases [4]. The temperature-responsive culture surface was grafted with a temperature-responsive polymer, poly(N-isopropylacrylamide) (PIPAAm). Carrier-free autologous oral mucosal epithelial cell sheets have

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been clinically utilized for cell therapies to treat corneal limbal stem cell deficiency [5] and esophageal ulcers [6]. In the case of autologous transplantation, there is a minimal risk of immune rejection; therefore, immunosuppressive medications are unnecessary. However, fabrication of an oral mucosal epithelial cell sheet requires a high seeding density. To obtain a sufficient number of cell sheets, a large amount of oral mucosal tissue is necessary, and collection of this tissue is invasive and can cause postoperative pain and oral scarring in patients. Therefore, the harvesting of sufficient tissue for covering a wide range of epithelial defects by surgery would be highly invasive for the patient. Moreover, additional oral mucosal tissue must be harvested from patients in order to fabricate cell sheets for transplantation in the case of emergency, such as the exacerbation of disease or unexpected changes in the scheduled date of transplantation.

In the case of stratified epithelial cells, such as keratinocytes, corneal epithelial cells, and oral mucosal epithelial cells, subculture methods often require the use of mouse feeder layers for preparing large numbers of cell sheets. Because the cultured keratinocytes can be used for subculture, a large quantity of cultured human epidermal grafts can be prepared from a small piece of human epidermis for treating severe burns [7]. However, human epithelial cell grafts co-cultured with mouse feeder layers are classified by the US Food and Drug Administration (FDA) as xenogeneic products.

Primary explant culture for the expansion of epithelial cells is a classical culture method for expanding stratified squamous epithelial cells without using mouse feeder layers [8]. For explant culture, small fragments of minced tissue are plated in culture dishes, and epithelial cells migrate from the fragments and proliferate. Thereby, explant culture yields a sufficient number of epithelial cells for the production of cultured epithelial cell grafts from a small amount of biopsy material obtained from a donor. Cell graft fabrication using an explant culture method has been developed for regenerative therapies using various substrates, such as an amniotic membrane [9–11], fibrin gel [12], and cadaver dermis [13]. There have also been clinical studies using such cell grafts for the treatment of corneal limbal stem cell deficiency [14–16]. We have also fabricated a cell sheet using an explant culture method from a small piece of tissue, such as the middle ear [17] or nasal mucosa [18]. A carrier-free cultured human nasal epithelial cell sheet can be successfully produced using subcultured epithelial cells derived from these explant cultures [18]. Moreover, the transplantation of cultured nasal mucosal epithelial cell sheets promoted considerable regeneration of the middle ear mucosa in a rabbit model [19] and reconstruction of the human middle ear mucosa in a clinical study [20]. Therefore, the purpose of this study was to evaluate the utility of oral mucosal epithelial cells expanded by explant culture in fabricating an adequate number of transplantable epithelial cell sheets without mouse feeder layers for potential use in regenerative medicine.

2. Methods

2.1. Preparation of human oral mucosal tissue

This study was conducted according to the principles of the Declaration of Helsinki and was approved by the Ethics Committee for Biomedical Research of the Institutional Review Board of Jikei University School of Medicine and Tokyo Women's Medical University. All patients provided written informed consent. Oral mucosal tissue was harvested from 11 patients who underwent tonsillectomy at the Jikei University Hospital Department of Otorhinolaryngology. Oral mucosal tissue, including the tonsil, was used in this study (Fig. 1A). The harvested tissue was sterilized twice with povidone-iodine and washed with phosphate-buffered saline.

2.2. Fabrication of oral mucosal epithelial cell sheets using explant culture method

Oral mucosal tissue was cut into approximately 2-mm³ cubes by scalpel, and four fragments of the minced tissues were placed in a culture dish coated with type I collagen (60-mm diameter; BD Biosciences, Franklin Lakes, NJ, USA) or Corning Primaria Tissue Culture Dishes (60-mm diameter; Corning Inc., Corning, NY, USA) for primary explant culture in keratinocyte culture medium (KCM) prepared as previously reported [21] (Fig. 1B–D). After 2 weeks of culture, the outgrown cells were treated with 2500 mg/L trypsin and 0.91 mM ethylenediaminetetraacetic acid (EDTA) (trypsin-EDTA, Invitrogen, Carlsbad, CA, USA) for approximately 20 min at 37 °C. The disaggregated cells, suspended in KCM, were filtered through a 100- μ m cell strainer (BD Biosciences) before seeding on a temperature-responsive cell culture insert (CellSeed, Tokyo, Japan) at a seeding density of 8, 12, 24, 36, 48, or 60 \times 10⁴ cells/cm². After subculture, the oral mucosal cells seeded on the culture inserts were cultured for approximately 1–2 weeks at 37 °C in a humidified atmosphere containing 5% CO₂. The cultured cells were harvested as an epithelial cell sheet from the temperature-responsive cell culture insert by reducing the temperature from 37 °C to 20 °C for 30 min (Fig. 1B). The appearance of cultured oral mucosal epithelial cells was observed under a phase contrast microscope.

2.3. Fabrication of oral mucosal epithelial cell sheets using enzymatic method

The harvested oral mucosal tissue was treated with 1000 U/mL dispase (Godo Shusei, Tokyo, Japan) for 2 h at 37 °C, and the oral mucosal epithelium was separated from the substantia propria using surgical forceps. Cell suspensions from the disaggregated epithelium were prepared by treatment with trypsin-EDTA for 20 min at 37 °C, and epithelial cells suspended in KCM were filtered through a 40- μ m cell strainer (BD Biosciences). The epithelial cells were seeded on a temperature-responsive cell culture insert (CellSeed) at densities of 1, 2, 4, or 8 \times 10⁴ cells/cm² and cultured for approximately 2 weeks at 37 °C in a humidified atmosphere containing 5% CO₂. After cultivation, the epithelial cell sheets were harvested from the temperature-responsive cell culture inserts by reducing the temperature to 20 °C. The cell shape and appearance were observed under a phase contrast microscope.

2.4. Fabrication of cell sheets using cryopreservation of oral mucosal cells expanded by primary explant culture

After 2 weeks of primary explant culture, the outgrown cells were harvested by treatment with trypsin-EDTA, and the disaggregated cells were suspended in CELLBANKER1 cryopreservation medium (3 \times 10⁶ cells in 1 ml; Zenoaq, Fukushima, Japan) and cryopreserved at –80 °C. After cryopreservation for 3 months, the cryopreserved cells were quickly thawed in a 37 °C water bath with shaking. The suspended cells were immediately diluted with 10 ml KCM, gently mixed by pipetting, and seeded on temperature-responsive cell culture inserts (CellSeed) for approximately 2 weeks at 37 °C in a humidified atmosphere containing 5% CO₂. After culture, epithelial cell sheets were harvested from the temperature-responsive cell culture insert by reducing the temperature from 37 °C to 20 °C for 30 min.

2.5. Serial explant culture of oral mucosal tissue

Oral mucosal tissue was cut into approximately 2-mm³ cubes, and four fragments of minced tissues were placed in culture dishes

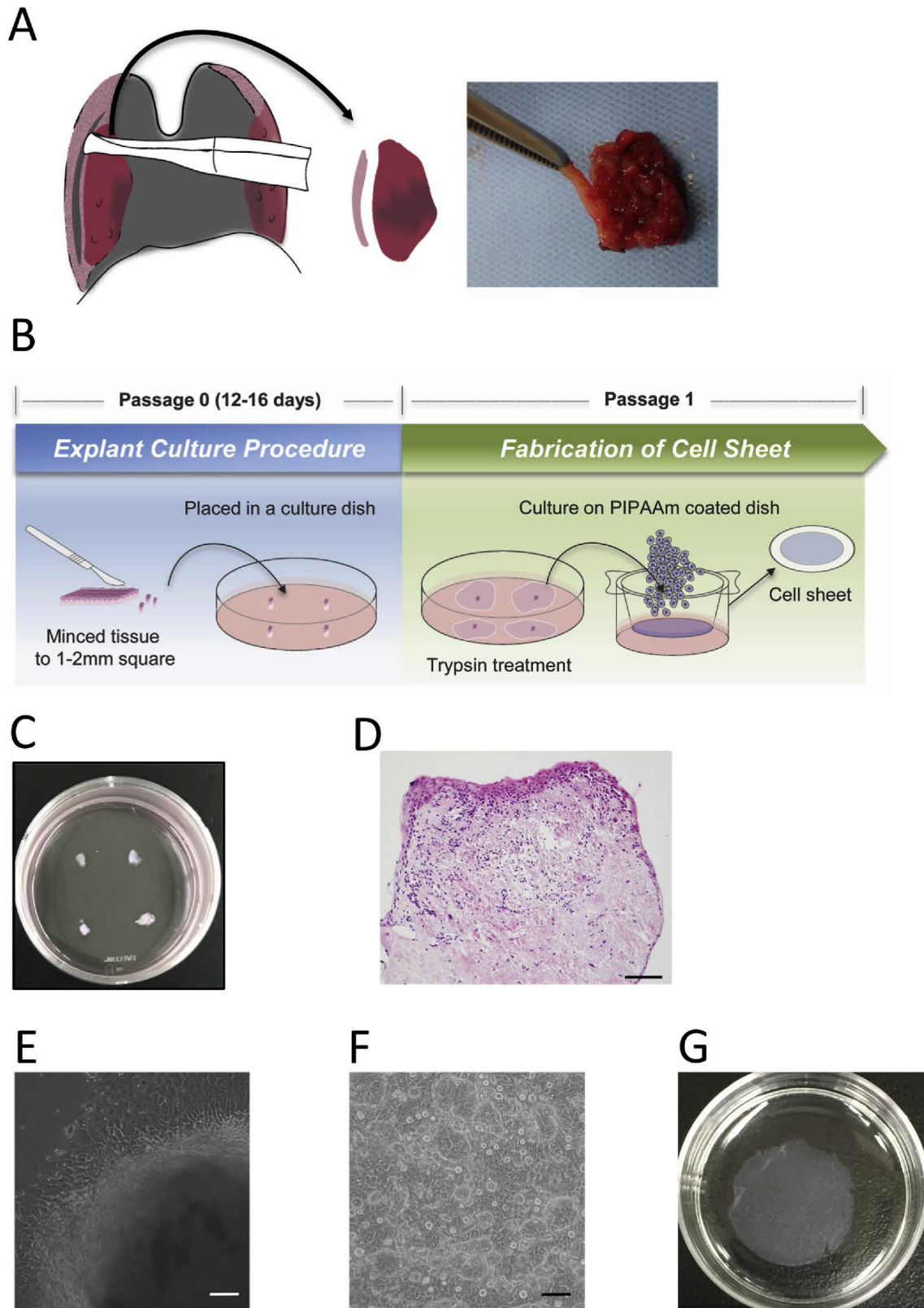


Fig. 1. Fabrication of oral mucosal epithelial cell sheets by primary explant culture. (A) Oral mucosal tissue used in this study. Mucosal tissue was harvested from patients who underwent tonsillectomy. (B) Schematic diagram of the culture method. Small fragments of minced tissues were placed in culture dishes. After primary explant culture, the outgrown cells were harvested with trypsin and seeded on temperature-responsive cell culture inserts. After subculture, cells were harvested as an epithelial cell sheet from the temperature-responsive cell culture insert by reducing the temperature from 37 °C to 20 °C for 30 min. (C) The tissue was cut into approximately 2-mm³ cubes, and four fragments of minced tissues were placed into a 60-mm culture dish. (D) Hematoxylin and eosin staining of oral tissue fragment from primary explant culture. Bar = 50 μm. (E) Small fragments grew and migrated outward from the oral mucosal tissue after primary explant culture. Bar = 100 μm. (F) Morphology of cultured oral mucosal epithelial cells after subculture. Bar = 100 μm. (G) Fabricated oral mucosal epithelial cell sheets.

coated with type I collagen (60-mm diameter; BD Biosciences) for primary explant culture in KCM. After culturing for 2 weeks, the plated oral tissue was replated in a new culture dish coated with type I collagen (60-mm diameter; BD Biosciences) to serially expand by explant culture in KCM. Explant cultures that were serially expanded were observed until the outgrowth of the epithelial cells from explanted tissue ceased. The epithelial cells expanded by explant culture were seeded on temperature-responsive cell culture inserts (CellSeed) for approximately 2 weeks at 37 °C in a humidified atmosphere containing 5% CO₂.

2.6. Histological and immunohistochemical analysis

Native oral mucosal tissue and harvested cell sheets were fixed with 4% paraformaldehyde, and paraffin-embedded tissue was processed into 5-µm-thick tissue sections. For cross-sectional observations, hematoxylin and eosin staining was performed by conventional methods. For immunohistochemistry, de-paraffinized sections were digested using proteinase K (Dako, Santa Clara, CA, USA) or heated with citrate (pH 6; Dako) at 125 °C for antigen retrieval. The sections were incubated with a peroxidase-blocking solution (S2023; Dako) for blocking endogenous peroxidase activity at 20–30 °C for 30 min and with a blocking reagent (Blocking One Histo; Nacalai Tesque, Kyoto, Japan) for prevention of nonspecific reactions at 20–30 °C for 1 h. The sections were treated with mouse monoclonal anti-pancytokeratin (1:100 dilution; AE1/AE3, Abcam, Cambridge, UK) or mouse monoclonal anti-p63 (1:100 dilution; 4A4, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight. On the following day, the sections were incubated with horseradish peroxidase-conjugated secondary antibodies (EnVision Detection Systems, Peroxidase/DAB, Rabbit/Mouse; Dako) at 20–30 °C for 30 min. The sections were incubated with 3,3'-diaminobenzidine (DAB) Solution (Dako) for approximately 1 min. Nuclear staining for immunohistochemistry was performed using hematoxylin.

2.7. Colony forming assay

Human oral mucosal epithelial cells were seeded at a density of 104 cells/cm² and co-cultured with NIH-3T3 cells treated with mitomycin C (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for detecting clonal growth of the seeded epithelial cells. Colony-forming efficiency (CFE) was calculated by dividing the

observed number of colonies by the initial cell number according to a previously reported method [21].

2.8. Transplantation of autologous oral mucosal epithelial cell sheet derived from explant culture

New Zealand White rabbits (n = 3, approximately 3 kg, male) were maintained in accordance with the Association for Research in Vision and Ophthalmology (ARVO) resolution on the use of animals in research. This animal study was approved by the Animal Care and Use Committee at the Tokyo Women's Medical University. Autologous oral mucosal epithelial cell sheets were transplanted into the corneal stroma in a rabbit limbal stem cell deficiency model, as previously reported [22]. Briefly, after induction of anesthesia by intramuscular injection of a mixture of midazolam (Astellas, Tokyo, Japan), medetomidine (Zenoaq), and butorphanol (Meiji Seika Pharma, Tokyo, Japan), the entire corneal epithelium, including the limbus and a 3-mm wide section of conjunctival tissue from the transitional zone between the cornea and the conjunctiva, was completely removed using scissors. The ocular surface, including the exposed corneal stroma, was treated with 1-n-heptanol (FUJIFILM Wako Pure Chemical Corporation). At 4–5 weeks after surgery, the corneal surface invading the conjunctival epithelium was surgically removed to expose the native transparent corneal stroma before cell sheet transplantation. Autologous oral mucosal epithelial cell sheets, derived from epithelial cells serially cultured after primary explant culture, were harvested by temperature reduction. The harvested autologous oral mucosal epithelial cell sheet, with a support ring, was transplanted onto the exposed transparent stroma, as previously reported [23]. For protection of the cornea, a hard contact lens (Meni-One Corporation, Aichi, Japan) was used to cover the ocular surface, and the upper and lower eyelids were sutured to stabilize the lens without moving. After surgery, a topical antibiotic (0.3% ofloxacin; Santen, Osaka, Japan) and a steroid (0.1% betamethasone; Shionogi, Osaka, Japan) were applied once a day for 1 week. The ocular surface was carefully observed by microscopy, and the presence of corneal epithelial defects was determined by fluorescein eye stain.

2.9. Statistical analysis

Statistical analysis was used to the aid of JMP Pro 11.0.0 (SAS, Cary, NC, USA) software. The mean differences compared with explant culture method and enzymatic method were performed by

Table 1
Fabrication of oral mucosal epithelial cell sheets using explant culture method.

ID	Age/Gender	Primary culture dish	Size of tissue (cm ²)	Cells/tissue area (×10 ⁴ cells/cm ²)	Seeding density (×10 ⁴ cells/cm ²)	Cell sheets (sheets/cm ²)	Total culture days	p	
								p0 (day)	p1 (day)
01	38/M	Coll	0.54	2088	12	41	21	14	7
		PRIMARIA	0.2	2340	16	34	21	14	7
02	28/M	Coll	0.75	2046	12	40	21	14	7
		PRIMARIA	0.67	686	12	13	21	14	7
03	31/F	Coll	0.45	3048	8	90	22	15	7
		PRIMARIA	0.45	2193	8	65	24	16	8
04	21/M	Coll	0.7	1800	12	35	21	14	7
05	48/M	Coll	1.21	1097	8	32	28	12	16
06	35/M	Coll	0.91	694	12	13	23	14	9
07	38/M	Coll	0.25	4260	16	63	21	14	7
08	35/M	Coll	0.32	1276	16	19	24	14	10
09	27/F	Coll	0.25	576	12	11	28	12	16
10	27/F	Coll	0.4	1500	8	44	30	12	18
11	22/F	Coll	0.15	3560	8	105	28	12	16

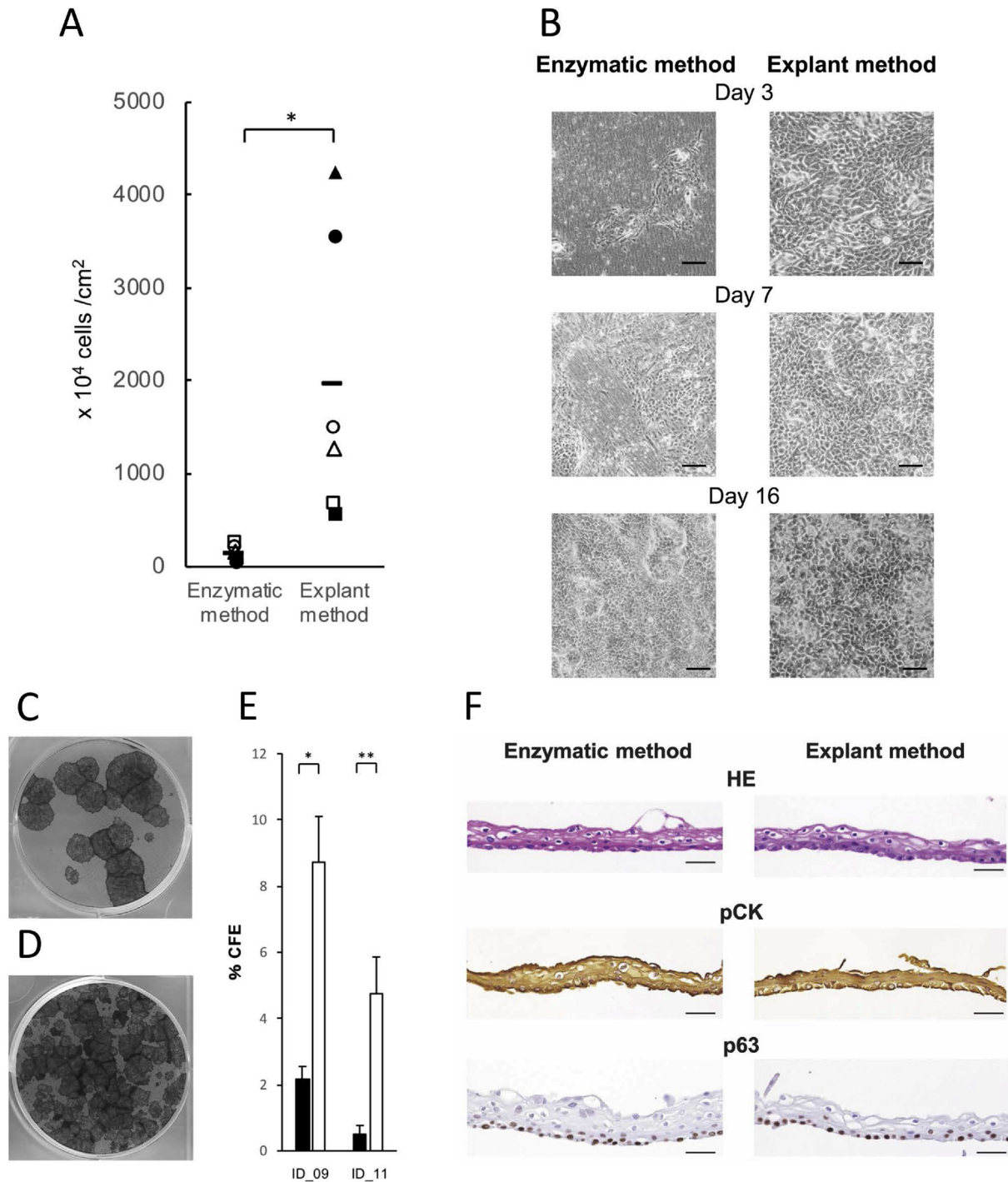


Fig. 2. Comparison of human oral mucosal epithelial cells derived from enzymatic and explant culture methods. (A) Numbers of cells isolated from human oral tissue using each method ($n = 6$), calculated by dividing the initial isolated cell number by the size of the tissue. $*p < 0.05$, paired t -test. (B) Observation using a phase contrast microscope of human oral mucosal epithelial cells cultured without mouse feeder layers. Epithelial cells harvested by enzymatic or primary explant culture method were seeded on temperature-responsive cell culture inserts at a density of 8×10^4 cells/cm² and cultured in KCM. Cultured cells were observed on days 3, 7, and 16 after seeding on the culture vessel. All bars = 100 μ m. (C–E) Colony-forming assay (CFA) of human oral mucosal epithelial cells seeded on six-well plates at a cell density of 113.6 cells/cm² and co-cultured with NIH-3T3 cells treated with mitomycin C. Colony-forming efficiency (CFE) was calculated by dividing the observed number of colonies by the initial cell number, multiplied by 100. Results of colony formation of primary epithelial cells (C) and harvested mucosal epithelial cells after explant culture (D). (E) Comparison of CFEs of primary epithelial cells derived from oral mucosal tissue and epithelial cells expanded by primary explant culture. CFE was calculated for each culture ($n = 3$). Black bars show primary epithelial cells derived from oral mucosal tissue; open bars show epithelial cells expanded by primary explant culture. Error bars indicate standard deviation. $*p < 0.05$, $**p < 0.01$, paired t -test. (F) Histological and immunohistochemical analyses of oral mucosal epithelial cell sheets derived from enzymatic or primary explant method. Oral mucosal epithelial cell sheets were subjected to paraffin-embedded sectioning and stained with hematoxylin and eosin (first row), anti-pan-cytokeratin (second row), and anti-p63 (third row). The photographs show the enzymatic (left) and explant methods (right). The cell sheets were composed of 3–5 stratified pan-cytokeratin-positive cells, well-differentiated cell layers, and contained p63-positive cells (a marker known to be positive in epithelial stem and progenitor cells) throughout the basal layer of the cell sheet. All bars = 50 μ m.

a paired two-tailed Student's t-test. A P-value of less than 0.05 ($P < 0.05$) was considered significant.

3. Results

3.1. Fabrication of oral mucosal epithelial cell sheets using explant culture method

The migration and rapid expansion of primary epithelial cells obtained from the periphery of oral mucosal tissue fragments placed in a culture dish were observed at 3 days after primary explant culture. These cells showed a polygonal cobblestone-like morphology characteristic of epithelial cells (Fig. 1E). Oral mucosal epithelial cells continued to exhibit the cobblestone morphology even after subculture from primary explant culture (Fig. 1F). After 7–10 days of subculture, confluent epithelial cells were successfully harvested as a carrier-free cell sheet from the temperature-responsive cell culture insert by reducing the temperature from 37 °C to 20 °C for 30 min (Fig. 1G). To confirm reproducibility, the total number of collected epithelial cells was enumerated and divided by the total area of the harvested oral mucosal tissue (Table 1). Epithelial cells expanded by primary explant culture were successfully prepared from all 11 patients, and the average numbers of cells per cm^2 of oral mucosal tissue were shown.

3.2. Comparison of oral mucosal epithelial cell sheets produced by enzymatic and explant culture methods

Oral mucosal epithelial cells for the fabrication of cell sheets were successfully expanded by primary explant culture, and the number of cells isolated from explant culture was significantly higher than that isolated using traditional enzymatic methods (Fig. 2A, Supplemental Table 1). When oral mucosal epithelial cells were seeded on culture inserts at a density of 8×10^4 cells/ cm^2 , those prepared by the enzymatic method became confluent in approximately 10 days (Fig. 2B), while those expanded by primary explant culture became confluent in 3 days (Fig. 2B). The colony sizes of epithelial cells expanded by explant culture were smaller than those derived from oral mucosal tissue (Fig. 2C and D). The CFE of oral epithelial cells expanded by primary explant culture was significantly higher than that of cells prepared by the enzymatic method (Fig. 2E). The epithelial cells prepared using each method were successfully harvested as transplantable cell sheets. All cell sheets were composed of 3–5 stratified pan-cytokeratin-positive cells and well-differentiated cell layers, and p63-positive epithelial stem and progenitor cells were observed throughout the basal layers of the cell sheets (Fig. 2F).

3.3. Utility of oral mucosal epithelial cells expanded by primary explant culture for fabrication of transplantable epithelial cell sheets

The seeding density and culture period for the fabrication of the cell sheets using each method were evaluated and are summarized in Supplemental Table 2. For the oral mucosal epithelial cells subcultured from primary explant culture, at seeding densities of 24, 36, 48, and 60×10^4 cells/ cm^2 on the temperature-responsive cell culture insert, epithelial cell sheets were successfully harvested after 4 or 5 days (Fig. 3A–E). The fabricated cell sheets seeded at a density of 60, 48, and 36×10^4 cells/ cm^2 showed a stratified epithelial structure. At a seeding density of 8×10^4 cells/ cm^2 on the culture insert (Fig. 3F), although a cell sheet could not be harvested after 5 days of culture, cell sheets were successfully harvested after 16 days of culture (Fig. 3E).

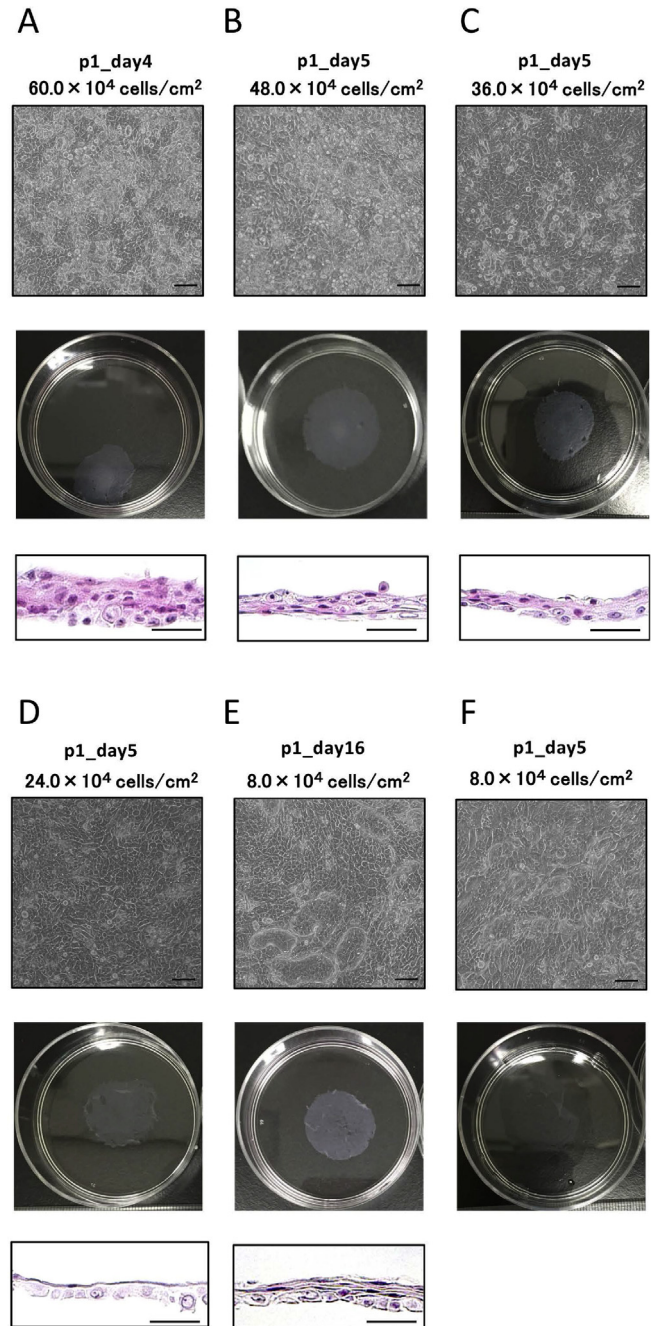


Fig. 3. Analysis of seeding density and culture period necessary for fabricating cell sheets using oral mucosal cells expanded by primary explant culture. At a seeding density of 60 (A), 48 (B), 36 (C), and 24 (D) $\times 10^4$ cells/ cm^2 on the temperature-responsive cell culture insert, the epithelial cell sheets were successfully harvested after 4 or 5 days. The fabricated cell sheets seeded at a density of 60, 48, and 36×10^4 cells/ cm^2 showed stratified epithelial structure. At a seeding density of 8×10^4 cells/ cm^2 on the culture insert, cell sheets were successfully harvested after 16 days of culture (E) and could not be harvested after 5 days of culture (F). Scale bars of phase contrast images indicate 100 μm . Scale bars of hematoxylin and eosin staining indicate 50 μm .

To examine the feasibility of cryopreservation of oral mucosal epithelial cells derived from primary explant culture, which could be used to prevent the additional harvest of oral mucosal tissue from patients, expanded epithelial cells from primary explant culture were examined for the ability to produce cell sheets following cryopreservation (Fig. 4A). After cryopreservation for 3 months, the cell viability of the thawed cells was more than 80%. Cells were

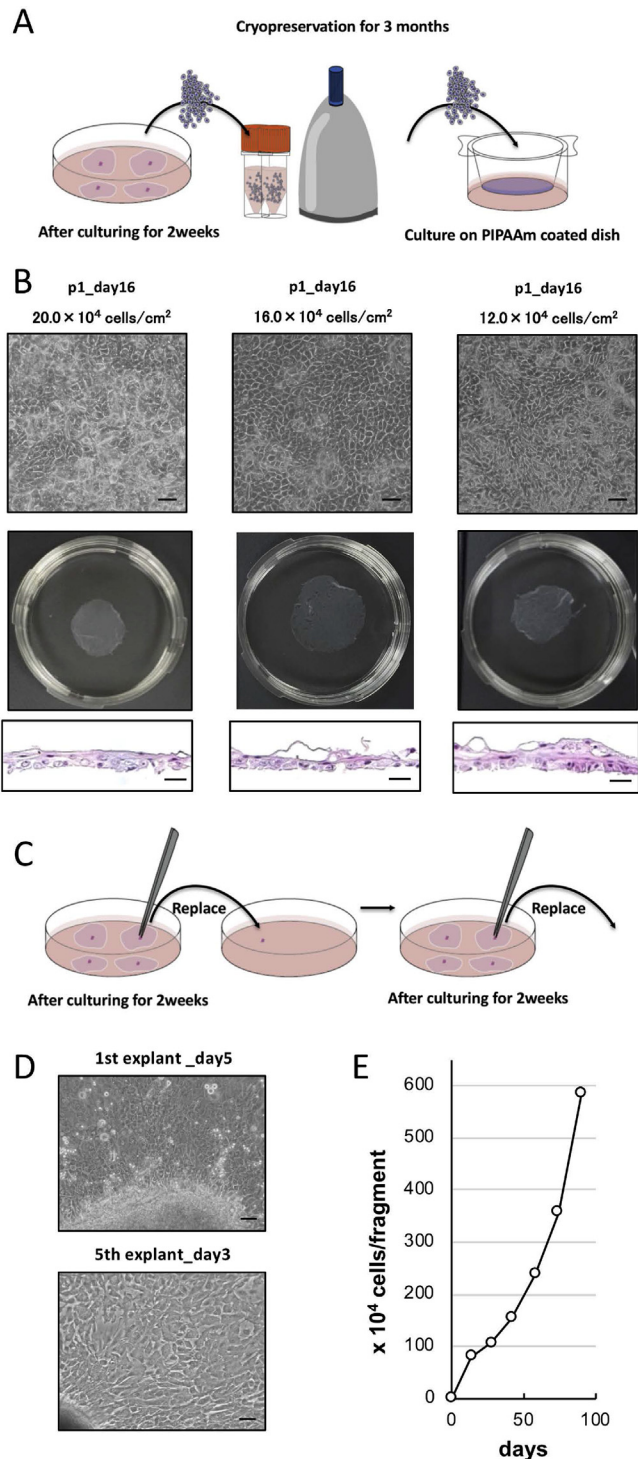


Fig. 4. Analysis of utility of oral mucosal epithelial cells expanded by explant culture. (A) Fabrication of human oral mucosal cell sheet using cryopreserved cells. After primary explant culture, outgrown cells were harvested, and disaggregated cells were suspended in cryopreservation medium and cryopreserved at -80°C . After cryopreservation for 3 months, cells were quickly thawed, and epithelial cells were seeded on temperature-responsive cell culture inserts. After culture, oral mucosal epithelial cell sheets were harvested by reducing the temperature. (B) Fabricated human oral mucosal cell sheet using cryopreserved cells. After subculture for 16 days, oral mucosal epithelial cell sheets were fabricated at seeding densities of 12, 16, and 20×10^4 cells/cm² using epithelial cells derived from primary explant culture. Scale bars of the phase contrast image indicate 100 μm . Scale bars of hematoxylin and eosin staining indicate 50 μm . (C) Serial explant culture of oral mucosal tissue using explant culture method. After primary explant culture, oral mucosal tissue fragments were plated in a new culture dish coated with type I collagen for serial explant culture in KCM. (D) Migration

seeded on temperature-responsive cell culture inserts, and the migration and proliferation of epithelial cells were observed on the culture insert. The cells showed a typical polygonal cobblestone-like appearance (Fig. 4B). Oral mucosal epithelial cell sheets were successfully fabricated using epithelial cells derived from primary explant culture after cryopreservation (Fig. 4B).

Next, we attempted serial explant culture of oral mucosal tissue using the explant culture method (Fig. 4C). After primary explant culture, the oral mucosal tissue fragments were plated in a new culture dish coated with type I collagen for serial explant culture in KCM. The migration and proliferation of epithelial cells obtained from the periphery of the tissue fragments were observed after serial explant culture, and the cells again showed a polygonal cobblestone-like appearance (Fig. 4D). The outgrowth of the epithelial cells from explant tissue was serially observed by replating the tissue fragments after each round of explant culture (Fig. 4E). The expanded epithelial cells from the second round of explant culture were seeded on temperature-responsive cell culture inserts. Although the oral mucosal epithelial cells also became confluent on the culture insert, cell sheets could not be harvested at any seeding density by reducing the temperature.

3.4. Transplantation of autologous oral mucosal epithelial cell sheet derived from explant culture in a rabbit corneal model

A rabbit limbal stem cell deficiency model was used to evaluate the efficacy of the autologous oral mucosal epithelial cell sheets in repairing an epithelial defect *in vivo*. In this experiment, after removing the corneal epithelium, the corneas of the model rabbits were treated with transplanted autologous epithelial cell sheets fabricated from oral mucosal epithelial cells expanded by primary explant culture (Fig. 5A).

Before transplantation, the rabbits' ocular surfaces were prepared by resection of the corneal and limbal epithelium. After surgery, the corneal surface was invaded by conjunctival scarring and new blood vessels, and corneal opacification was observed. Oral mucosal epithelial cell sheets were fabricated from rabbit biopsies using the explant culture method in a manner similar to that used for humans. The transplanted epithelial cell sheets comprised 3–5 stratified cell layers (Fig. 5B–F). Pan-CK, a known epithelial cell marker, was expressed in all cell layers of the epithelial cell sheets. There were also p63-positive cells, consistent with putative stem/progenitor cells in the basal layer. These expression patterns are similar to those in normal human oral mucosal epithelial cell sheets.

Sheets were then transplanted onto the ocular surfaces after surgically re-exposing the native transparent corneal stroma (Fig. 5G). After transplantation of the oral mucosal epithelial sheet, the ocular surface prevented the permeation of the fluorescein dye in the corneal stroma. The reconstructed corneal surface was clear and smooth without observable corneal defects at 2 weeks after transplantation, which was confirmed by fluorescein staining (Fig. 5G). Although invasion of new blood vessels was observed in a cornea, the cornea was transparent and exhibited no scarring and no inflammation at 12 weeks after transplantation (Fig. 5G).

4. Discussion

Explant culture is a useful manufacturing method for preparing an adequate number of oral mucosal epithelial cells for the

and proliferation of oral epithelial cells from the periphery of the tissue fragments were observed after 1 day of serial explant culture, and cells showed a polygonal cobblestone-like appearance. All bars = 100 μm . (E) Total cell numbers from serial explant culture.

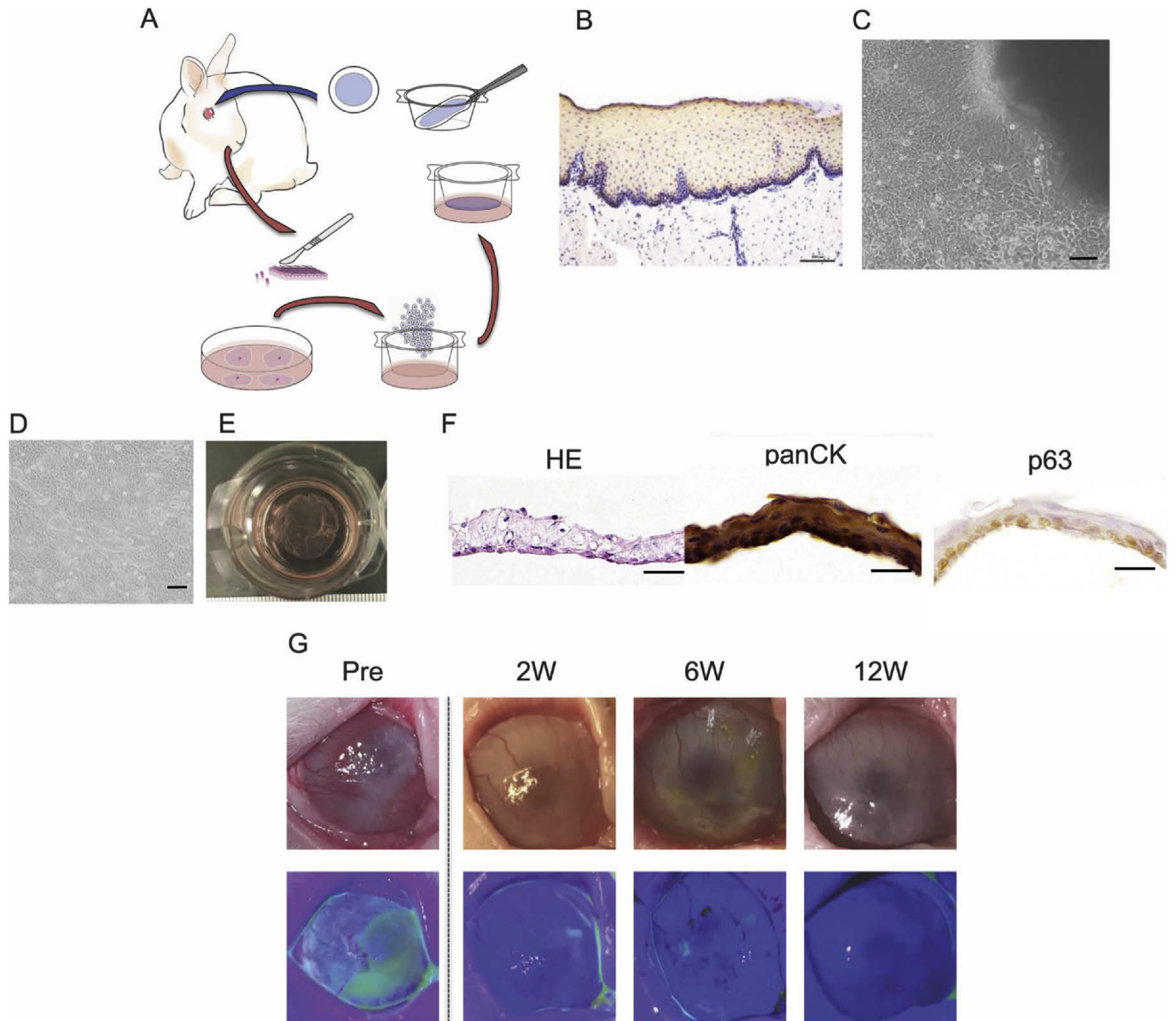


Fig. 5. Analysis of autologous transplantation of cell sheet fabricated from oral mucosal epithelial cells expanded by primary explant culture in a rabbit corneal model. (A) Autologous oral mucosal epithelial cell sheet was transplanted onto the keratectomized ocular surface of the rabbit limbal stem cell deficiency model. After 4–5 weeks, the conjunctivalized ocular surface was surgically removed to re-expose the native transparent corneal stroma before cell sheet transplantation. Autologous oral mucosal epithelial cell sheets, derived from epithelial cells serially cultured after primary explant culture, were harvested by temperature reduction. The harvested autologous oral mucosal epithelial cell sheet was placed on the re-exposed transparent stroma immediately. (B) Pan-cytokeratin was detected in the epithelial layer of normal rabbit oral mucosal tissue. Bar = 100 μ m. (C) Migration and proliferation of epithelial cells from the periphery of the tissue fragments were observed after 3 days. Bar = 100 μ m. (D) The oral mucosal epithelial cell sheet showed polygonal cobblestone-like appearance under a phase contrast microscope. Bar = 100 μ m. (E) Fabricated cell sheet. (F) The sheet was comprised of 3–5 stratified cell layers. Pan-cytokeratin was expressed in all cell layers, and expression of p63, a putative stem/progenitor cell marker, was observed in the basal layer. Bar = 25 μ m. (G) Autologous transplantation of harvested epithelial cell sheets on keratectomized corneal surfaces. Limbal stem cell deficiency model 5 weeks after surgery, and transplantation of autologous oral mucosal epithelial cell sheets without (upper row) and with (lower row) fluorescein staining. After transplantation, the ocular surface prevented the permeation of the fluorescein dye into the corneal stroma. The reconstructed ocular surface was clear and smooth by 2, 6, and 12 weeks after transplantation without observable corneal defects, as confirmed by fluorescein staining.

fabrication of cell sheets via culture without the necessity of mouse feeder layers. In the present study, carrier-free human oral mucosal epithelial cell sheets were fabricated in all human cases, and autologous transplantation of the harvested cell sheets showed rapid epithelial regeneration to cover epithelial defects in an *in vivo* rabbit model. Moreover, higher seeding densities of oral mucosal epithelial cells expanded by explant culture increased the success rate for harvesting cell sheets and shortened the culture period

required for fabrication of the cell sheet. Thus, the culture period needed for successful harvesting of the cell sheet was correlated with the seeding density of the subculture on temperature-responsive culture vessels. Additionally, cryopreservation of oral mucosal epithelial cells after primary explant culture also yielded a useful cell source for the fabrication of transplantable cell sheets. Therefore, the use of primary explant culture to obtain epithelial cells for fabricating cell sheets can enable the manufacturing plan

for the preparation of cultured oral mucosal epithelial cell sheets to be easily adapted to suit the patients and surgeons using the cell grafts.

In a previous study of esophageal epithelial regeneration, the transplantation of human oral mucosal epithelial cell sheets prevented esophageal stenosis after endoscopic resection of esophageal cancer [6]. In order to prepare the autologous cell sheets, oral mucosal tissue had to be obtained from a patient. According to a clinical study of the re-epithelialization of esophageal ulcers after aggressive endoscopic resection, approximately 10 sheets of autologous oral mucosal epithelial cells were required for transplantation [24]. In the clinical study, the average size of the oral mucosal tissue needed to prepare 10 sheets was 2.8 cm² (range: 2.19 cm²–3.86 cm²) [21]. Resection of oral mucosal tissues of this size causes severe oral pain, discomfort, and scarring. Moreover, conventional culture methods that do not use mouse feeder layers are fundamentally limited by the amount of resectable tissue that can be used in an autologous manner. Before the fabrication of cell sheets from culture on temperature-responsive cell culture inserts, expansion of oral mucosal epithelial cells by primary explant culture can be used to obtain >10 sheets from <1 cm² of biopsy material. These results indicate that, unlike tissue prepared for primary culture using proteinases, the explant culture method provides a sufficient number of cells from small oral mucosal tissue biopsies for regenerative medicine.

Previous studies have compared explant culture methods and enzymatic methods for the primary culture of mucosal epithelial cells, and both have been demonstrated to be successful for the expansion of human oral mucosal epithelial cells, regardless of age or sex [25–28]. Cultured mucosal epithelial cells expanded by both methods express cytokeratin, exhibit similar percentages of p63-positive cells, and contain BrdU-labeled cells [25,27]. Consistent with these findings, in the present study, cell sheets of oral mucosal epithelial cells expanded by primary explant culture expressed cytokeratin in all cell layers and p63 in the basal layer, indicating that cells expanded by explant culture successfully resulted in the fabrication of an epithelial cell sheet.

When seeded on temperature-responsive cell culture inserts at a density of 8×10^4 cells/cm², the oral mucosal epithelial cells harvested from primary explant culture covered the entire culture surface faster (<3 days) than epithelial cells derived from oral mucosal tissue prepared by the enzymatic method (12 days). Moreover, the CFE of epithelial cells expanded by primary explant culture was significantly higher than that of primary epithelial cells derived from oral mucosal tissue. Conversely, the colony sizes of epithelial cells expanded by explant culture were smaller than those derived from oral mucosal tissue. The epithelial cells derived from mucosal tissue included approximately 1% highly proliferative cells, which formed holoclone-like colonies, and the cells seeded on temperature-responsive cell culture insert needed about 2 weeks to reach confluence. The results from the Colony-forming assay (CFA) indicated that the cells expanded by explant culture included many adherent epithelial cells, which successfully became confluent on the culture surface in <3 days. In the explant culture method, tissue fragments without enzymatic treatment maintained cell–cell junctions and cell adhesion to the basement membrane. Therefore, the results of serial explant culture may indicate that the characteristics of oral mucosal epithelial stem/progenitor cells are maintained in the tissue fragments without treatment with proteinases. Oral mucosal epithelial cells expanded by primary explant culture is a technical method that efficiently harvests stem cell-like transient amplified cells, which is meaningful since transplantable epithelial cell sheets can be fabricated; however, further studies are required to analyze the cells expanded by primary explant culture.

As an *in vivo* test of the oral mucosal epithelial cell sheets prepared by primary explant culture, rabbit oral mucosal epithelial cell sheets were prepared and subcultured on temperature-responsive cell culture inserts. Subsequent transplantation of these autologous epithelial cell sheets to the exposed transparent corneal stroma resulted in an ocular surface that was clear and smooth, with no observable epithelial defects. After resection of the corneal and limbal epithelium, re-epithelialization of the entire cornea normally takes approximately 5–6 weeks. Moreover, strong inflammation after resection often produces granulation tissue, which impairs re-epithelialization. In contrast, after transplantation, the cell sheet immediately covered the epithelial defect and promoted regeneration of the ocular surface, consistent with the transplantation of epithelial cell sheets fabricated by primary culture [23,29]. Therefore, oral mucosal epithelial cell sheets created using the explant culture method appear to be appropriate for transplantation onto an epithelial defect and useful as cell products for clinical applications.

In summary, our results demonstrate that an explant culture method involving the use of small fragments derived from minced tissue for primary culture, was able to adequately prepare a large number of oral mucosal epithelial cells. Moreover, the harvested cells were successfully subcultured for the fabrication of epithelial cell sheets. This new method provides a novel basic culture strategy for fabricating mucosal epithelial cell grafts for clinical application of regenerative therapies. Therefore, this culture method is applicable for a wide range of epithelial defects, such as those in the pharyngeal, laryngeal, esophageal, and rectal areas.

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Appendix A. Supplementary data

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