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New culture technique of human eliminable feeder-assisted target cell sheet production

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ABSTRACT

Cultured cell sheets for transplantation generally have been co-cultured with animal feeder cells, which carry risks because of different species and results in non-contact culture between the feeder and target cells. We developed a new technique to produce human eliminable feeder-assisted target cell sheets by novel human-derived genetically modified feeder cells. Three genes (*human-derived telomerase reverse transcriptase gene*, *enhanced green fluorescent protein gene*, and *herpes simplex virus thymidine kinase gene*) were transducted into human stromal cells, which enabled genetically modified feeder cells to be immortalized, labeled, and eliminated as needed. A target cell sheet was produced as one sheet by assisting the genetically modified feeder cells and successfully transplanted *in vivo* without their contamination. Genetically modified human eliminable feeder cells could be a promising tool for cultivated cell sheet transplantation.

1. Introduction

Clinical applications of regenerative medicine have been performed recently in many fields, in which the potential for a cultivated cell sheet transplantation technique has been investigated for keratinocytes [1,2], corneal epithelial cells [3–9], oral mucosal epithelial cells [10], corneal endothelial cells [11], urothelial cells [12], and cardiomyocytes [13]. Among the cultivated cell sheets, animal-derived feeder cells, such as keratinocytes [1,2], corneal epithelial cells [3–9], oral mucosal epithelial cells [10], and urothelial cells [12], are needed to produce cell sheets. However, these culture methods using animal feeder cells can cause unknown infections and contamination from xenogeneic feeder cells [14]. Furthermore, using animal-derived feeder cells resulted in non-contiguous culture between feeder cells and target cultured cells, enabling direct stimulation for proliferation or differentiation [15,16].

We developed a technique of human eliminable feeder-assisted target cell sheet production. We focus on the production of cultivated corneal epithelial cell sheets as a model which has been used clinically to treat severe ocular diseases [5–8], and report the efficacy of a novel genetically modified human-derived feeder cell line with the properties of immortalization, labeling, and elimination as needed.

2. Materials and methods

2.1. Culture of human corneal stromal cells

Corneas were obtained from an eye bank in the USA. Epithelial and endothelial cells were removed with sterile surgical forceps from the remaining corneal scleral rims after keratoplasty. Corneal stroma was cut into a few pieces and placed endothelial cell-side down on 35 mm culture dishes containing Dulbecco's modified Eagle's medium (DMEM; Nikken Biomedical Laboratory) with 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen). Corneal stromal cells were cultured at 37 °C in 5% CO_2 and 95% air, and the medium was changed every 2–4 days.

2.2. Construction of lentiviral vector and preparation of lentivirus

Lentiviral vectors were constructed using Gateway Technology (Invitrogen). Replication-defective, self-inactivating lentiviral vectors were prepared with EF1α as an internal promoter, a phosphoglycerate kinase (PGK) promoter-neomycin resistance gene (pLentiNeo) or a PGK promoter-puromycin resistance gene (pLentiPuro) [17], and these were attR-containing destination vectors. The entry vectors containing attL were prepared as follows. Human-derived *TERT* and an internal ribosome entry site (IRES)–*EGFP*

Abbreviations: TERT, human-derived telomerase reverse transcriptase; HSV-TK, herpes simplex virus thymidine kinase; EGFP, enhanced green fluorescent protein; MMC, mitomycin C.

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genes were cloned into pENTR1A vector (Invitrogen), resulting in pENTR–TERT–IRES–EGFP. The HSV–TK gene was cloned into pENTR1A vector, resulting in pENTR–TK. LR recombination reactions were performed with the entry vectors (pENTR–TERT–IRES–EGFP and pENTR–TK) and the destination vectors (pLentiNeo and pLentiPuro), respectively, and the expression vectors (pLenti–TERT–IRES–EGFP–Neo and pLenti–TK-Puro) were created. The lentivirus was produced by co-transfecting 293T cells with the lentiviral expression vector and pLP/VSVG (encoding the VSV-G envelope protein), along with the packaging constructs pLP1 and pLP2 (Invitrogen), and the concentrated lentivirus was prepared by ultracentrifugation for 2 h at 20,000 rpm.

2.3. Infection of lentiviral vector

TERT + EGFP + TK-transducted human corneal stromal cells were produced in two steps. First, human corneal stromal cells were infected with lentivirus (pLenti–TERT–IRES–EGFP–Neo). After infection, selection by 800 μg/mL G418 (Invitrogen) was performed and *TERT + EGFP*-transducted human corneal stromal cells were produced. Second, *TERT + EGFP*-transducted human corneal stromal cells were infected with lentivirus (pLenti–TK-Puro). After infection, selection by 1 μg/mL puromycin (Sigma) was performed and *TERT + EGFP + TK*-transducted human corneal stromal cells were produced. The cells were maintained in DMEM/10% FBS.

2.4. Ganciclovir treatment

TERT + EGFP- or *TERT + EGFP + TK*-transducted human corneal stromal cells (2×10^4 cells/cm^2) were cultured in 24-well cell culture dishes. After 1 day culture, ganciclovir (0, 10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M) was added to each cell. Viable cells were counted following trypan blue staining at 0, 2, 4, 6, 8, 10, and 12 days after the start of ganciclovir treatment. The medium containing ganciclovir was changed every 2 days. The cell numbers are shown as the percentage of each value at time 0. Each value is expressed as the mean ± standard error (SE.) of three wells.

2.5. Preparation of human corneal epithelial cell sheet

Corneas were obtained from an eye bank in the USA. The remaining corneal scleral rims after keratoplasty were incubated with 2.4 U/mL Dispase solution (BD Biosciences) for 1 h at 37 °C and treated with 0.02% EDTA solution (Nacalai Tesque) for 2 min at room temperature. The epithelial cells including limbal zones were scraped with sterile surgical forceps. The collected cells were incubated with 0.25% trypsin–EDTA (Invitrogen) for 15 min at 37 °C; culture medium (DMEM/F-12 [Invitrogen] with 5% FBS, 1 nM cholera toxin [Calbiochem], 10 ng/mL human recombinant epidermal growth factor [Sigma], insulin–transferrin–selenium-G supplement [Invitrogen], 100 U/mL penicillin, and 100 μg/mL streptomycin [Invitrogen]) then was added to stop the enzyme activity. The epithelial cell suspensions ($1–7 \times 10^5$ cells/mL) were co-cultured with MMC-treated *TERT + EGFP + TK*-transducted human corneal stromal cells (2×10^4 cells/cm^2) on type I collagen gel (Collagen Gel Culturing Kit; Nitta Gelatin). MMC-treated *TERT + EGFP + TK* transducted human corneal stromal cells were prepared by treatment with MMC (8 μg/mL) for 2 h at 37 °C. After 2 to 3 weeks in culture, the epithelial cells were treated with 10^{-4} M ganciclovir for 6 or 7 days. The medium containing 10^{-4} M ganciclovir was changed every 2 to 3 days.

2.6. PCR analysis of feeder cell contamination

After treatment with 10^{-4} M ganciclovir, part of the human corneal epithelial cell sheet was harvested. *TERT + EGFP + TK*-transducted human corneal stromal feeder cells were collected as a control. Genome DNAs were extracted with QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The PCR reaction was performed as follows. The thermal cycling conditions were 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C, and 10 min at 72 °C. The long terminal repeat primers were specific for lentivirus vector integrated into human corneal stromal feeder cells. The forward primer was 5′-AAGGGCTAATTCACTCCCAA-3′, and the reverse primer was 5′-TGCGTCGAGAGAGCTCTGGTTT-3′. The GAPDH control primers were as follows. The forward and reverse primers were 5′-TCCAGAACATCATCCCTGCCTCTA-3′, and 5′-TGTTGAAGTCAGAGGAGACCACCTG-3′, respectively. PCR products were electrophoresed on a 2% agarose gel.

2.7. Transplantation of human corneal epithelial cell sheet in mice

Animal experiments were performed according to the Guidelines for Animal Experiments of Osaka University. Eight-week-old male ICR mice were obtained from SLC. Corneas were wounded using standard protocols [18,19]. Briefly, the entire corneal surface including the limbal region was exposed for 30 s to 50% ethanol, and a superficial keratectomy was performed. A human corneal epithelial cell sheet 5 mm in diameter then was trephinated and transplanted onto the cornea; the lids were closed using 8.0 nylon sutures (Alcon). Antibiotics and steroids were applied postoperatively. The mice were sacrificed 7 days after transplantation.

2.8. Immunohistochemistry

Cultivated human corneal epithelial cell sheets and mouse eyes transplanted with human corneal epithelial cell sheets were fixed with 4% paraformaldehyde in PBS for 30 min and for 3 to 4 h, respectively. After fixation, the specimens were embedded in Technovit 8100 (Heraeus Kluzer) according to the manufacturer's instructions. The sections (5 μm) were processed for hematoxylin and eosin (HE) staining and indirect immunohistochemistry. The sections were incubated in 0.05% trypsin–EDTA (Invitrogen) for 10 min at 37 °C and washed in PBS. The sections then were incubated in blocking buffer (Brockace [DS Pharma Biomedical] and 0.3% TritonX-100 in PBS) for 1 h at room temperature. The sections then were incubated overnight at 4 °C with the following primary antibodies: mouse monoclonal anti-cytokeratin 3 (1:50, Progen), and murine monoclonal anti-human mitochondria (1:10, Millipore). The secondary antibody was Alexa Fluor 488 goat anti-mouse IgG (1:200, Invitrogen). Cell nuclei were counterstained with 0.1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI, Sigma).

3. Results

3.1. Development of genetically modified human corneal stromal cells

To eliminate unknown infections and contamination from xenogeneic feeder cells, three genes (the *human-derived telomerase reverse transcriptase* (*TERT*) gene, the *enhanced green fluorescent protein* (*EGFP*) gene and the *herpes simplex virus thymidine kinase* (*HSV-TK*) gene) using two lentiviral vectors (Fig. 1A) were transducted into human corneal stromal cells anatomically present in contact with a target cultured epithelial cell layer. Fig. 1B shows the developmental procedures of *TERT + EGFP + TK*-transducted human feeder cells. Feeder cells infected with two lentiviral vectors exhibited the same fibroblast-like morphology and contact inhibition as primary human corneal stromal cells (Fig. 1C, D and F).

We then studied the proliferation and visualization of human feeder cells transducted with the *TERT* and *EGFP* genes. The primary human corneal stromal cells had a limited lifespan and were