

Original Research Article

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ESTABLISHMENT OF PRODUCTION METHOD OF SMALL INTESTINAL EPITHELIAL CELL SHEETS UNDER TWO-DIMENSIONAL CELL CULTURE

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ABSTRACT: The aim of this study is to establish the new method of small intestinal epithelial cell culture in order to make intestinal epithelial cell sheets. Cell tissue engineering and cell sheet are very unique technology for cell culture. In the field of regenerative medicine, clinical studies have already attempted to use this technology. In our study, proper cell culture condition for small intestinal cells and the method of making the cell sheet were examined. Conventional intestinal cell culture method was the three-dimensional culture. Cultured cells were unique form and called spheroid. However, this form was not appropriate for making the cell sheet. Therefore, new cell culture condition which was called the two-dimensional culture was necessary to make cell sheets. In our study, cell culture method for using rLaminin-521 coating dish and the mouse embryonic fibroblast which was co-cultured with cultured intestinal cells were the proper way to make small intestinal cell sheets. Indeed, cultured cells under two-dimensional method adhered and stretched on the dish for horizontal direction for two weeks. Gene expressions of culture cells showed that they proved to survive under that condition by confirming the stem cell markers. As to the intestinal cell sheets, the method of co-cultured with MEF enabled the making of small intestinal cell sheet. In conclusion, establishment of small intestinal cell culture in two-dimensional method and making of the intestinal cell sheet. Cell sheets will enable us to provide the efficient cell therapy from its features.

KEYWORDS: Small intestinal stem cell, cell sheet, two-dimensional culture, cell tissue engineering

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1. INTRODUCTION

Cell sheet engineering is a promising new technology for fabricating transplantable cell grafts by using thermo-responsive cell culture dishes (*UpCell*[®]) which use covalently grafted *N*-isopropylacrylamide (PIPAAm) known as thermo-responsive polymer. The grafted polymer reacts due to temperature variations and changes to its molecular form affecting cell adhesion on the surface of the culture dish *in vitro*. [1] The dish surface is hydrophobic and kept at 37 °C in order to optimize cultured cell's ability to adhere onto the surface. On the other hand, the grafted polymer takes a hydrophilic form at 20 °C, and the cultured cells easily detach from the surface of the dish without degradation of intercellular adhesion molecules [2] or extracellular matrix (ECM). [3] Since this technique requires no use of proteolytic enzyme, cells are harvested as a transplantable cell sheet with the intercellular adhesion molecules and ECM intact. [4] Having such features, cell sheets have been used for ours and a variety of other clinical studies based on new regenerative therapy. Prevention of esophageal stenosis after endoscopic submucosal dissection [5], improvement of heart function against chronic heart dysfunction [6] and improvement of corneal opacity [7] have been performed with human autologous cell sheets as clinical studies in Japan and the outcome in most cases was very favorable.

Small intestinal epithelium is a columnar monolayer and composed of two parts, consisting of crypt and villus sides. [8] Paneth cells existing in the bottom of the crypt maintain a leucine-rich environment repeat-containing G protein-coupled receptor (Lgr5)-expressing stem cells that are especially named crypt based columnar (CBC) cells. [9-11] These cells can construct a special niche [12, 13] for maintaining a stable environment for the stem cells. [12] Small intestinal stem cells are differentiated into 4 types of cells. [14, 15] Except for Paneth cells, goblet, enteroendocrine and absorptive epithelial cells exist in the villus and migrate toward the tip of villus with proliferation and differentiation. [16] Finally, these cells finish their rapid turnover life-cycle and shed themselves on the tip of villus in order to renew the epithelium with a short life-cycle of 3-5days. [17] However, Paneth cells can survive at the bottom of the crypt for approximately 6 weeks. [18] Lgr5-expressing stem cells also exist in the bottom of crypt and supply the differentiated cells to maintain the balance among these 4 types of cells. [19] In this manner, intestinal epithelium constantly repeats self-renewal for maintaining their function and treating damage to the epithelium due to radiation, inflammation, etc. [20]

In recent research, the methods for intestinal stem cell cultures have been established. [12, 21-24] Intestinal stem cells were isolated and cultured in a gel called a three-dimensional cell culture and cultured cells formed like spheroids. In a recent report, a study of intestinal epithelial regeneration has been found. Yui et al. [25] reported that intestinal stem cells were cultured using a three-dimensional culture method and cultured spheroids were instilled by enema into animal

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models of inflammatory bowel diseases (IBDs) such as ulcerative colitis (UC) and Crohn's disease (CD) and survived on the lesions.

IBDs are the intermittently and chronic epithelial inflammatory diseases. In these diseases, the extent of mucosal damage and mucosal healing are the important factors to ascertain the efficacy of the treatment. Moreover, damaged mucosa would cause colitic cancer. So mucosal healing is a substantial factor for preventing future disease and responds to treatment in IBDs.[26] If we succeed to make the intestinal epithelial cell sheets, there's a possibility that this will help the cell transplantation to treat the intestinal mucosal damage. Therefore, the aim of this research was to make small intestinal epithelial stem cell sheets.

2. MATERIALS AND METHODS

Animal preparations

Animal care was performed according to protocol approved by the Tokyo Women's Medical University Animal Experimentation Committee. C57BL/6NCrSlc mice (6-week-old males) purchased from Sankyo Labo Service Corporation (Tokyo, Japan) were used for this study.

Isolation of small intestinal crypts

Isolation of small intestinal crypts was performed in accordance with the method shown by Sato T and Clevers H.[21] Subtotal small intestines were separated from mice. To wash the surface of intestinal epithelium, 1000 μ L micropipette was used to inject cold PBS into the small intestines. After washing with PBS, the intestines were opened longitudinally and placed in a 50 mL conical tube to shake them for washing the luminal surface again with cold PBS. These intestines were cut into 2-5 mm blocks with scissors and transferred to 50 mL conical tubes with 20 mL cold PBS. The tissues were agitated with a 10 mL pipette, and the supernatant was discarded after the fragments settling down. This step was performed for 3 times. For isolating crypts from the blocks, a 20 mL of 2 mM EDTA (DOJINDO LABORATORIES, Kumamoto, Japan) buffer was added to the tube, and the tube was gently shaken at 4°C for 30 minutes. Then, the supernatant was discarded and 20mL cold PBS was added to the tube. Pipetting was performed several times and the supernatant was again discarded. After this wash, 20mL cold PBS was added to the tubes and the tissues were pipetted several times. The fragments settled down in the bottom of this tube, and the supernatant in which contained the crypts passed through a 70 μ m cell strainer (Falcon, Fisher Scientific, MA, US) to collect into a new 50 mL conical tube coated with 1% (w/v) Bovine Serum Albumin (BSA) (Sigma-Aldrich, MO, US) in PBS. This protocol after mixing the 2 mM EDTA buffer for isolating the crypts was repeated two or more times in order to collect them. The supernatant, rich with crypts was then centrifuged at 300 \times gravity for 5 minutes. The pellets settled into the bottom and the

supernatant was then discarded. Advanced Dulbecco's Modified Eagle Medium/Ham's F-12(DMEM/F-12) (Life TechnologiesTM, MA, US) containing 10% penicillin/streptomycin (Invitrogen, CA, US) was added to this tube, and the pellets in the bottom of these tubes were suspended and transferred into a 15mL conical tube. The tubes were centrifuged at $200 \times$ gravity for 3 minutes, and the supernatant was then discarded. DMEM/F-12 was added into the tube and the pellets were suspended. Crypts became single cells after going through this process twice. After this protocol, a new medium was added to suspend the cells for counting the number of cells by using a phase contrast microscope.

Three-dimensional (3D) culture

Isolated cells were suspended into MatrigelTM (Corning Inc., NY, US) at 50×10^4 cells/mL on ice. The MatrigelTM in which the cells were embedded were applied onto 12 wells plates at 500 μ L/well and incubated in 5% CO₂ incubator at 37 °C for 30 minutes to form a hemisphere like shape. Stem cell culture medium was as follows. DMEM/F-12 consisted of several supplements that were N2 supplements (Life technologiesTM), B27 supplements (Life technologiesTM), *N*-acetylcysteine (Sigma-Aldrich), murine recombinant Epithelial Growth Factor (EGF) (Life technologiesTM), murine recombinant noggin (Pepro Tech, NJ, US) and human recombinant R-spondin-1 (Stem RD Inc., CA, US). The epithelial cells were cultured for approximately one week. The cells were observed by phase contrast microscope at 7days and 14days after cell embedding.

Two-dimensional (2D) culture

Human recombinant Laminin-521 (rLaminin-521, BioLamina, Sundbyberg, Sweden) coated dishes were prepared. Human rLaminin-521 of which the concentration was 20 μ g/mL was coated on a 35 mm cell culture dish in 5% CO₂ incubator at 37 °C for one hour. After incubation, that dish was washed twice with PBS. The cells with a density of 100×10^4 cells / dish were seeded on that culture dish and cultured for two weeks with the same medium which was previously described. The cells were observed by phase contrast microscope at 14days.

RNA Extraction and RNA reverse transcription

To compare DNA expression level among these three groups (Non-culture (NC) primary cells, 3D culture cells, 2D culture cells), RNA sampling for each group was performed by RNeasy Plus Mini Kit (50) (QIAGEN, Hilden, Germany) and measurement of the concentration of the total RNA level was performed by NanoDrop 2000c Uv-Vis Spectrophotometer (Thermo SCIENTIFIC, MA, US). Three kinds of RNA were reverse-transcribed into cDNA with PrimeScript[®] RT reagent Kit (Perfect Real Time) (TAKARA BIO INC., Shiga, Japan) and iCyclerTM (Bio-Rad Laboratories, Inc., CA, US).

Real-time PCR analysis

TaqMan probes were used to analyze the gene expressions of these cells by primer Express Software (Applied Biosystems, Foster City, CA). TaqMan probes designed for mouse GAPDH (housekeeping gene), Lgr5 [27] andBmi1[28] (intestinal stem cell marker), Muc2 [29, 30] (goblet cell marker), Chga [25] (enteroendocrine cell marker), Cd24a [12] (Paneth cell marker), MKi67 [31] (proliferation marker), Epcam[32, 33] (intestinal epithelial cell marker) were used (Table1). The GAPDH was used as an internal standard for the integrity and the quantity of the RNA.

Table 1: Taqman's probe and Gene symbol

Molecular name	gene symbol	probe ID
<i>Small intestinal cell markers, Stem cell marker</i>		
Leucine-rich repeat-containing G-protein coupled receptor 5	Lgr5	Mm00438890_m1
B lymphoma Mo-MLV insertion resion 1	Bmi1	Mm00776122_gH
<i>Goblet cell marker</i>		
Intestinal mucin 2	Muc2	Mm01276696_m1
<i>Enteroendocrine cell marker</i>		
Chromogranin A	Chga	Mm00514341_m1
<i>Paneth cell marker</i>		
Nectadrin heat stable antigen	Cd24a	Mm01191887_g1
<i>Proliferation marker</i>		
Antigen identified by monoclonal Antibody by Ki67	MKi67	Mm01278617_m1
<i>Epithelial marker</i>		
Epithelial cell surface antigen	Epcam	Mm00493214_m1

Statistical analysis

The results of RT-PCR were presented as the mean \pm SD, and the statistical analyses were carried out, according to ANOVA and Fisher's PLSD by using StatView 5.0 software. AP-value of less than 0.05 ($P < 0.05$) was considered significant.

Fabrication of cell sheets

To distinguish cell species on the same dish, the C57BL/6-Tg (CAG-EGFP) mice (Charles River Laboratories Japan, Tokyo, Japan) were used to harvest the small intestinal epithelial cells. Procedure of the cell preparation was previously described. The epithelial cells were cultured on 35 mm thermo-responsive cell culture dishes (CellSeed, Tokyo, Japan) which were coated with human rLaminin-521 for two weeks. After two weeks, mouse embryo fibroblast (MEF) feeder cells (Repro CELL Inc. Tokyo Japan) were used to co-culture with small intestinal epithelial cells for 2-3 days. The cell culture dish was incubated at 20°C for several minutes to collect cultured cells as a cell sheet. This cell sheet was analyzed by using a fluorescence

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microscope. This was treated with 5%, 10%, 20%, 30%, and 50% acetone every 3 minutes at room temperature and fixed with 70% acetone for 10 minutes at 4°C. After the fixation, it was rinsed with PBS and incubated with rhodamine-phalloidin (Thermo Fisher SCIENTIFIC, 1:250) for 10 minutes at room temperature and enclosed with ProLong[®] Diamond Antifade Mountant with DAPI (Thermo Fisher SCIENTIFIC).

It was then observed by using a phase contrast microscope and scanning laser microscope (FV1200, IX83, OLYMPUS, Tokyo, Japan).

3. RESULTS AND DISCUSSION

Phase contrast Microscopic Examinations in 3D and 2D cultures

Cultured cells became round in shape after a few days and eventually these cells expanded and formed a spheroid for two weeks in 3D culture (Fig 1).

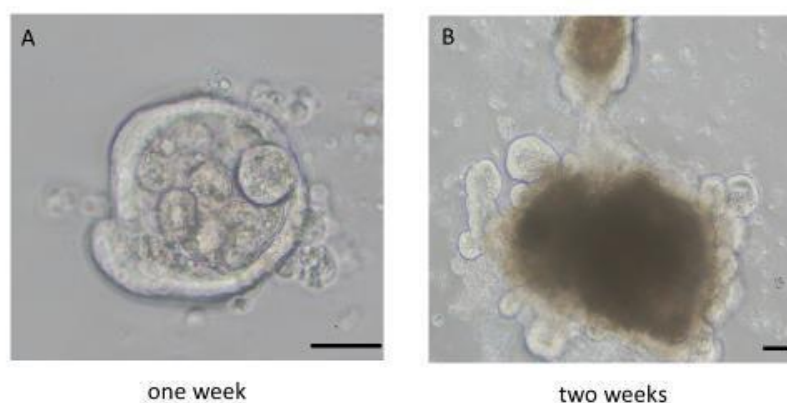


Figure 1. Three dimensional (3D) intestinal cell culture.

A: Small intestinal epithelial cells from mice were cultured in the gel for one week to form a round in shape. Scale bar: 50µm

B: Small intestinal stem cell culture in the gel for two weeks. Their size became larger than one week and formed like a spheroid. Scale bar: 50µm

However, in 2D cultures, epithelial cells were stretched in a horizontal direction and survived on the dish, which was coated with rLaminin-521, for two weeks (Fig 2A). In the case of using non-coated dishes, isolated cells were not able to adhere to the surface (Fig 2B).

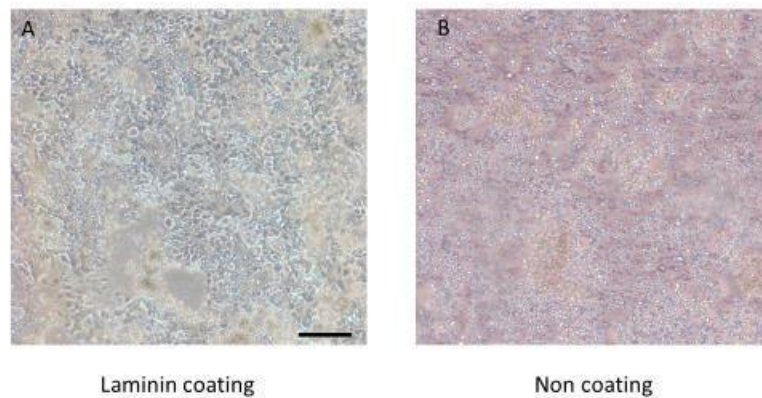


Figure 2. Two dimensional (2D) intestinal cell culture

A: Isolated intestinal epithelial cells adhered and survive on the rLaminin-521 coated culture dish. These cells spread to the horizontal direction.

B: On the normal dish, isolated intestinal epithelial cells could not adhere and survive.

All cells floated on the medium. Scale bar: 100 μ m

Real-time PCR analysis and Gene Expression

Comparative analyses of gene expressions in three groups (NC cells, cultured cells in 3D and 2D) are shown in Fig 3.

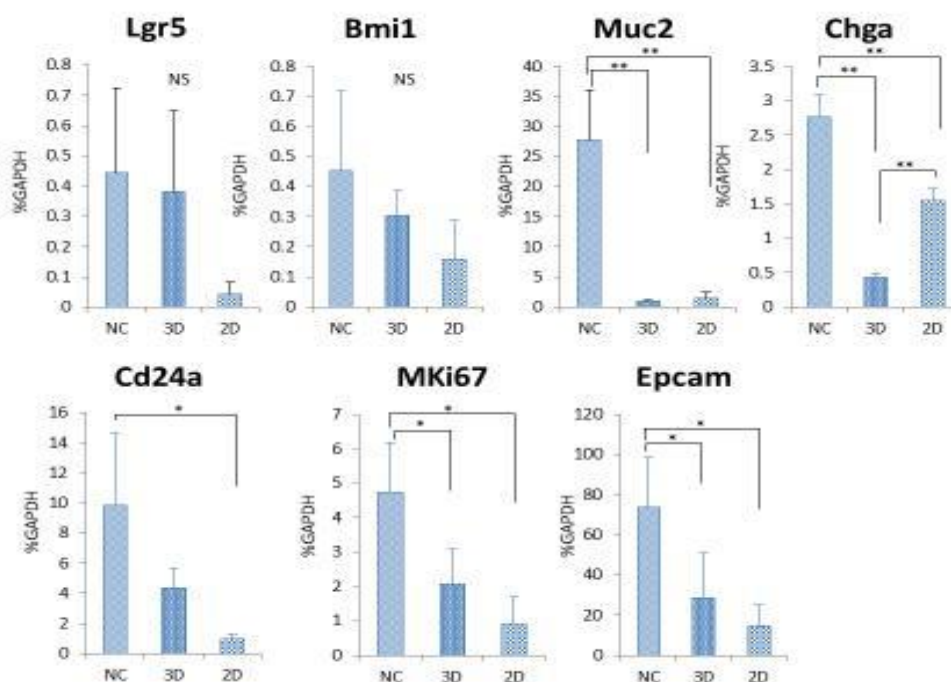


Figure 3. Comparative data of gene expressions among three types of cells showed as follows.

Stem cell markers (Lgr5 and Bmi1) had no significant difference among three groups.

This result represented that two culture methods were effective for intestinal epithelial cell culture.

Other markers of these cultured cells had significant differences compared with NC cells. Cultured stem

cells that were not differentiated could survive under these culture conditions. Data were expressed as mean \pm SD.

(n=3, * $P<0.05$, ** $P<0.005$, NS: no significant) (NC: non-cultured primary cells, 3D: cultured cells under a three-dimensional culture, 2D: cultured cells under a two-dimensional culture)

As for these two cultured cells, the level of all gene expressions except for chromogranin A showed no significant differences. However, the level of gene expressions in each cultured cell tended to decrease and had significant differences with several kinds of gene symbols as compared with NC cells.

Small intestinal cell sheets

Co-cultured small intestinal epithelial cells with MEF cells formed cell sheet about 10mm in diameter (Fig 4).

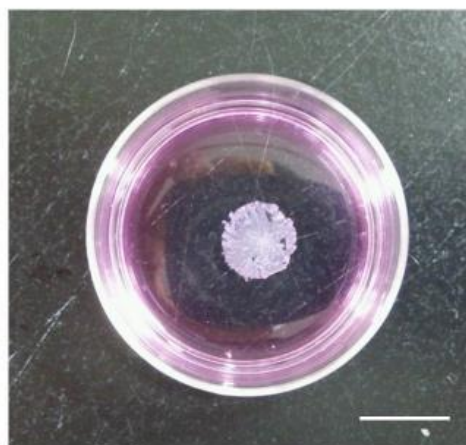


Figure .4. Intestinal epithelial cell sheet

Isolated intestinal epithelial cells co-cultured with Murine Embryonic Fibroblasts (MEF) on temperature-responsive cell culture dish. In this figure, cell sheet was finished to incubate at 20°C for several minutes. Cell sheet floated in the medium and it was dipped up by plastic film to transplant. Scale bar: 1cm

Under a phase-contrast microscope, intestinal epithelial cells which were composed of GFP-positive cells were surrounded by the MEF cells to bridge the space among them (Fig 5). As the result of this phenomenon with an aid of MEF, cell sheets increased their strength and were harvested easily from temperature-responsive cell culture dish upon temperature reduction.

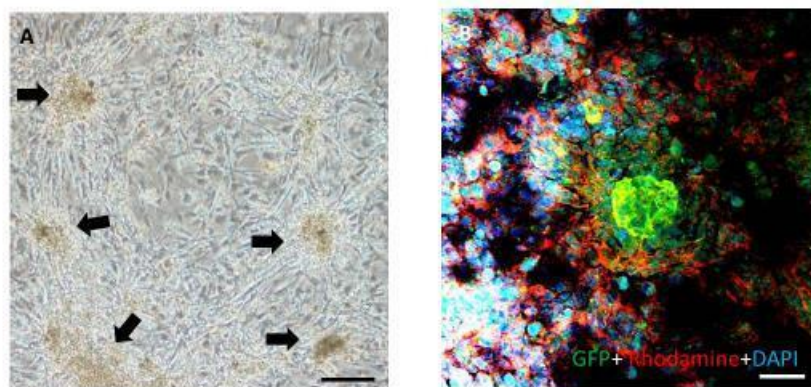


Figure.5. The images of phase-contrast microscopic and scanning laser microscopic examinations

A: In phase-contrast microscope, intestinal epithelial cells (black arrow) were surrounded by the MEF cells. MEF cells bridged the space among intestinal cells. Scale bar: 50 μ m

B: In scanning laser microscope, GFP positive cells from C57BL/6-Tg (CAG-EGFP) mouse's intestine were the cultured intestinal cells.

Scale bar: 50 μ m

DISCUSSION

The intestinal epithelium has been reported to have a self-renewal characteristic *in vivo* [18, 34, 35]. In recent reports, the intestinal epithelium could be isolated from the organ and culture *in vitro*, and cultured cells have retained their characteristics [21, 25]. In our study, the reproducibility of their culture method was first taken. In an actual experiment, isolated intestinal epithelial cells became a round-shaped structure in a 3D culture. This phenomenon has been reported by Sato et al. [16, 21, 36] and it was called a crypt-like organoid. The central part of this organoid represented a cyst-like structure and the marginal region represented a crypt-like budding structure [21]. In this organoid, stem cells and Paneth cells both existed [10, 12, 37] at the bottom of this crypt-like structure.

It was noted that the intestinal epithelial stem cells were very fragile and became anoikis [21]. To reduce anoikis, 3D cultures with the Matrigel[®] were used as a substitute for scaffold stabilized the environment for stem cell growth. This method enabled us to culture intestinal stem cells without a mesenchymal cellular niche, the essential mechanism for the differentiation and proliferation of intestinal stem cell incorporating Paneth cells [12, 38, 39]. The form of cultured stem cells became a round-shape called "spheroid" and differed from the original shape. This spheroid was surrounded with epithelium and a basement membrane component was placed inserted [40]. When cultured stem cells were transplanted into organs, the school of thought was that contact with their basement membrane and adhesive surface was better for cell transplantation to increase its organ acceptance rate. As a result, the structural features of cell sheets were thought

to be very suitable for transplantation. For this reason, we attempted to make small intestinal cell sheets. In previous reports, adhesiveness of cell sheets has proven to have a strong ability to survive within organs as compared with other method of transplantation *in vitro* [6, 41, 42]. Cell sheets retained their adhesion molecules consisting of ECM and intercellular adhesion molecules. These proteins assisted them in attaching to organs. Therefore, this led to a substantial success rate improvement in cell transplantation. Indeed, Ohki et al [5] reported to use human autologous oral mucosal epithelial cell sheets for grafting onto the esophageal ulcerative lesions as new regenerative therapy to prevent the stenosis after endoscopic submucosal dissection (ESD). In this study, cell sheets prevented the stenosis through the promotion of epithelialization at the lesion. The important aspect of cell therapy or cell transplantation is to survive on the site to produce the effect against diseases. Moreover, in order to increase cell transplantation efficiency, a grafting device needed to be developed [43]. This device enabled transplantation of cell sheets into the organs repeatedly and easily after an esophageal ESD. For these reasons, it was thought that produce and transplantation of intestinal cell sheets were an efficient method for intestinal epithelial regeneration.

As to the making of cell sheets, it was necessary to examine the cell culture conditions. There were some problems we encountered making the small intestinal cell sheets, because of their spherical structure. Therefore, new requirement studies for stem cell cultures in 2D culture conditions were performed. Previously in reports, several methods of 2D cultures were examined. [44-46] However, reproducibility of these reports was insufficient. In our study, rLaminin-521 which was the basement membrane component acted as a scaffold to maintain the cell adhesion [47, 48] were used in an attempt to improve cell adhesion factor on the dish in 2D culture. This component was capable of adhering intestinal cells on the dish. Moreover, in this method, chemically defined material was used as compared with previous methods.

Comparative study of gene expression among NC cells, cultured cells in 3D and 2D conditions from mice small intestines was examined and there were some significant differences in the level of gene expression between NC and cultured cells. However, existence of several types of intestinal genes in 2D culture was clearly beneficial for the making of cell sheets and this technique will be suitable for the making of intestinal cell sheets *in vitro*. In our actual experiment, cultured intestinal cells adhered sparsely on the dish and the filling of space between cells was necessary to collect them as cell sheets. MEF cells were valuable ingredients to fill in the space in this regard. According to these results, intestinal stem cells have become cell sheets. However, the quality and purity of these sheets would not be adequate. To have a better effect on intestinal diseases if these sheets will be used to treat, this problem should be solved. Adjustment of reagent, the number of seeding cells and concentration of laminin are thought to be the examination items in our next study.

As tools of new regenerative therapy, our cell sheets and cell tissue engineering will be useful for grafting cells to the organs. In our next report, we intend to use and examine our intestinal epithelial cell sheets as critical tools for conducting mucosal regeneration and healing in animal models. If higher quality intestinal stem cell sheets of greater purity can be made, this will be a new catalyst to treat intestinal diseases, as tissue engineered using regenerative therapy.

CONFLICT OF INTEREST

Teruo Okano, Ph.D is a founder and member of the board of Cell Seed Inc., which has licences for certain cell sheet-related technologies and patents from Tokyo Women's Medical University.

Masayuki Yamato, Ph.D is the ownership and partnership: Share holder of CellSeed Inc., Tokyo, Japan.

Other authors have no financial relationship to disclose.

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