**Figure 1** Feeder layers. Human dermal fibroblasts (a) and NIH/3T3 cells (b) were examined using phase-contrast microscopy. Gene expression was analysed by reverse transcription PCR. Both human dermal fibroblasts and NIH/3T3 cells expressed many factors for the maintenance of stem/progenitor cells and the growth of epithelial cells (c). Scale bars: 100 μm (a, b). CC, cystatin C; EPR, epiregulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; IGF1a, insulin-like growth factor 1a; KGF, keratinocyte growth factor; N-cad, N-cadherin; PTN, pleiotrophin; Shh, sonic hedgehog.
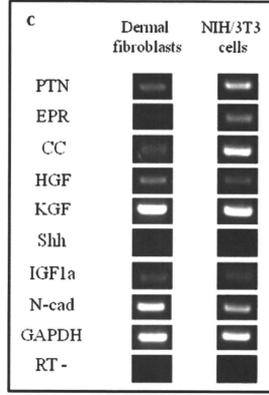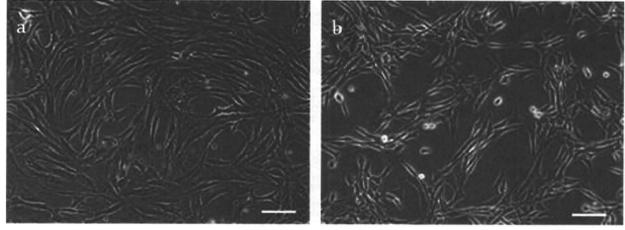
3-μm thick frozen sections. Cryosections from the cell sheets were immunostained with monoclonal antibodies against keratin 1 (K1, LHK1; Abcam, Cambridge, UK), keratin 3/76 (K3/76, AE5; Progen), keratin 4 (K4, 6B10; Abcam), keratin 10 (K10, DE-K10; DakoCytomation, Glostrup, Denmark), keratin 13 (K13, 1C7; American Research Products, Belmont, Massachusetts, USA), keratin 15 (K15, LHK15; Millipore), p63 (4A4; Santa Cruz Biotechnology), ZO-1 (1A12; Zymed, South San Francisco, California, USA), MUC16 (Ov185; Abcam), a polyclonal antibody against keratin 12 (K12, L-15; Santa Cruz Biotechnology), followed by incubation with Alexa488-labelled secondary antibodies (Molecular Probes, Eugene, Oregon, USA). Nuclei were co-stained with Hoechst 33342 (Sigma), and the cell sheets were mounted with PermaFluor (Beckman Coulter, Miami, Florida, USA). Slides were observed using confocal laser scanning microscopy (LSM-710; Carl Zeiss). The same concentration of corresponding normal, non-specific IgG was used as negative control. The percentage of p63 and K15-positive cells in each cultured cell sheet was calculated.

Statistical analysis

Data were analysed using t tests; $p < 0.05$ was considered statistically significant.

RESULTS

Human dermal fibroblasts had morphological characteristics similar to those of NIH/3T3 cells (figure 1a,b). The gene expression pattern of dermal fibroblasts was similar to that of NIH/3T3 cells (figure 1c). Although dermal fibroblasts did not express epiregulin (EPR), other genes including pleiotrophin (PTN), cystatin C (CC), hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), insulin-like growth factor 1a (IGF1a) and N-cadherin (N-cad) were expressed by both dermal fibroblasts and NIH/3T3 cells.

Colony-forming assays revealed that human dermal fibroblasts as well as NIH/3T3 cells are able to support the ex-vivo expansion of oral mucosal epithelial cells (figure 2a–d). The mean colony-forming efficiency of the primary cultures was $1.5 \pm 0.8\%$ in the fibroblast group and $2.3 \pm 0.8\%$ in the 3T3 group (mean±SD, n=3) (figure 2e), and the difference was not statistically significant (p=0.266, t test). The colony size in the fibroblast group (15.0 ± 11.5 mm^2) was larger than that in the 3T3 group(6.4 ± 2.1 mm^2). However, the difference was not statistically significant (p=0.271, t test).

Oral mucosal epithelial cell sheets were successfully cultured with human dermal fibroblasts and NIH/3T3 cells (figure 3a,b), and all of the cell sheets were successfully harvested by reducing the temperature to 20°C for 30 min. Therefore, all of the cell sheets passed the recovery test. The harvested cell sheets in both groups, flattened at their basal and apical surfaces, were composed of four to five layers of small basal cells, flattened middle cells and polygonal flattened superficial cells (figure 3c,d).

Immunofluorescence analyses revealed that cell sheets in both groups have a similar marker expression pattern (figure 4). K3/76, a marker for corneal and oral mucosal differentiated epithelial cells,[10] was positive in both groups. K12,

Figure 2 Colony-forming assay. Human dermal fibroblasts (a) as well as NIH/3T3 cells (b) supported the ex-vivo expansion of human oral mucosal epithelial cells. Cells were cultured for approximately 10 days, followed by fixation and staining with rhodamine B (c, dermal fibroblasts; d, NIH/3T3 cells). Colony-forming efficiency (CFE) was calculated, and no statistically significant differences were found between the human dermal fibroblasts and NIH/3T3 cells (e). Scale bars 100 μm (a, b).
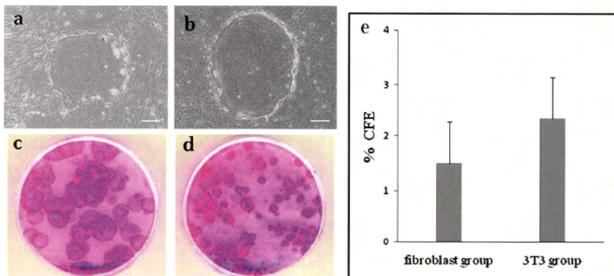
a corneal-epithelium-specific marker,[10] was not expressed in either group. Although K4 and K13 are markers for mucosal stratified squamous epithelia,[11][12] only K4 was detected in the superficial cells in both groups. K1 and K10, markers for supra-basal cells in the epidermis,[13] were negative in both groups. ZO-1, a marker of tight junctions,[14] and MUC 16, a membrane associated-mucin specific to ocular surfaces, were expressed in both groups.

p63, which has been proposed to be a corneal epithelial stem/progenitor cell marker,[15] was expressed in the basal cells of both groups (figure 5a,b). The percentage of p63-positive cells in the fibroblast group (46.1±4.2%) was significantly higher than that in the 3T3 group (30.7±7.6%) (p=0.038, t test) (figure 5e). K15, a specific basal cell component of the epidermis and other stratified squamous epithelia,[16] was positive in basal cells in both groups (figure 5c,d). There were no significant differences between the percentages of K15-positive cells in the fibroblast group (24.0±3.7%) and the 3T3 group (20.6±2.5%) (p=0.257, t test) (figure 5f).

The cell viability of the cultured cell sheets in the fibroblast group and the 3T3 group was 88.7±4.1% and 85.9±3.5%, respectively. The purity of the epithelial cells in the cultured sheets was 98.2±1.9% and 96.3±3.6%, respectively. There were no statistical differences in cell viability (p=0.408, t test) or purity (p=0.466, t test) between the groups.

Figure 3 Human oral mucosal epithelial cell sheets. Examination of cell morphology was performed using phase-contrast microscopy (a, dermal fibroblasts; b, NIH/3T3 cells) and H&E staining (c, dermal fibroblasts; d, NIH/3T3 cells). Scale bars 100 μm (a, b), 50 μm (c, d).
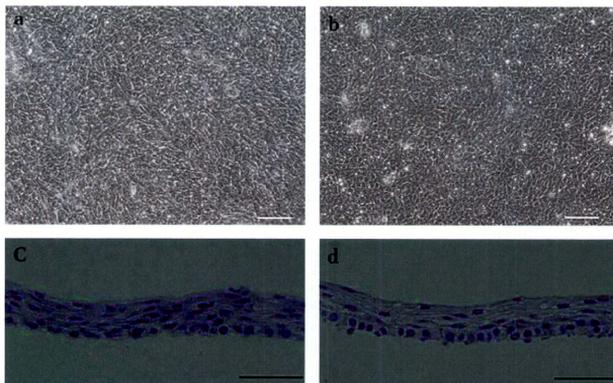
DISCUSSION

Dermal fibroblasts were shown to express many genes required for the maintenance of epithelial stem/progenitor cells and the proliferation of epithelial cells. Sugiyama et al[4] reported the expression of PTN, EPR, CC, HGF, KGF and IGF1a by human mesenchymal stem cells. In the current study, human dermal fibroblasts were confirmed to express N-cadherin in addition to these factors. The colony-forming efficiency with human dermal fibroblasts was similar to that with NIH/3T3 cells, and a colony-forming assay revealed that human dermal fibroblasts can expand oral mucosal epithelial cells well. In addition, immuno-fluorescence analyses revealed that cell sheets cultured with human dermal fibroblasts, as well as with NIH/3T3 cells, expressed markers such as K3/76, ZO-1, MUC16, p63, and K15. Moreover, cell sheets cultured with human dermal fibroblasts contained more p63-positive cells than those cultured with NIH/3T3 cells. Therefore, it was suggested that human dermal fibroblasts can maintain stem/progenitor cells in expansion more efficiently than NIH/3T3 cells.

The cultivation of epithelial cells with 3T3 feeder layers has been already established.[18] Also, a number of investigators has reported positive results for clinical treatments with cultured epithelial cells using 3T3 feeder layers.[1][18][19] However, 3T3 cells [3] have the potential risk of transmitting murine infectious diseases. The use of xeno-free feeder cells, especially autologous

Figure 4 Immunohistochemical analyses of human oral mucosal epithelial cell sheets. Staining of human oral mucosal epithelial cell sheets cultured with dermal fibroblasts and NIH/3T3 cells with anti-keratin 3/76 (K3/76), anti-keratin 12 (K12), anti-keratin 4 (K4), anti-keratin 13 (K13), anti-keratin 1 (K1), anti-ZO-1 and anti-MUC16 antibodies. Nuclei were co-stained with Hoechst 33342. ZO-1 expression is marked with arrows. Scale bars 50 μm.
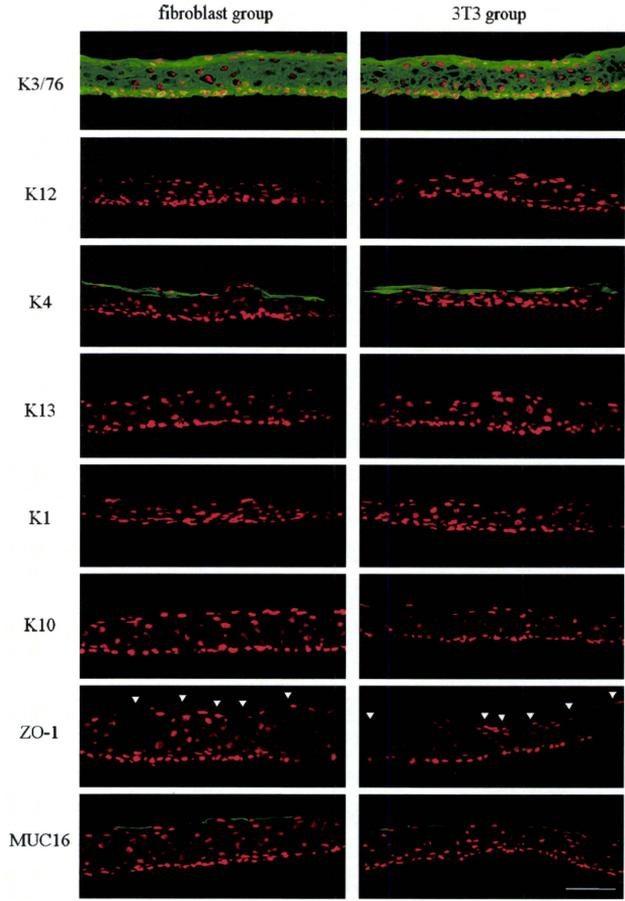

feeder layers, can prevent this problem. Although human adipose tissue-derived or bone marrow-derived mesenchymal stem cells can be used to generate transplantable epithelial cell sheets, dermal fibroblasts can be obtained with much less invasion to patients. Therefore, dermal fibroblasts are more desirable as an autologous feeder cell source than mesenchymal stem cells. Whereas the colony-forming efficiency of human limbal epithelial cells was 1.9±1.8% with bone marrow-derived mesenchymal stem cells,[5] that of human oral mucosal epithelial cells was 1.5±0.8% with human dermal fibroblasts in the current study. The colony-forming efficiency in these two reports cannot be compared directly, because of differences in

the cultured epithelial cell type, media and sera. However, both feeder layers are thought to be able to generate transplantable epithelial cell sheets.

A xeno-free culture method of keratinocytes derived from skin using human dermal fibroblast has already been reported.[7] Therefore, it is well known that human dermal fibroblasts have a feeder effect on keratinocytes. Here, we cultured oral mucosal epithelial cells using human dermal fibroblast feeder layers. We are planning to use the cultured cell sheets for ocular reconstruction in future experiments. Zakaria et al[21] recently reported a new culture and transplantation method of limbal epithelial cells without xenogenic materials. If oral mucosal epithelial cells

Figure 5 Analyses of human oral mucosal epithelial cell sheets for stem/progenitor markers. Anti-p63 staining (a, b) and anti-keratin 15 (K15) staining (c, d) of human oral mucosal epithelial cell sheets cultured with dermal fibroblasts and NIH/3T3 cells. Nuclei were co-stained with Hoechst 33342. Scale bar 50 μm. The percentage of p63-positive cells in the cell sheets cultured with dermal fibroblasts was significantly higher than that in cell sheets cultured with NIH/3T3 cells (e). The percentage of K15-positive cells was not significantly different between the groups (f). *p<0.05, t test.
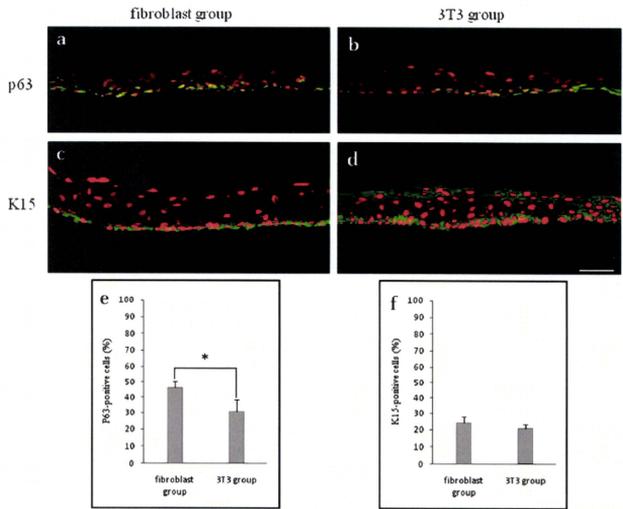
can be cultured successfully, this method can also be an alternative to the method using 3T3 cells.

We recently developed a validation system for tissue-engineered epithelial cell sheets to be used in corneal regenerative medicine.[22] There has been no other established evaluation method for epithelial cell sheets before transplantation to date. However, the quality of cell sheets for clinical use can be standardised even in different facilities. We evaluated cell sheets using our validation method and obtained positive results. We thus believe that the oral mucosal epithelial cell sheets cultured with this method can be successfully used for ocular reconstruction.

It was previously reported that fibroblasts can affect the phenotypic characterisation of keratinocytes in co-culture.[22 23] However, epithelial cell sheets cultivated in the current study did not express K1 or K10, markers for suprabasal cells in the epidermis. Therefore, we propose that the phenotypic characterisation of keratinocytes cultured in the current study did not reflect that of the epidermis.

We also demonstrated that modified KCM worked well to generate oral mucosal epithelial cell sheets. Many methods using cholera toxin have been reported for the cultivation of human corneal or oral mucosal epithelial cells and human epidermal keratinocytes.[17 18 24] Agents known to increase the level of cellular cyclic AMP, including cholera toxin and isoproterenol, have been reported to increase the growth of colonies of cultured human epidermal cells and keratinocytes derived from other stratified squamous epithelia.[25] We also demonstrated the effectiveness of modified KCM with isoproterenol in the current study.

In conclusion, our novel culture system with post-mitotic human dermal fibroblast feeder cells with clinically approved products is effective and safe. Therefore, this system can be used as an alternative cultivation method for human oral mucosal epithelial cell sheets.

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