Histological and immunohistochemical analyses

For histological examination, native rat oral mucosa, native rat skin, harvested oral mucosal epithelial cell sheets, and transplanted cell sheets were fixed with 10% neutral buffered formalin (Wako Pure Chemicals, Osaka, Japan), routinely processed into 3-μm thick paraffin-embedded sections, and stained with hematoxylin and eosin. For immunohistochemistry, deparaffinized sections were washed with PBS and digested by heat treatment. Sections were then treated with one of the following antibodies: anti-cytokeratin (CK) 4 (1:200, 6B10, Progen Biotechnik, Heidelberg, Germany), anti-CK13 (1:200, 1C7, Progen Biotechnik), anti-CK 14 (1:200, L002, Santa Cruz Biotechnology, Santa Cruz, CA), anti-CK 10 (1:10 LHP1, Biomeda Corp., Foster City, CA), anti-p63 (1:500, 4A4, Santa Cruz Biotechnology) or anti-PCNA (proliferating cell nuclear antigen) (1:500, 5A10, Santa Cruz Biotechnology), overnight at 4°C. All sections were then peroxidase stained using the LSAB 2 kit (DakoCytomation, Glostrup, Denmark) according to the manufacturer's suggested protocol.

Electron microscopy

Glutaraldehyde-fixed specimens were rinsed in phosphate buffer and post fixed in 2% osmium tetroxide for 2 h at 4°C. The specimens were then dehydrated through a graded series of ethanol and methyl glycidyl ether, and embedded in epoxy resin according to standard techniques. Semi-thin sections (1.5 μm thickness) were then stained with toluidine blue and a suitable area was chosen. The blocks were trimmed and thin-sectioned (80 nm thickness), stained with 4% uranyl acetate-Reynolds lead nitrate, and examined by transmission electron microscopy (TEM) (JEM2000EX, JEOL, Tokyo, Japan) at 80 kV.

RESULTS AND DISCUSSION

Fabrication of oral mucosal epithelial cell sheets

Buccal mucosal epithelial cells were isolated from rat oral cavities, and seeded on temperature-responsive culture dishes with mitomycin C-treated NIH/3T3 feeder layers. During *in vitro* culture, the epithelial cells formed cell colonies and then grew to confluence within 5 days. After an additional 2–3 days of culture, the tissue-engineered epithelial cell sheets were harvested by temperature reduction to 20°C for 30 min. All the cultured epithelial cells spontaneously detached from the dish surface as a single contiguous cell sheet, and the epithelial cell sheets were stratified, consisting of 2–3 cell layers [Fig. 3(c)]. Compared to oral mucosal epithelial cells of different species including human,[9,15] rabbit, [11] and canine,[16] rat oral mucosal epithelial cells showed increased proliferation under the same culture conditions. Colony forming assay of epithelial cell sheets fabricated under these culture conditions revealed that these cell sheets retained epithelial stem/progenitor cells.[11] However, when the cultured epithelial cells were subjected to temperature reduction after prolonged

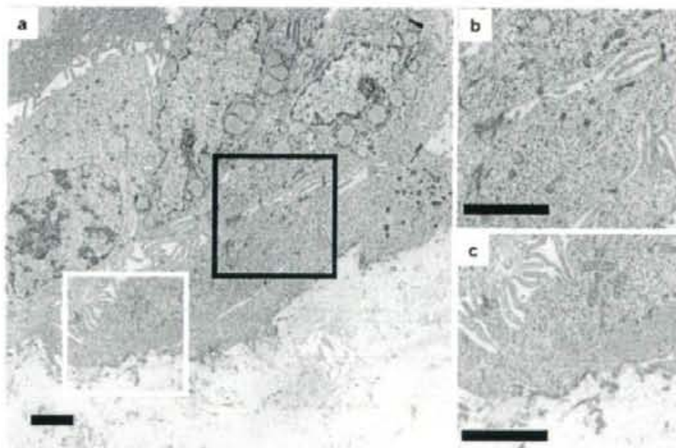

Figure 2. Transmission electron microscopy of the transplanted oral mucosal epithelial cell sheets. Oral mucosal epithelial cell sheets 1 day after subcutaneous transplantation (a). The area enclosed by the black box in A shows the presence of cell-to-cell junctions (b). The area enclosed by the white box in A reveals the presence of an immature basement membrane along the basal surface of the transplanted cell sheets (c). Scale bars represent 2 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

culture (over 10 days), the epithelial cell layers became extremely fragile and transplantable cell sheets could not be obtained (data not shown).

Subcutaneous transplantation of autologous tissue-engineered oral mucosal epithelial sheets

We transplanted autologous oral epithelial cell sheets under the skin to examine the effects of the ectopic sites on proliferation and differentiation of tissue-engineered epithelial cell sheets. When the oral mucosal epithelial cell sheets were transplanted subcutaneously, the epithelial tissues were clearly visible throughout the follow-up period. However, while the borders of the transplanted cell sheets remained clearly defined, the size of grafts decreased over time, to ~20% of the original size at day 7. One day after transplantation, the epithelial cell sheets showed a similar appearance to the harvested epithelial cell sheets prior to transplantation and maintained stable attachment with the host connective tissue of the skin flaps [Fig. 3(d)]. Interestingly, TEM revealed that both desmosomes and hemidesmosomes were present within the transplanted cell sheets [Fig. 2(a,b)], and an immature basement membrane was regenerated beneath the cell sheets within 24 h after transplantation [Fig. 2(c)]. This rapid regeneration of the epithelial basement membrane was not observed when epithelial cell sheets were harvested by proteolytic treatment with dispase,[17] suggesting that the ECM deposited during culture and maintained during cell harvest from temperature-responsive culture surfaces,[18,19] promoted basement membrane regeneration after transplantation.

Strong inflammatory responses within the subcutaneous tissues were observed during the first 3 days following cell sheet transplantation. Coincidentally, during this period, the transplanted epithelial cells showed intense proliferation and cell stratification. At day 3, the regenerated epithelial tissues consisted of cuboidal basal cells, flattened middle cells, and flattened polygonal superficial cells, similar to the native oral mucosa [Fig. 3(e)]. Over time, the regenerated epithelia increased in thickness reaching up to 15 cell layers at day 7 [Fig. 3(g)]. After 1 week, however, the regenerated epithelia gradually decreased in thickness, with loss of cell nuclei in the upper layers [Fig. 3(h,i)]. After 3 weeks, the transplanted oral mucosal epithelial cell sheet degenerated and no remnants were observed on the skin flaps (data not shown).

Cytokeratin expression profiles

To examine the possibility of phenotypic modulation of the grafted oral mucosal epithelial cells

TABLE I
Immunohistochemistory Expression Profiles

		CK4	CK13	CK10	CK14	p63	PCNA
Oral mucosal epithelium	S	+	+	−	−	+	+
	IM	+	+	−	−		
	B	−	−	−	+		
Skin epidermis	S	−	−	+	−	+	+
	IM	−	−	+	+		
	B	−	−	−	+		
Cell sheet	S	+	+	−	+	+	±
	IM	−	−	−	+		
	B	−	−	−	+		
Day 1	S	+	+	−	+	+	+
	IM	−	−	−	+		
	B	−	−	−	+		
Day 3	S	+	+	−	+	++	++
	IM	−	−	−	+		
	B	−	−	−	+		
Day 5	S	+	+	−	+	+	+
	IM	+	+	−	−		
	B	−	−	−	+		
Day 7	S	+	+	−	−	+	−
	IM	+	+	−	+		
	B	−	−	−	+		
Day 10	S	+	+	−	−	+	−
	IM	+	+	−	+		
	B	−	−	−	+		
Day 14	S	+	−	−	−	±	−
	IM	+	−	−	+		
	B	−	−	−	+		

after subcutaneous implantation, we examined cytokeratin expression within the transplanted tissues (Table I). CK4 and CK13, which are markers of nonkeratinized, stratified epithelia,[20] were expressed in the middle and superficial layers of the native oral mucosal epithelium [Figs. 4(a) and 5(a)] but showed faint expression in the tissue-engineered cell sheets [Figs. 4(c) and 5(c)] after harvest from the temperature-responsive dishes. Interestingly, CK4 and CK13 were observed in all suprabasal cell layers of the regenerated epithelia. CK4 and CK13 were detected in regenerated epithelia for 14 and 7 days after transplantation, respectively, followed by a gradual loss of expression for both markers [Figs. 4(d–i) and 5(d–i)]. CK10, a marker of the keratinized skin epidermis,[20] was not detected in the tissue-engineered epithelial cell sheets or any of the regenerated epithelia (Fig. 6). CK14 which is considered a marker of basal progenitor cells,[21,22] was strongly expressed in the fabricated epithelial cell sheets [Fig. 7(c)] and all cell layers in the regenerated epithelia at day 3, indicating that the cultured oral mucosal epithelia and the early regenerated tissues possessed an activated phenotype [Fig. 7(e)]. However, CK14 could only be detected in the basal and lower cell layers at days 5, 7, 10, and 14 [Fig. 7(f–i)].

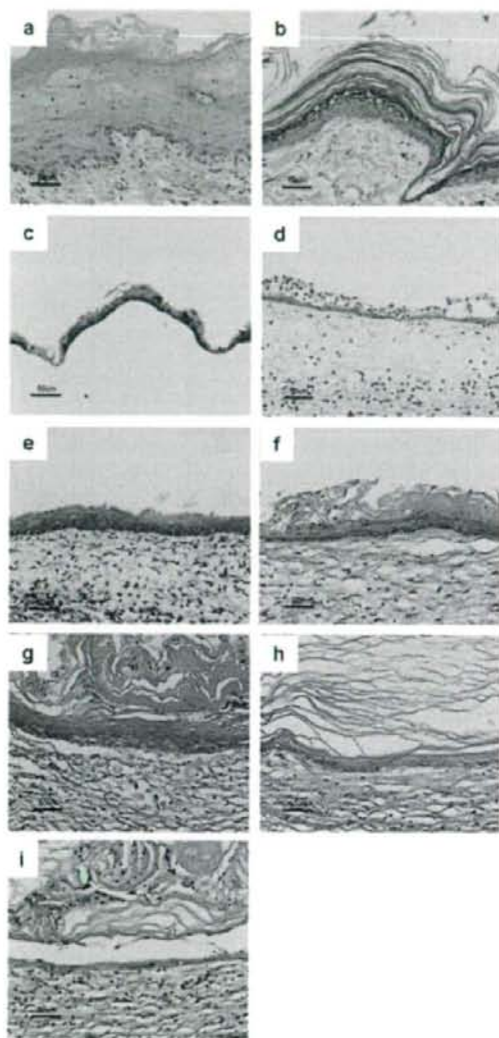

Figure 3. Histological examination of subcutaneously transplanted autologous oral mucosal epithelial cell sheets. Hematoxylin and eosin staining was performed in normal oral mucosa (a), normal skin (b), and in the fabricated oral mucosal epithelial cell sheets after cell sheet harvest (c), and 1 (d), 3 (e), 5 (f), 7 (g), 10 (h), or 14 days (i) after transplantation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Presence of stem and progenitor cells

It has been established that epithelial cells cultured in the presence of 3T3 feeder layers can provide long-term restoration to damaged tissues due to the presence of stem/progenitor cells within the epithelial grafts.[14,23–25] Therefore, to determine the fate of epithelial stem/progenitor cells within the oral mucosal epithelial cell sheets, we also examined the presence of p63, which is a homolog of the p53 transcription factor and an essential factor in both epi-

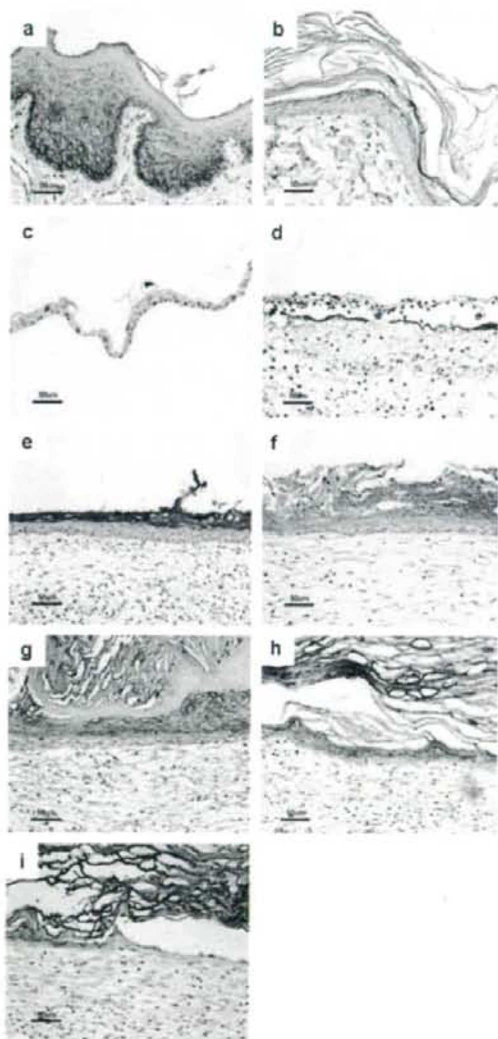

Figure 4. Expression of cytokeratin 4 within transplanted oral mucosal epithelial cell sheets. Immunohistochemistry was performed in normal oral mucosa (a), normal skin (b), and in the fabricated oral mucosal epithelial cell sheets after cell sheet harvest (c), and 1 (d), 3 (e), 5 (f), 7 (g), 10 (h), or 14 days (i) after transplantation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

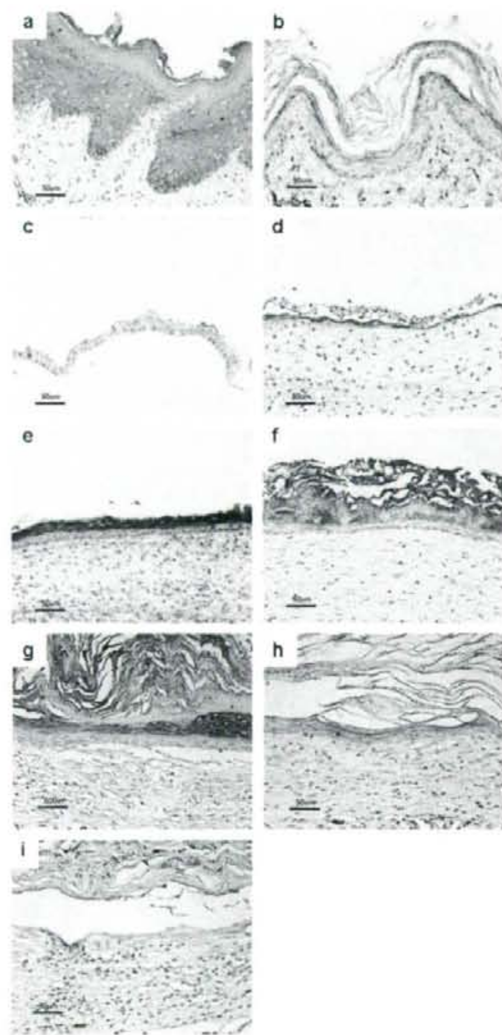

Figure 5. Expression of cytokeratin 13 within transplanted oral mucosal epithelial cell sheets. Immunohistochemistry was performed in normal oral mucosa (a), normal skin (b), and in the fabricated oral mucosal epithelial cell sheets after cell sheet harvest (c), and 1 (d), 3 (e), 5 (f), 7 (g), 10 (h), or 14 days (i) after transplantation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

thelial development and stem cell maintenance.[26–29] p63 was detected throughout the basal layer of the native oral mucosal epithelium [Fig. 8(a)] as well as in the fabricated epithelial cell sheets by the 4A4 anti-p63 antibody [Fig. 8(c)], which recognizes all isoforms of p63 including ΔNp63.[27,30] Three days after transplantation, strong expression of p63 was detected [Fig. 8(e)], however the number of p63-positive cells decreased over time [Fig. 8(f–i)]. Interestingly, p63 expression appeared to be colocalized with CK14-positive areas at day 14. Additionally, only very faint expression of PCNA was occasionally

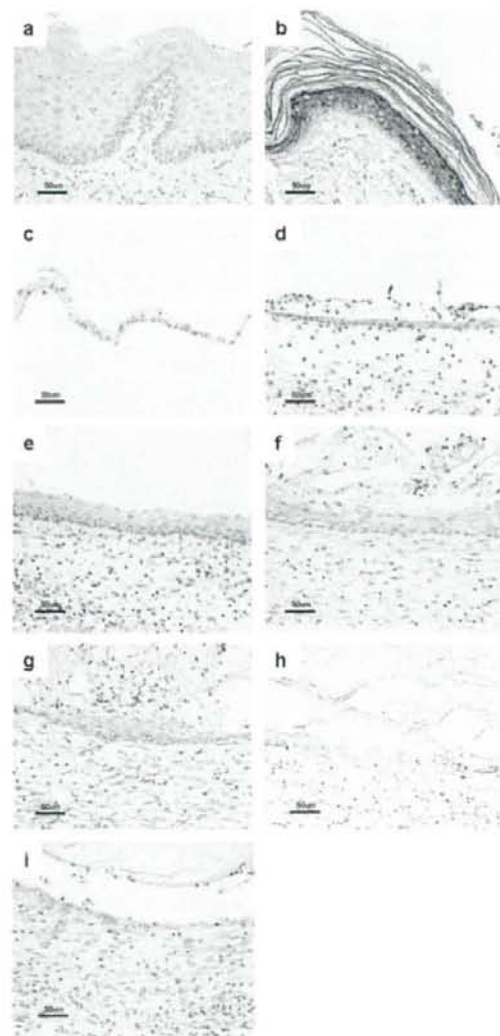

Figure 6. Expression of cytokeratin 10 within transplanted oral mucosal epithelial cell sheets. Immunohistochemistry was performed in normal oral mucosa (a), normal skin (b), and in the fabricated oral mucosal epithelial cell sheets after cell sheet harvest (c), and 1 (d), 3 (e), 5 (f), 7 (g), 10 (h), or 14 days (i) after transplantation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

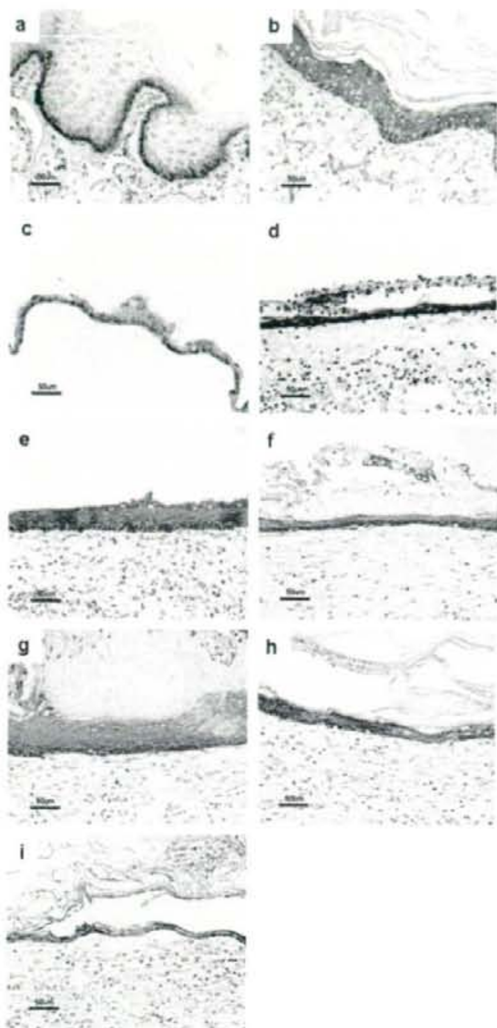

Figure 7. Expression of cytokeratin 14 within transplanted oral mucosal epithelial cell sheets. Immunohistochemistry was performed in normal oral mucosa (a), normal skin (b), and in the fabricated oral mucosal epithelial cell sheets after cell sheet harvest (c), and 1 (d), 3 (e), 5 (f), 7 (g), 10 (h), or 14 days (i) after transplantation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

observed in the fabricated cell sheets [Fig. 9(c)]. PCNA is a nuclear protein that is vital for cellular DNA synthesis and cell cycle progression.[31] After transplantation, strong expression of PCNA was detected in regenerated epithelia within 3 days [Fig. 9(e)], indicative of cell proliferation during the stratification process. However, the number of PCNA-positive cells decreased, with no detection of PCNA by day 10 [Fig. 9(f–i)].

The long-term follow-up of fabricated epithelial cell grafts transplanted to skin wounds in clinical settings shows that transplanted autologous epider-

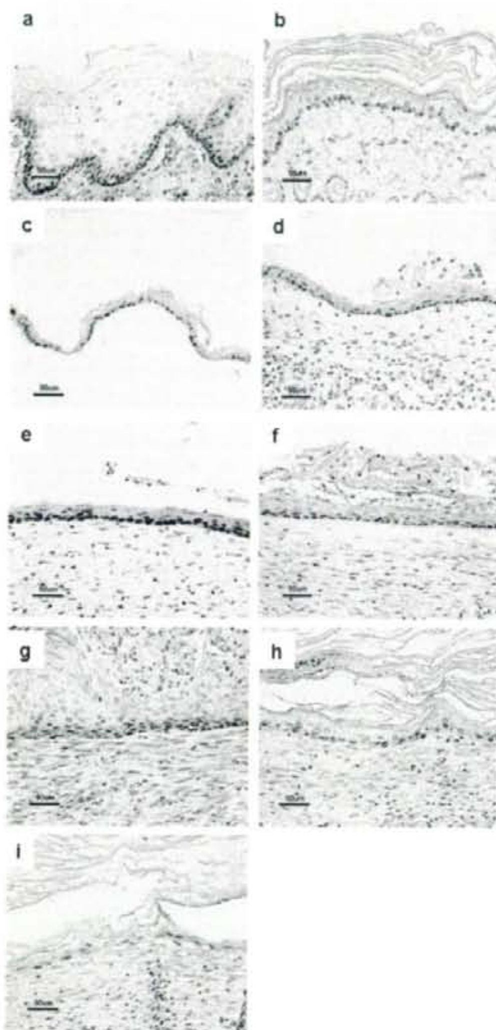

Figure 8. Expression of p63 within transplanted oral mucosal epithelial cell sheets. Immunohistochemistry was performed in normal oral mucosa (a), normal skin (b), and in the fabricated oral mucosal epithelial cell sheets after cell sheet harvest (c), and 1 (d), 3 (e), 5 (f), 7 (g), 10 (h), or 14 days (i) after transplantation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

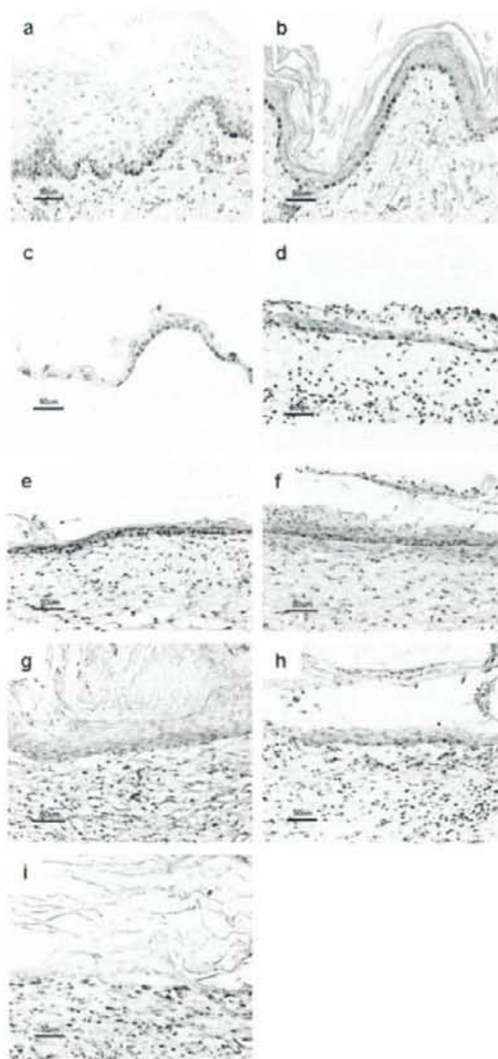

Figure 9. Expression of PCNA within transplanted oral mucosal epithelial cell sheets. Immunohistochemistry was performed in normal oral mucosa (a), normal skin (b), and in the fabricated oral mucosal epithelial cell sheets after cell sheet harvest (c), and 1 (d), 3 (e), 5 (f), 7 (g), 10 (h), or 14 days (i) after transplantation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mal cells can survive on the dermis for more than 20 years.[32] In contrast, the subcutaneously transplanted epithelial cells showed significant proliferation within 1 week after transplantation, but the epithelia degenerated over time and disappeared by 3 weeks in the present study. This cell fading was also reported in the original report of subcutaneous transplantation of cultured epidermal cells, although the authors concluded that it was caused by immunorejection. In the present study, the subcutaneously transplanted cells were unable to survive for more than 1 month even with the use of an autologous model. Similar observations have been reported with different cell types transplanted into the subcutaneous space.[33–35] For example, under these conditions, transplanted hepatocytes survived for less than 2 weeks in the absence of capillary network induction. Therefore, tissue survival in the subcutaneous space may require increased vascularization. Similarly, epithelial tissues are normally exposed to air and may not be able to survive in the subcutaneous space where oxygen supply is limited. Finally, the observation that the epithelial cell proliferation coincided with inflammation in the subcutaneous connective tissue suggests that inflammatory cytokines play a critical role in epithelial regeneration.

In addition, the gradual loss of the regenerated epithelia after 1 week implies a possibility that the absence of the stem/progenitors cell niche in the subcutaneous space leads to degeneration of the epithelial grafts. Therefore, it also appears that the lack of appropriate mesenchymal signal transduction results in the autologous oral mucosal epithelial cell sheets developing an activated phenotype, without changes in cytokeratin expression after transplantation. Nevertheless, improved understanding of epithelial cell differentiation after subcutaneous transplantation can provide a useful methodology for investigation into the mechanisms of various regenerative medicine approaches and epithelial stem cell biology.

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