Figure 5 Real-time RT-PCR analysis of the phenotype of transplanted oral mucosal epithelial cell (OEC) sheets. In rabbit nos. 1 and 2, cytokerain 6 (K6), an OEC marker, was detected, but neither of the corneal epithelial cell markers, cytokeratn 12 (K12) nor Pax6, were detected. In rabbit no. 3, K6, K12, and Pax6 were all detected, suggesting that the ocular surface was a heterogeneous mix of transplanted OEC sheets and corneal epithelial cells. Cytokeratin 14 (K14), a basal marker, and ΔNp63, a putative stem/progenitor marker, were detected during the 24-week follow-up period in all three rabbits. Vimentin, a mesenchymal marker, was detected in the transplanted OEC sheets, whereas cultivated OEC sheets and normal OECs only expressed these markers slightly. The red bars indicate the mRNA expression in rabbit no. 1; the green bars, rabbit no. 2; and the blue bars, rabbit no. 3; the yellow bars represent the control which was a normal cornea treated with 10 scrapings. Black bars indicate the mRNA expressions of oral mucosal, corneal, limbal, and conjunctival epithelial cells obtained from normal rabbit.

Reconstructed epithelia were thinner than native cornea and comprised three to four epithelial cell layers, while epithelial cell layers showed slightly increased thickness through the corneal periphery to the limbus and conjunctiva (Figure 6e). Muc5 was used to identify the conjunctival epithelial invasion into the cornea region.[27] In transplanted ocular surfaces, Muc5-expressing cells were observed in the periphery of the cornea and in the conjunctiva (Figure 6f). K4 was expressed in the superficial layer of the reconstructed corneal epithelium, which did not express Muc5, as observed in normal central corneal and limbal epithelia

(Figure 6g,h). On the contrary, all of the peripheral epithelial layers of the reconstructed cornea and conjunctiva expressed K4 (Figure 6g,i,j). The same expression pattern was obtained in all three rabbits.
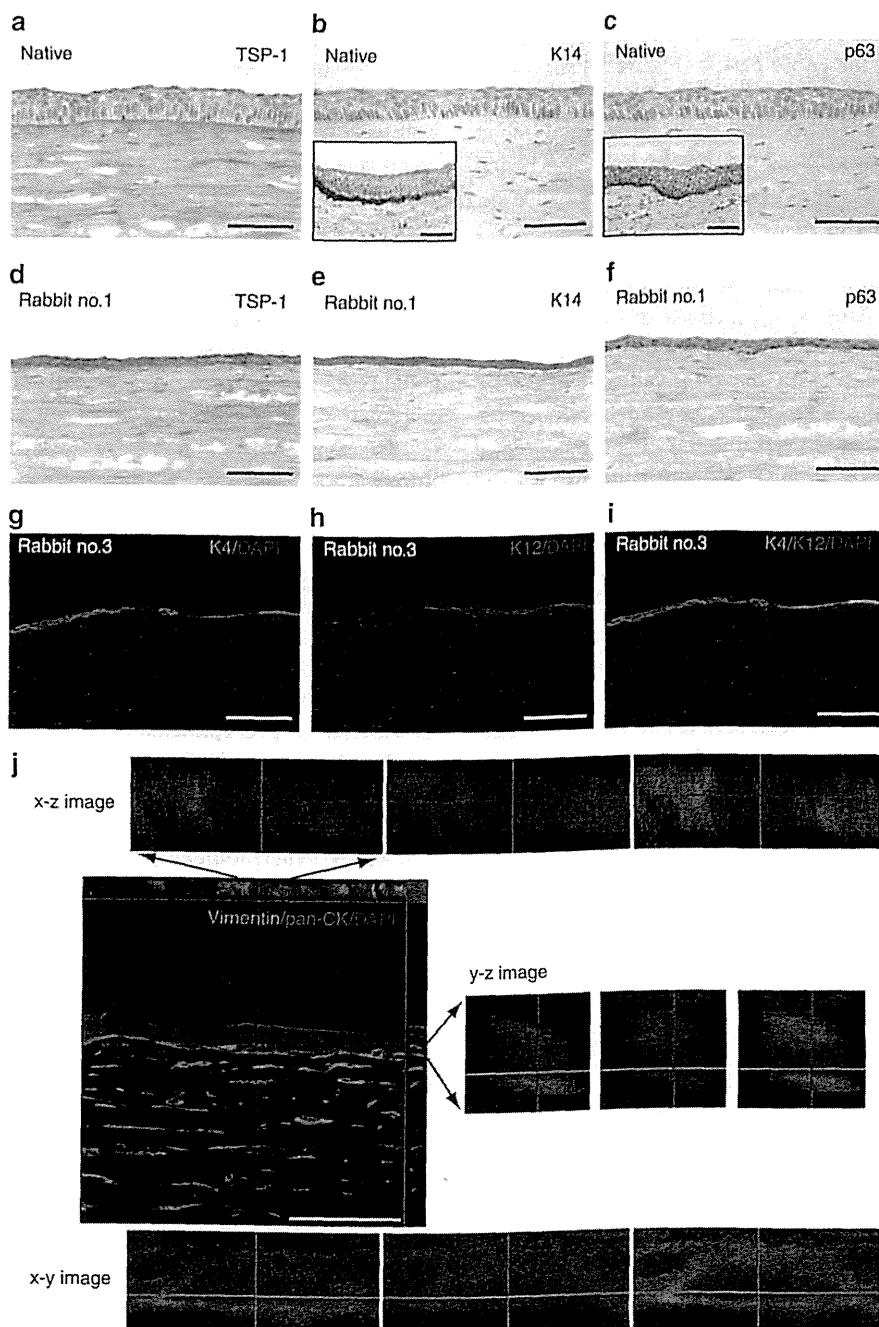
TSP-1 was deposited in the basement membrane and the upper part of the stroma beneath the basement membrane in normal rabbit cornea (Figure 7a). TSP-1 expression was detected in the epithelial cytoplasm and the upper part of the stroma in transplanted rabbits, while it was not detected in normal corneal epithelium (Figure 7a, d). K14, a basal epithelial cell marker, and p63, a putative
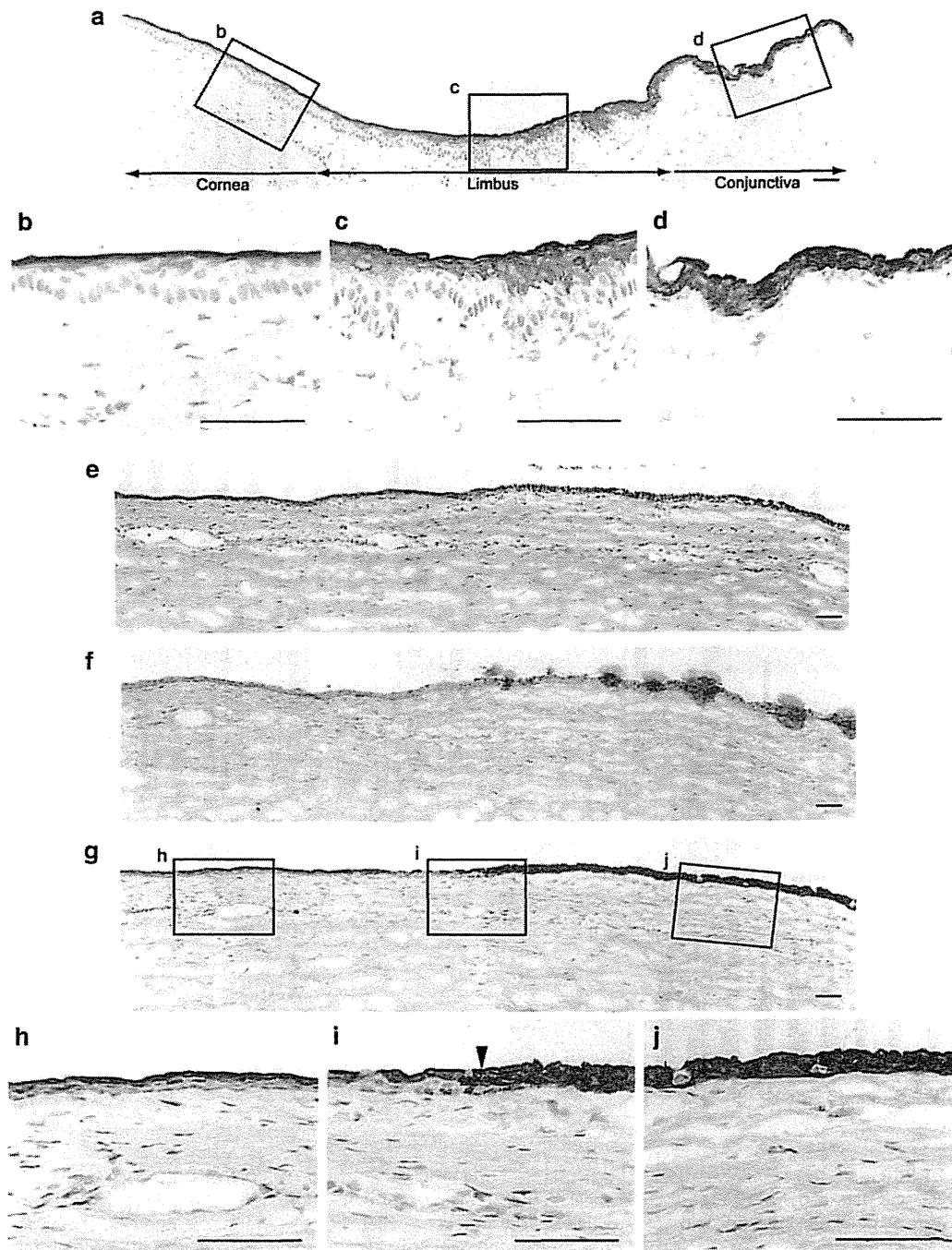
Figure 6 Investigation of the survival of transplanted oral mucosal epithelial cell (OEC) sheets by histological analysis. (a–d) In the normal rabbit ocular surface, K4 was expressed in all conjunctival epithelial cell layers, while only the superficial layer of the limbal and corneal epithelia expressed K4. The transplant rabbits were sacrificed after 10 scrapings, and the ocular surfaces were subjected to histological analysis. **(e)** Reconstructed epithelia were thinner than native cornea and were comprised three to four epithelial cell layers, while the epithelial cell layer was slightly thicker through the corneal periphery to the limbus and conjunctiva. **(f)** Mucin 5 (Muc5), a goblet cell marker, was used to identify conjunctival epithelial invasion into the cornea region. In transplanted ocular surfaces, Muc5-expressing cells were observed at the periphery of the cornea as well as the conjunctiva. **(g–j)** Interestingly, K4 was expressed in the superficial layer of ocular surface which did not express Muc5. These K4 expression patterns were similar to that of the transitional zone between the cornea and bulbar conjunctiva. Bars = 50 µm.

stem/progenitor cell marker, were not detected in normal central cornea epithelium, while limbal basal epithelial cells were stained with K14 and p63 antibodies (**Figure 7b,c**). However, all the epithelial layers of the transplanted corneal surfaces were intensely stained with the antibodies (**Figure 7e,f**). These expression patterns of TSP-1, K14, and p63 were observed in all three rabbits. In line with the results of the gene expression analysis, K12 was detected only in the central reconstructed cornea of rabbit no. 3 (**Figure 7g**). The central ocular epithelium of rabbit no. 3 was a chimera of K12-positive and K4-positive cells (**Figure 7g–i**). Immunostaining of serial sections revealed that some cells were K12 and K4 double positive as the superficial layers of the peripheral native cornea and limbus.

Figure 7 Characterization of transplanted oral mucosal epithelial cell (OEC) sheets by immunohistochemical analysis. (a) In normal cornea, thrombospondin 1 (TSP-1) expression was observed in the epithelial basement membrane and the upper part of the stroma beneath the basement membrane, while TSP-1 expression was not detected in normal corneal epithelium. (d) However, in transplanted ocular surfaces, TSP-1 expression was observed in all epithelial layers and diffused in the corneal stroma. (b,c) Cytokeratin 14 (K14) and p63 were not detected in normal central cornea epithelium, while limbal basal epithelial cells expressed K14 and p63 (inset). (e,f) However, K14 and p63 were observed in all the epithelial layers of the transplanted ocular surfaces. (g–i) In one of the rabbits (rabbit no. 3), K4-expressed transplanted OEC sheets and K12-expressed cornea epithelial cells were observed in a heterogeneous pattern. (j) Vimentin, a mesenchymal cell marker, was co-expressed in the basal layer of the cytokerain-expressed epithelial cells, suggesting that the basal cells of transplanted OEC sheets might induce epithelial–msenchymal transition. Bars = 100 µm.

In all three transplant rabbits, the basal epithelial layer of the central ocular surface contained vimentin-positive cells (Figure 7j). Interestingly, double immunofluorescent staining and confocal laser scanning microscopy revealed that these vimentin-positive cells were also pan-cytokeratin (pan-CK) positive. However, vimentin-positive keratocytes in the corneal stroma were pan-CK negative.

DISCUSSION

This study reported that (i) the corneal surface of a rabbit LSCD model was successfully reconstructed by transplantation of cultivated autologous OEC sheets fabricated on a temperature-responsive culture surface and (ii) gene expression analysis and immunostaining after repeated scraping of the central ocular surfaces revealed two different types of ocular reconstruction.

In two of the three rabbits (rabbit nos. 1 and 2), neither K12 nor Pax6 was detected over the entire period (**Figure 5**), suggesting that the ocular surfaces were reconstructed with only the transplanted OEC sheets. On the other hand, in the other rabbit (rabbit no. 3), the ocular surface expressed not only K6 but also K12 and Pax6 after the second scraping (**Figure 5**), implying that the ocular surface was reconstructed with K6-positive cells derived from the transplanted OEC sheet and K12- and Pax6-positive corneal epithelial cells derived from residual limbal epithelial stem cells. Since neither K12 nor Pax6 were expressed in the ocular surface of rabbit no. 3 at the first scraping, the ocular surface was initially reconstructed only with the transplanted OEC sheet. Since the expressions of K6, K12, and Pax6 were detected after the second scraping, the ocular surface appeared to be composed of two kinds of epithelial cells. Double immunostaining of K4 and K12 also revealed that the central reconstructed ocular surface of rabbit no. 3 was a chimera of K4-positive cells and K12-positive cells. However, the possibility of transdifferentiation of OECs to corneal epithelial cells cannot be excluded, although we previously reported that K12 expression was not detected in the ocular surface of a rabbit LSCD model with transplanted OEC sheets.[15] If the K12- and Pax6-positive epithelial cells were derived from limbal epithelial stem cells, transplantation of OEC sheets likely provided an ideal environment for corneal epithelial cells derived from residual limbal epithelial stem cells in the LSCD model by reducing inflammation and neovascularization. Rapid epithelial cell proliferation for wound healing after scraping of the central ocular surface would evoke proliferation and differentiation of any residual limbal epithelial stem cells. The condition of the ocular surfaces such as the severity of inflammation, neovascularization, opacity, and conjunctival epithelium invasion was shown to be varied among human LSCD patients.[28,29] In some patients, limbal epithelial stem cells might remain in the limbus but could not differentiate into corneal epithelial cells due to the lack of an appropriate environment. Limbal allografts are reported to be most successful in cases that show only recipient DNA in the reconstructed ocular surface, suggesting that paracrine factors from allogeneic transplantation stimulate proliferation of residual stem cells of the patient.[30] For patients who do have residual stem cells, the primary role of transplanted OEC sheets would be to provide an appropriate environment for the proliferation of any residual limbal epithelial stem cells.

K14 and ΔNp63 were constantly expressed in the ocular surfaces of transplanted rabbits during the entire follow-up period, and in the fabricated OEC sheets (**Figures 2c** and **5**), suggesting the presence of epithelial basal and putative stem/progenitor cells. In immunohistochemical analysis, OEC sheet–transplanted ocular surfaces were covered with approximately three layers of similar sized epithelial cells (**Figure 7e,f**), while the native cornea epithelial cell layer had cuboidal basal cells and flat cells at the upper cell layer (**Figure 7b,c**). Since K14 and p63 staining were observed in all cell layers of the transplanted OEC sheet, the transplanted OECs were considered to be in a growth phase induced by the physical scraping. Although the localization of epithelial stem cells in transplanted ocular surfaces still needs to be elucidated, the transplanted stem cells could be maintained, and it appears that the stem cell niche would be reconstructed.

Although ΔNp63α,[22,26] C/EBPδ,[31] ABCG2,[32] cytokeratin 15 (K15),[33,34] and others have been reported as a putative valuable candidates for epithelial stem cell markers, genuine epithelial stem cells have not been identified. Since stem cells have a replication competence and pluripotency,[35,36] clonal analysis has been often utilized to examine the proliferative capacity and the differentiation potential of epithelial stem cells *in vitro*[37] and *ex vivo*.[38] In this study, the cornea reconstruction of the rabbit LSCD model was successfully observed for a total follow-up period of 24 weeks, even when scraping of central cornea was performed every 2 weeks (**Figure 3** and **Supplementary Figures S2–S4**). This observation should provide strong evidence for the presence of epithelial stem cells on the ocular surface.

K6 expression is induced in hyperpoliferative epithelium during wound hearing even in epidermis which lacks its expression in the normal condition, and the expression level returns to normal after healing is completed.[39] In this study, K6 expression was not observed in the central corneal surface of the control rabbit 2 weeks after every scraping. K6 expression in OECs was significantly higher than that in cornea and conjunctival epithelial cells of native human or rabbit. Therefore, K6 was chosen as the marker for OECs. In the wound-healing process, the K6 expression level of corneal epithelial cells might increase just after scraping, but this has not been examined. In this study, K6 expression was hardly detected in the central corneal surface of the control rabbit 2 weeks after scraping. Therefore, the elevated K6 expression due to hyperproliferation was considered to have returned to normal levels at 2 weeks after scraping. This phenomenon is also reported in a mouse skin model.[39] Moreover, it showed that the wound healing was completed within 2 weeks after every scraping. Fluorescein staining results also showed complete wound healing within 2 weeks after every scraping. However, K6 expression was observed in the central ocular surface of all three transplanted rabbits during the 24-week follow-up period (**Figure 5**). Furthermore, transplanted OEC sheet survival was investigated not only by K6 expression but also by K12 and Pax6 and corneal epithelial cell marker expression (**Figure 5**). This might be indirect evidence that epithelial stem cells contained in the transplanted OEC sheets could survive on the transplanted ocular surface during the investigation period.

It was reported that survival of a functional ocular surface decreases progressively until ~6 months after OEC sheet transplantation in LSCD patients.[40] Kaplan–Meier analysis of the stability of ocular surfaces with transplanted OEC sheets revealed an early decline over the first 6 months, remaining comparatively stable thereafter.[40] Therefore, the follow-up period in this study would be relevant to applications in clinical settings.

TSP-1 was deposited only at the basement membrane and the underlying stroma in normal rabbit cornea (**Figure 7a**), while it was detected in the epithelia and the upper part of the stroma in transplant rabbits (**Figure 7a,d**). Although TSP-1 is known as an antiangiogenic factor,[41,42] it has also been reported that TSP-1 expression is induced in wound healing to support the proliferation of cornea epithelium.[43] Furthermore, in the investigation into the manner of cultivated oral mucosal epithelial gene expression, TSP-1 expression is reported to increase during the culture period.[44] In this study, TSP-1 was either faint or hardly expressed

in both normal oral mucosa epithelium and fabricated OEC sheets before transplantation (**Figure 5**). Therefore, the significant upregulation of TSP-1 in transplanted ocular surfaces would be due to the wound healing. Interestingly, the high expression of TSP-1 was maintained in rabbit nos. 1 and 2 for a long period, while it returned to the normal corneal level in rabbit no. 3 after the second scraping. This expression pattern might resemble K6 expression.

In two of the three OEC sheets, a small amount of vimentin expression was detected before transplantation (**Figure 5**). The upregulation of vimentin expression was observed in reconstructed ocular surfaces after the transplantation of OEC sheets in all three rabbits, and the expression was detected during the entire follow-up period (**Figures 5 and 7j**). Double immunostaining and confocal laser scanning microscopy revealed that vimentin-positive cells were pan-CK positive in ocular surface epithelial cells. Vimentin is one of the mesenchymal cell-specific markers.[45] Several vimentin-expressing cells including dendritic cells, melanocytes, and Langerhans cells are localized only in the limbal and conjunctival epithelia.[46] Furthermore, some basal epithelia express vimentin in the limbal region, but the central corneal epithelium does not contain vimentin-expressing cells.[46] In real-time RT-PCR analysis of native rabbit epithelium, vimentin expression was detected only in the limbal and conjunctival epithelium and was hardly detected in the cornea and oral epithelium (**Figure 5**). Since vimentin expression was not detected in the central corneal epithelium of the scraped control rabbit, the vimentin- and pan-CK double-positive cells would have been derived from the transplanted OEC sheets. Previously, Tseng's group[47,48] reported that some basal epithelial cells in the limbus were vimentin- and pan-CK double positive. By the epithelial–mesenchymal transition, epithelial cells obtain mesenchymal phenotypes and increase their cell motility.[49] It was also shown that epithelial–mesenchymal transition has a crucial role in gastrulation, tumor metastasis, and wound healing.[49] Tseng's group[50] also reported that limbal epithelial cells showed epithelial–mesenchymal transition and migrated to the stroma in organ culture with airlifting. Although, in this study, it is unclear whether the double-positive cells were the result of epithelial–mesenchymal transition or not, this is the first finding of the presence of vimentin- and pan-CK-double positive cells in central ocular surfaces.

This study successfully demonstrated that fabricated autologous OEC sheets had the ability to reconstruct LSCD ocular surfaces and maintain ocular surface homeostasis for a long period. The regulation of the fate of stem cells and the constitution of a stem cell niche should be elucidated to establish an effective therapy for reconstructing the ocular surface. In addition, the origin and role of pan-CK- and vimentin-double positive cells should also be investigated.

MATERIALS AND METHODS

Autologous OEC sheet transplantation in a rabbit LSCD model. New Zealand White rabbits were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and with an experimental procedure approved by the Animal Care and Use Committee at the Tokyo Women's Medical University. To fabricate this rabbit LSCD model, the entire cornea epithelium including limbus and conjunctival tissue from the transitional zone between the cornea and the conjunctiva by 3 mm was completely removed surgically under anesthesia to expose the stroma. After keratectomy, the ocular surface was treated with the topical application of 1-n-heptanol for 5 minutes. An antibiotic (0.3% ofloxaxin; Santen, Osaka, Japan) and steroid (0.1% betamethasone; Shionogi, Osaka, Japan) were applied once a day for 1 week. Three weeks after surgery, a 5-by-10 mm specimen of oral mucosal biopsy was obtained from the interior buccal mucosa. After treatment with dispase II (Godo Shusei, Tokyo, Japan) at 37 °C for 1 hour, OEC layers were isolated and trypsinized for 20 minutes at 37 °C to produce single cell suspensions. The suspended OECs were cultivated on a temperature-responsive cell-culture insert (UpCell; CellSeed, Tokyo, Japan) at an initial cell density of 1.2×10^5 cells/cm^2 with mitomycin C-treated (Wako, Osaka, Japan) NIH/3T3 feeder cells. The culture medium was a mixture of Dulbecco's modified Eagle's medium (Sigma, St Louis, MO) and Ham's F-12 (Sigma) at a ratio of 3:1 supplemented with 10% fetal bovine serum (Japan Bioserum, Hiroshima, Japan), 0.4 µg/ml hydrocortisone (Wako), 2 nmol/l triiodothyronine (Wako), 1 nmol/l cholera toxin (Wako), 5 µg/ml insulin (Life Technologies, Carlsbad, CA), 5 µg/ml transferring (Life Technologies), 10 ng/ml epidermal growth factor (Life Technologies), 100 U/ml penicillin (Life Technologies), and 100 µg/ml streptomycin (Life Technologies). Approximately 14 days later, the cell sheets were harvested by reducing the temperature. Five weeks after the preparation of LSCD model, the conjunctival and subconjunctival scar tissue of the model was removed to reexpose the corneal stroma. A harvested autologous OEC sheet with the supporting membrane ring was placed directly onto the exposed transparent stromal bed. For protection, the ocular surface was covered with a contact lens, and a tarsorrhaphy was performed. An antibiotic (0.3% ofloxacin) and steroid (0.1% betamethasone) were applied locally once a day for 1 week.

Follow-up and clonal analysis of transplanted cell sheets in vivo. One week after transplantation, the ocular surfaces were carefully observed and stained with fluorescein dye to determine if there were any defects. Four weeks after transplantation, the central ocular surface was scraped circularly (5 mm in diameter) by a scalpel under local anesthesia and stained with fluorescein dye to confirm that the corneal stroma was reexposed. The wounded ocular surface was covered with a contact lens, and an antibiotic (0.3% ofoxacin) and steroid (0.1% betamethasone) were applied locally once every few days. Two weeks after the artificial wounding, reepithelialization was identified by fluorescein staining and again re-scraped circularly. This 2-week procedure was then repeated 10 times for a total follow-up period of 24 weeks.

Microarray analysis. Human oral mucosal tissues were obtained in compliance with institutional review board regulations, informed consent regulations, and the tenets of the Declaration of Helsinki. An oral mucosal biopsy was obtained from a healthy volunteer, and the epithelial layers were carefully removed after treatment with dispase I (1,000 PU/ml; EIDIA, Tokyo, Japan) at 37 °C for 1 hour. Human conjunctiva and limbal tissues were isolated from whole human globes (Northwest Lions Eye Bank, Seattle, WA) using scissors, and the 8.0-mm-diameter portions of the central corneas were obtained by trephination. Excised tissues were incubated individually by treatment with 2.4 units/ml dispase II (Life Technologies) at 37 °C for 1 hour. Total RNA was extracted using an RNeasy Total RNA Kit (Qiagen, Hilden, Germany) from isolated human oral mucosal, corneal, limbal, and conjunctival epithelium. For microarray analysis, cDNA hybridization to Human Genome U133 Plus2.0 (47,000 probe sets, transcription products) was performed by RNA amplification procedure with a GeneChip 3' IVT Express Kit (Affymetrix, Santa Clara, CA). The expression data were analyzed by GeneChip Operating Software using standard protocols.

Real-time PCR analysis. Total RNA was extracted from normal human oral mucosal, corneal, limbal, and conjunctival epithelial cells, and a piece of transplanted epithelium was obtained by scraping. Real-time PCR analysis was performed in the standard manner. Primer pairs were designed by Primer Express Software v2.0 (Applied BioSystems) to detect

the rabbit mRNA expressions of K6a, K14, ΔNp63, TSP-1 and β-actin (Supplementary Table S1). All assays were performed in duplicate for individual samples.

Histological analysis. Normal human oral mucosa, human corneoscleral rims, and transplanted OEC sheets on rabbit cornea tissues were embedded in an optimum cutting temperature compound and processed into 5-μm frozen sections. Tissue sections were stained with mouse monoclonal anti-K6 (1:100 dilution; Lab Vision, Fremont, CA), mouse monoclonal anti-K4 (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-K13 (1:100 dilution; Abcam, Cambridge, UK), mouse monoclonal anti-Muc 5 (1:100 dilution; Life Technologies), mouse monoclonal anti-K14 (1:50 dilution; Santa Cruz Biotechnology), mouse monoclonal anti-p63 (1:100 dilution; Santa Cruz Biotechnology), and mouse monoclonal anti-TSP-1 (1:50 dilution; Abcam). For double immunofluorescence staining, tissue sections were stained with anti-K4, goat polyclonal anti-K12 (1:100 dilution; Santa Cruz Biotechnology), mouse monoclonal anti-pan-CK (1:20 dilution; Abcam), and mouse monoclonal anti-vimentin antibody (Santa Cruz Biotechnology). These sections were observed by a confocal laser-scanning microscope (LSM-510, Carl Zeiss, Jena, Germany).

SUPPLEMENTARY MATERIAL

Table S1. Primer pairs and probes for rabbit mRNA expression analysis by real-time RT-PCR.
Figure S1. Evaluation of rabbit limbal stem cell deficiency model by immunohistochemical analyses of pannus.
Figure S2. Time course images of transplanted ocular surface with repeated wound healing (rabbit 1).
Figure S3. Time course images of transplanted ocular surface with repeated wound healing (rabbit 2).
Figure S4. Time course images of transplanted ocular surface with repeated wound healing (rabbit 3).
Figure S5. Time course images of normal corneal surface with repeated wound healing (the control).
Materials and Methods

REFERENCES

1. Takahashi, K, Tanabe, K, Ohnuki, M, Narita, M, Ichisaka, T, Tomoda, K *et al.* (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**: 861–872.
2. Nishida, K, Yamato, M, Hayashida, Y, Watanabe, K, Yamamoto, K, Adachi, E *et al.* (2004). Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *N Engl J Med* **351**: 1187–1196.
3. Sawa, Y, Miyagawa, S, Sakaguchi, T, Fujita, T, Matsuyama, A, Saito, A *et al.* (2012). Tissue engineered myoblast sheets improved cardiac function sufficiently to discontinue LVAS in a patient with DCM: report of a case. *Surg Today* **42**: 181–184.
4. Ohki, T, Yamato, M, Ota, M, Takagi, R, Murakami, D, Kondo, M *et al.* (2012). Prevention of esophageal stricture after endoscopic submucosal dissection using tissue-engineered cell sheets. *Gastroenterology* (epub ahead of print).
5. Ito, S, Sato, M, Yamato, M, Mitani, G, Kutsuna, T, Nagai, T *et al.* (2012). Repair of articular cartilage defect with layered chondrocyte sheets and cultured synovial cells. *Biomaterials* **33**: 5278–5286.
6. Hamahashi, K, Sato, M, Yamato, M, Kokubo, M, Mitani, G, Ito, S *et al.* (2012). Studies of the humoral factors produced by layered chondrocyte sheets. *J Tissue Eng Regen Med* (epub ahead of print).

7. Iwata, T, Yamato, M, Tsuchioka, H, Takagi, R, Mukobata, S, Washio, K *et al.* (2009). Periodontal regeneration with multi-layered periodontal ligament-derived cell sheets in a canine model. *Biomaterials* **30**: 2716–2723.
8. Iwata, T, Yamato, M, Zhang, Z, Mukobata, S, Washio, K, Ando, T *et al.* (2010). Validation of human periodontal ligament-derived cells as a reliable source for cytotherapeutic use. *J Clin Periodontol* **37**: 1088–1099.
9. Thoft, RA and Friend, J (1983). The X, Y, Z hypothesis of corneal epithelial maintenance. *Invest Ophthalmol Vis Sci* **24**: 1442–1443.
10. Schermer, A, Galvin, S and Sun, TT (1986). Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol* **103**: 49–62.
11. Cotsarelis, G, Cheng, SZ, Dong, G, Sun, TT and Lavker, RM (1989). Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. *Cell* **57**: 201–209.
12. Tsubota, K, Satake, Y, Kaido, M, Shinozaki, N, Shimmura, S, Bissen-Miyajima, H *et al.* (1999). Treatment of severe ocular-surface disorders with corneal epithelial stem-cell transplantation. *N Engl J Med* **340**: 1697–1703.
13. Tseng, SC (1989). Concept and application of limbal stem cells. *Eye (Lond)* **3** (Pt 2): 141–157.
14. Ti, SE, Anderson, D, Touhami, A, Kim, C and Tseng, SC (2002). Factors affecting outcome following transplantation of ex vivo expanded limbal epithelium on amniotic membrane for total limbal deficiency in rabbits. *Invest Ophthalmol Vis Sci* **43**: 2584–2592.
15. Hayashida, Y, Nishida, K, Yamato, M, Watanabe, K, Maeda, N, Watanabe, H *et al.* (2005). Ocular surface reconstruction using autologous rabbit oral mucosal epithelial sheets fabricated ex vivo on a temperature-responsive culture surface. *Invest Ophthalmol Vis Sci* **46**: 1632–1639.
16. Ramirez-Miranda, A, Nakatsu, MN, Zarei-Ghanavati, S, Nguyen, CV and Deng, SX (2011). Keratin 13 is a more specific marker of conjunctival epithelium than keratin 19. *Mol Vis* **17**: 1652–1661.
17. Tanioka, H, Kawasaki, S, Yamasaki, K, Ang, LP, Koizumi, N, Nakamura, T *et al.* (2006). Establishment of a cultivated human conjunctival epithelium as an alternative tissue source for autologous corneal epithelial transplantation. *Invest Ophthalmol Vis Sci* **47**: 3820–3827.
18. Nakamura, T, Endo, K, Cooper, LJ, Fullwood, NJ, Tanifuji, N, Tsuzuki, M *et al.* (2003). The successful culture and autologous transplantation of rabbit oral mucosal epithelial cells on amniotic membrane. *Invest Ophthalmol Vis Sci* **44**: 106–116.
19. Murakami, D, Yamato, M, Nishida, K, Ohki, T, Takagi, R, Yang, J *et al.* (2006). Fabrication of transplantable human oral mucosal epithelial cell sheets using temperature-responsive culture inserts without feeder layer cells. *J Artif Organs* **9**: 185–191.
20. Presland, RB and Dale, BA (2000). Epithelial structural proteins of the skin and oral cavity: function in health and disease. *Crit Rev Oral Biol Med* **11**: 383–408.
21. Figueira, EC, Di Girolamo, N, Coroneo, MT and Wakefield, D (2007). The phenotype of limbal epithelial stem cells. *Invest Ophthalmol Vis Sci* **48**: 144–156.
22. Pellegrini, G, Dellambra, E, Golisano, O, Martinelli, E, Fantozzi, I, Bondanza, S *et al.* (2001). p63 identifies keratinocyte stem cells. *Proc Natl Acad Sci USA* **98**: 3156–3161.
23. Shiraishi, A, Converse, RL, Liu, CY, Zhou, F, Kao, CW and Kao, WW (1998). Identification of the cornea-specific keratin 12 promoter by in vivo particle-mediated gene transfer. *Invest Ophthalmol Vis Sci* **39**: 2554–2561.
24. Collinson, JM, Hill, RE and West, JD (2000). Different roles for Pax6 in the optic vesicle and facial epithelium mediate early morphogenesis of the murine eye. *Development* **127**: 945–956.
25. Cursiefen, C, Masli, S, Ng, TF, Dana, MR, Bornstein, P, Lawler, J *et al.* (2004). Roles of thrombospondin-1 and -2 in regulating corneal and iris angiogenesis. *Invest Ophthalmol Vis Sci* **45**: 1117–1124.
26. Di Iorio, E, Barbaro, V, Ruzza, A, Ponzin, D, Pellegrini, G and De Luca, M (2005). Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. *Proc Natl Acad Sci USA* **102**: 9523–9528.
27. Kawashima, M, Kawakita, T, Satake, Y, Higa, K and Shimazaki, J (2007). Phenotypic study after cultivated limbal epithelial transplantation for limbal stem cell deficiency. *Arch Ophthalmol* **125**: 1337–1344.
28. Saito, T, Nishida, K, Sugiyama, H, Yamato, M, Maeda, N, Okano, T *et al.* (2008). Abnormal keratocytes and stromal inflammation in chronic phase of severe ocular surface diseases with stem cell deficiency. *Br J Ophthalmol* **92**: 404–410.
29. Kawasaki, S, Nishida, K, Sotozono, C, Quantock, AJ and Kinoshita, S (2000). Conjunctival inflammation in the chronic phase of Stevens-Johnson syndrome. *Br J Ophthalmol* **84**: 1191–1193.
30. Pellegrini, G, De Luca, M and Arsenijevic, Y (2007). Towards therapeutic application of ocular stem cells. *Semin Cell Dev Biol* **18**: 805–818.
31. Barbaro, V, Testa, A, Di Iorio, E, Mavilio, F, Pellegrini, G and De Luca, M (2007). C/EBPdelta regulates cell cycle and self-renewal of human limbal stem cells. *J Cell Biol* **177**: 1037–1049.
32. Watanabe, K, Nishida, K, Yamato, M, Umemoto, T, Sumide, T, Yamamoto, K *et al.* (2004). Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2. *FEBS Lett* **565**: 6–10.
33. Porter, RM, Lunny, DP, Ogden, PH, Morley, SM, McLean, WH, Evans, A *et al.* (2000). K15 expression implies lateral differentiation within stratified epithelial basal cells. *Lab Invest* **80**: 1701–1710.
34. Yoshida, S, Shimmura, S, Kawakita, T, Miyashita, H, Den, S, Shimazaki, J *et al.* (2006). Cytokeratin 15 can be used to identify the limbal phenotype in normal and diseased ocular surfaces. *Invest Ophthalmol Vis Sci* **47**: 4780–4786.
35. Lajtha, LG (1979). Stem cell concepts. *Differentiation* **14**: 23–34.
36. Morrison, SJ, Shah, NM and Anderson, DJ (1997). Regulatory mechanisms in stem cell biology. *Cell* **88**: 287–298.
37. Pellegrini, G, Golisano, O, Paterna, P, Lambiase, A, Bonini, S, Rama, P *et al.* (1999). Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. *J Cell Biol* **145**: 769–782.

38. Notara, M, Schrader, S and Daniels, JT (2011). The porcine limbal epithelial stem cell niche as a new model for the study of transplanted tissue-engineered human limbal epithelial cells. *Tissue Eng Part A* **17**: 741–750.

39. Inada, R, Matsuki, M, Yamada, K, Morishima, Y, Shen, SC, Kuramoto, N *et al.* (2000). Facilitated wound healing by activation of the Transglutaminase 1 gene. *Am J Pathol* **157**: 1875–1882.

40. Satake, Y, Higa, K, Tsubota, K and Shimazaki, J (2011). Long-term outcome of cultivated oral mucosal epithelial sheet transplantation in treatment of total limbal stem cell deficiency. *Ophthalmology* **118**: 1524–1530.

41. Sekiyama, E, Nakamura, T, Cooper, LJ, Kawasaki, S, Hamuro, J, Fullwood, NJ *et al.* (2006). Unique distribution of thrombospondin-1 in human ocular surface epithelium. *Invest Ophthalmol Vis Sci* **47**: 1352–1358.

42. Sekiyama, E, Nakamura, T, Kawasaki, S, Sogabe, H and Kinoshita, S (2006). Different expression of angiogenesis-related factors between human cultivated corneal and oral epithelial sheets. *Exp Eye Res* **83**: 741–746.

43. Uno, K, Hayashi, H, Kuroki, M, Uchida, H, Yamauchi, Y, Kuroki, M *et al.* (2004). Thrombospondin-1 accelerates wound healing of corneal epithelia. *Biochem Biophys Res Commun* **315**: 928–934.

44. Alaminos, M, Garzón, I, Sánchez-Quevedo, MC, Moreu, G, González-Andrades, M, Fernández-Montoya, A *et al.* (2007). Time-course study of histological and genetic patterns of differentiation in human engineered oral mucosa. *J Tissue Eng Regen Med* **1**: 350–359.

45. Franke, WW, Schmid, E, Osborn, M and Weber, K (1978). Different intermediate-sized filaments distinguished by immunofluorescence microscopy. *Proc Natl Acad Sci USA* **75**: 5034–5038.

46. Schlötzer-Schrehardt, U and Kruse, FE (2005). Identification and characterization of limbal stem cells. *Exp Eye Res* **81**: 247–264.

47. Chen, SY, Hayashida, Y, Chen, MY, Xie, HT and Tseng, SC (2011). A new isolation method of human limbal progenitor cells by maintaining close association with their niche cells. *Tissue Eng Part C Methods* **17**: 537–548.

48. Li, GG, Zhu, YT, Xie, HT, Chen, SY and Tseng, SC (2012). Mesenchymal stem cells derived from human limbal niche cells. *Invest Ophthalmol Vis Sci* **53**: 5686–5697.

49. Kalluri, R and Weinberg, RA (2009). The basics of epithelial-mesenchymal transition. *J Clin Invest* **119**: 1420–1428.

50. Kawakita, T, Espana, EM, He, H, Li, W, Liu, CY and Tseng, SC (2005). Intrastromal invasion by limbal epithelial cells is mediated by epithelial-mesenchymal transition activated by air exposure. *Am J Pathol* **167**: 381–393.

Review Article   Open Access

A New National Framework for Clinical Trials and Evaluation of Innovative Medical Care Technologies Using Living Cell Transplantation in Japan

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Abstract

Following the global initiative to develop medical innovative technologies such as living cell transplantation, the Japanese promotion plan, "Five-year Clinical Trial Vitalization Plan 2012" was launched in 2012. Subsequently, "Clinical Trials Core Hospitals" or medical care institutions that performed research and development of innovative technologies were identified, which were regularly evaluated by the "Evaluation System for Investigational Medical Care." In addition, the regulatory guidelines for pharmaceuticals and medical devices have been reexamined and the revised Pharmaceutical Affairs Law (PAL), which was renamed as the Pharmaceuticals, Medical Devices and Other Therapeutic Products Act (PMD Act), and the Act on the safety of regenerative medicine were developed in 2013 and is scheduled for implementation in November 2014. Based on these changes in the national framework for innovative medical care in Japan, this article aims to explain and evaluate the possibility for this framework to be utilized as the universal case model for stem cell and living cell transplantation.

Keywords: Regenerative medicine; Living cell transplantation; Regulation; Evaluation system

Introduction

Efforts in developing innovative medical care such as living cell transplantation techniques, also known as regenerative medicine, has recently increased in the past several years. The global interest in developing innovative medical technologies has thus prompted the Japanese government to establish the "Five-year Medical Innovation Strategy" in 2012 [1], which was subsequently followed by the conception of the "Health and Medical Strategy" in 2013 [2] as one of the centers of economic policy. The regulatory guidelines for pharmaceuticals and medical devices have been reexamined and the revised Pharmaceutical Affairs Law (PAL), which was renamed as the Pharmaceuticals, Medical Devices and Other Therapeutic Products Act (PMD Act) [3], and the Act on the Safety of Regenerative Medicine [4] were developed in November 2013 and is scheduled for implementation in November 2014 [5]. Based on this new legal framework, Japan will have the potential to become the prime venue for international medical researchers and industries. This article aims to explain and examine the possibility for this framework to be utilized as the universal case model for stem cell and living cell transplantation efforts.

Present Status of Clinical Researches and Trials in Japan and the Five-Year Clinical Trials Vitalization Plan 2012

The Medical Service Law, Medical Practitioners Law, and the Ethical Guidelines for Clinical Studies are responsible in allowing clinical researches and trials to be conducted in Japan [6-8]. In addition, PAL [9] stipulates that clinical trials should be performed in accordance with the guidelines for Good Clinical Practice (GCP) [10], which was established by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. This system thus leads investigators in non-commercial clinical studies to consider that their research does not need to be based on GCP guidelines. The Ethical Guidelines for Clinical Studies has been developed on the basis of the Declaration of Helsinki [11] and defines the protective measures for safety, rights, and the compensation of patients according to the GCP guidelines. However, the quality control and assurance, as well as the reliability of the results of clinical trials have not been clearly defined. Based on the differences between GCP guidelines and the Ethical Guidelines, the results of non-commercial clinical trials, particularly those performed in academia; generally do not contribute to the marketing strategies of a novel treatment. Therefore, this results in failure to further develop and utilize innovative medical care technologies involving regenerative medicine and living cell transplantation after the completion of the non-commercial clinical trials. The inability to provide supporting evidence on the efficacy and safety of a novel medical technology further impedes product development. To decrease the gap between the GCP guidelines and the Ethical Guidelines, a reexamination of clinical research guidelines has been performed. These assessments have indicated that monitoring and auditing should be conducted to determine the efficacy or safety of pharmaceuticals and medical devices.

In parallel with these regulatory changes to clinical trials, the government of Japan has improved the conditions of clinical trials by implementing a promotion policy and budget in the medical innovation scheme. Subsequently, the Ministry of Health, Labour and Welfare (MHLW) formulated the Five-Year Clinical Trial Vitalization Plan 2012 in March, 2012 [12]. The goal of this plan was to increase the accessibility of new pharmaceuticals and medical devices and to assist in the search for the best treatments using a combination of

marketed pharmaceuticals and medical devices. MHLW thus launched the project, "Clinical Trial Core Hospital," which supports investigator-initiated high quality trials. Fifteen hospitals that were selected by MHLW as "Clinical Trials Core Hospitals" were granted priority to perform clinical studies. In addition, support was provided to each "Clinical Trials Core Hospital" to improve its quality of education, which may subsequently influence the quality and success of clinical trials and increase the employment rates. Furthermore, this project promoted the centralization of research and increased the national budget allowing high-quality trials that required smaller research funds to be performed. In June 2014, the revised medical service law was enacted and the "Clinical Trials Core Hospital" project was launched to identify eligible institutions [13]. It is planned to select up to 15 institutes as "Clinical Trials Core Hospital" in 2015, which have been granted by MHLW, and the criteria of eligibility is now under consideration.

In parallel with the creation of "Clinical Trials Core Hospital," the Japanese government established a new funding agency that would centralize medical research grant funding from the three ministries, namely, MHLW, Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Economy, Trade and Industry. The medical research core was called the "Japanese Organization for Medical Research and Development," and its corresponding act was approved in "Diet" in May 2014. This is considered the Japanese "National Institutes of Health (NIH)," which unifies research grants from around the country.

Improving the evaluation system for investigational medical care

In Japan, it has become a national rule that sufficient medical treatment, as part of the social security system, must be protected under the public health insurance system, so that people can receive suitable medical treatment regardless of taxation capacity. However, there has been an increase in the medical needs of patients, which is probably the consequence of recent advances in medical treatment schemes. Based on these needs, MHLW has established an evaluation system that combines public health insurance with innovative therapy and has been named as the "Evaluation System for Investigational Medical Care" [14]. The system is mandated to evaluate techniques that utilize unapproved or off-label pharmaceuticals and medical devices in select hospitals such as the "Clinical Trials Core Hospitals." These hospitals have been approved by the system to perform innovative and investigational medical care in combination with the health insurance, thus accelerating the collection of data that is essential in order to be covered by national insurance. This is the Japanese style of "Hospital Exemption" that has been implemented in EU, and the system has been improved in October, 2012. The results of the evaluation can also be used as reference when reviewing new or off-label products submitted for marketing approval. With this improvement, investigational medical care has been divided into two categories. Category A is allowed to perform trials only if the institution is deemed eligible. However, it can only use a limited amount of pharmaceuticals, medical devices, or clinical reagents. Category B is allowed to employ non-limited (unapproved or off-label) materials that have been determined to be safe and effective based on the GCP guidelines. Furthermore, the total insurance coverage for this special investigational medical care, such as regenerative medicine or personalized medicine, is also examined with a focus on its social validity and effectiveness. Several technologies in regenerative medicine or personalized medicine using an individual's own living cells may be too difficult to review in terms of marketing approval because the specifications of the final products vary with each case. In this evaluation system, investigational medical care is reviewed as a total technology, not as separate products. Therefore, the system does not focus on the specifications of the products but evaluates the entire technique, starting from the collection stage to the transplantation phase. Thus, the evaluation system is regarded as a more suitable system for regenerative medicine or personalized medicine.

Regenerative medical care using living cell transplantation and the new legal framework in Japan

Based on the need to develop innovative medical care, the field of regenerative medicine using living cell transplantation has been the global focus of research. Using living cells, regenerative medicine aims to regain the function of organs that have been damaged by illness or injury. This area of medicine thus increases the possibility of finding a treatment for diseases that have long been considered incurable.

Because regenerative medicine utilizes living cells, this innovative technology increases the risk for bacterial or viral infection and tumorigenicity. It is therefore imperative that sufficient safety measures are established. In the emergency economic policy measures (January 11, 2013; Japanese Cabinet decision), a reexamination of the special quick reviewing system for regenerative medicine was initiated. Based on the policy, the Regenerative Medicine Promoting (RMP) Act was established, which defines the duty of the government and citizens in Japan to utilize regenerative medicine, and a scheme for receiving regenerative medicine more quickly and safely was enacted by "Diet" on April 26, 2013 [15]. The RMP Act aims to comprehensively promote the use of regenerative medicine by ensuring that it is safe and does not present any ethical issues. On the basis of this act, the government has submitted the two acts relating to regenerative medicine [16]; one is the Act on Safety of Regenerative Medicine [4], and the other is the PMD Act [3]. In the Act on Safety of Regenerative Medicine, hospitals that wish to perform regenerative medical care using living cells should be reviewed by a certified special review board member and notify MHLW of the provision. On the other hand, hospitals can request a certified industry to manufacture specific cellular components that would be used in regenerative medical care. The regenerative medical care is divided into three categories on the basis of the risk of the technologies.

The PMD Act defines the category of regenerative medical products and conditional/time-limited authorization system. To perform an adaptive post-authorized clinical trial for determining the efficacy of a technology, the investigator is allowed to conduct an exploratory study. If the results clearly show efficacy and safety, the product is given conditional/time-limited authorization for use, which then facilitates in determining more suitable conditions for regenerative medical products. Regenerative medical products possess heterogeneous characteristics and are manufactured for a limited number of patients, particularly those who have developed incurable diseases. Based on this background, a major randomized and blinded pre-marketing clinical trial may not be feasible especially in orphan diseases, and prior authorization based on the epidemiological concept for effectiveness and public demand is considered. On the other hand, the products with conditional/time-limited authorization will have a duty to collect all patients' data and submit it to national patient's registry, which is planned to be established by MHLW. This is one type of adaptive licensing that has been utilized to improve product

accessibility to patients [17]. The limitation of this framework is that the concrete condition of approval is now under consideration and I think conditional/time-limited approval may be limited for the products for the intractable disease or orphan diseases.

Discussion

To eliminate medical disparities and to ensure "safe medical treatment," a secure system from which the Japanese society could receive the medical treatment with excellent hospitality from any facility is necessary [18]. On the other hand, the healthcare system of Japan is incapable of "specialization and centralization," thus becoming a weak point by which many resources invested in the field are later determined to be cost-ineffective. This present state of health care in Japan has become less attractive for researchers who want to conduct innovative product development. On the other hand, international researchers and sponsors who are interested in highly innovative fields such as regenerative medicine are constantly searching for optimal places to perform their research, as well as develop and market their novel technology. The Japanese government has embarked in transforming the medical institution by marketing these facilities as attractive venues for researchers and sponsors. The "Japan Agency for Medical Research and Development" and the "Clinical Trials Core Hospital" were established to promote research and development of medical innovative technologies. In the field of regenerative medicine using living cell transplantation, each "Clinical Trials Core Hospital" is assessed by a certified special review board and supported by national grants, with the vision of later becoming a world-renowned center for regenerative medicine. Within three years, some cellular- and tissue-based products will be submitted for marketing authorization based of the results of research cooperation between a sponsor and "Clinical Trials Core Hospital." The sponsor who aims at marketing regenerative medical products will be able to cost-effectively progress in technology or product development. These changes are envisioned to create an attractive place in Japan where researchers and developers could conduct investigations and research and development activities in regenerative medicine. In addition, "Clinical Trials Core Hospital" is planned to be granted and supervised by "Japan Agency for Medical Research and Development", which controlled the plan-do-check-act cycle of the development in "Clinical Trials Core Hospitals" and supported the collaboration with industries.

In addition to promoting innovative medical care and regenerative medicine, a new regulatory framework has been recently established. This framework is represented by three complementary acts, namely, the RMP Act, Act on the Safety of Regenerative Medicine, and the PMD Act. The Act on the Safety of Regenerative Medicine regulates the use of living cell transplantation techniques and supports a medical practitioner's research by permitting him or her to entrust the cell processing part of the research to an external industry. The PMD Act promotes the use of cellular- and tissue-based products by acting as the approving body that reviews the characteristics of these items. The RMP Act is responsible for investing in the field of regenerative medicine and in supporting the development of the field of regenerative medicine. This regulatory framework may facilitate in the development and the establishment of the safety of regenerative medicine and living cell transplantation techniques. This framework is also beneficial to patients because it increases their chance to receive a new treatment for an incurable disease, as well as the industry that develops the new cellular- and tissue-based products, or supports the medical practitioner to perform the living cell transplantation.

Through all plans, Japanese government aim to serve the new treatment for intractable disease's patients and increase the market scale of regenerative medicine to 10 billion dollars until 2020. It is our hope that the new regulatory framework is effectively applied and serves as the universal case model for the development of innovative medical care such as regenerative medicine using living cell transplantation.

References

1. (2012) Five-Years Medical Innovation Strategy.
2. (2013) Health and Medical Strategy [in Japanese].
3. (2013) Pharmaceuticals,Medical Devices, and Other Therapeutic Products Act [in Japanese].
4. (2013) Act on the Safety of Regenerative Medicine [in Japanese].
5. Hara A, Sato D, Sahara Y (2014) New Governmental Regulatory System for Stem Cell-Based Therapies in Japan. Therapeutic Innovation & Regulatory Science.
6. (2014) Medical Service Law [in Japanese].
7. (2014) Medical Practitioners Law [in Japanese].
8. Ministry of Health, Labour and Welfare (2003) Ethical Guidelines for Clinical Studies.
9. (2013) Pharmaceutical Affairs Law [in Japanese].
10. (1996) ICH Harmonised Tripartite Guideline Guideline for Good Clinical Practice E6 (R1).
11. World Medical Association (2013) World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA 310: 2191-2194.
12. (2012) 5-Year Clinical Trials Vitalization Plan 2012 [in Japanese].
13. (2014) Law on Establishment of the relevant Act to promote ensure overall care and medical care in the region.
14. (2012) Notificaiton of Evaluation System for Investigational Medical Care.
15. Cyranoski D (2013) Japan to offer fast-track approval path for stem cell therapies. Nat Med 19: 510.
16. (2013) Unknown territory Nature 494: 5.
17. Eichler HG, Oye K, Baird LG, Abadie E, Brown J, et al. (2012) Adaptive licensing: taking the next step in the evolution of drug approval. Clin Pharmacol Ther 91: 426-437.
18. Nomura H, Nakayama T (2005) The Japanese healthcare system. BMJ 331: 648-649.

Maintenance and Distribution of Epithelial Stem/ Progenitor Cells after Corneal Reconstruction Using Oral Mucosal Epithelial Cell Sheets

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Abstract

We assessed the maintenance and distribution of epithelial stem/progenitor cells after corneal reconstruction using tissue-engineered oral mucosal cell sheets in a rat model. Oral mucosal biopsy specimens were excised from green fluorescent protein (GFP) rats and enzymatically treated with Dispase II. These cells were cultured on inserts with mitomycin C-treated NIH/3T3 cells, and the resulting cell sheets were harvested. These tissue-engineered cell sheets from GFP rats were transplanted onto the eyes of a nude rat limbal stem cell deficiency model. Eight weeks after surgery, ocular surfaces were completely covered by the epithelium with GFP-positive cells. Transplanted corneas expressed p63 in the basal layers and K14 in all epithelial layers. Epithelial cells harvested from the central and peripheral areas of reconstructed corneas were isolated for a colony-forming assay, which showed that the colony-forming efficiency of the peripheral epithelial cells was significantly higher than that of the central epithelial cells 8 weeks after corneal reconstruction. Thus, in this rat model, the peripheral cornea could maintain more stem/progenitor cells than the central cornea after corneal reconstruction using oral mucosal epithelial cell sheets.

Introduction

Corneal epithelial stem cells are located in the basal layer of the limbus, which is the narrow transition zone between the cornea and the conjunctiva [1,2]. The limbal epithelium is a reservoir for replacing corneal epithelial cells that are normally continuously lost from the corneal surface [3]. Severe corneal diseases, such as Stevens–Johnson syndrome, or chemical burns destroy the limbus and cause limbal stem cell deficiency (LSCD). In these cases, corneal epithelial cell sources are exhausted, the peripheral conjunctival epithelium invades inwardly, and the corneal surface becomes enveloped by vascularized conjunctival scar tissue, which results in corneal opacification that leads to severe visual impairment [4,5].

In cases of severe LSCD, we and others recently demonstrated the successful application of constructs involving ex vivo expansion of autologous oral mucosal epithelium [6–8]. This method averts the risks of immune rejection and long-term immunosuppression, and thus offers clinical advantages over conventional allogeneic corneal transplantation [9]. We have performed transplantation of oral mucosal epithelial cell sheets in over 20 patients. For these patients, corneal transparency was restored and postoperative visual acuity remained improved for 2–8 years, whereas abnormal

corneas were successfully reconstructed using conventional allogeneic transplantation in only 20–30% of patients for 2–3 years [10,11].
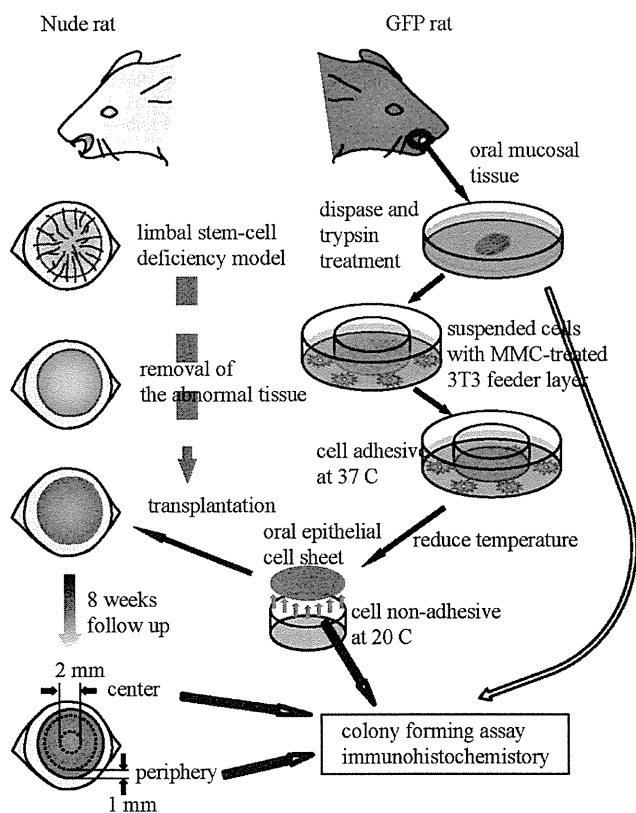
Cultured oral mucosal epithelial cell sheets contain stem/ progenitor cells, as demonstrated by colony-forming assays (CFAs) and immunohistochemistry [6,7]. Clinically successful long-term reconstruction after cell sheet transplantation suggests that these transplanted stem/progenitor cells are maintained in vivo postoperatively [12–14]. Although a few studies have investigated the existence of stem/progenitor cells in reconstructed cornea [15,16], this has yet to be established. Thus, in this study, we assessed the maintenance and distribution of epithelial stem/progenitor cells after corneal reconstruction using oral mucosal epithelial cell sheets in a rat model.

Materials and Methods

2.1. Primary culture of GFP rat oral mucosal epithelium

Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Our experimental procedures were approved by the Committee for Animal Research of Osaka University Graduate School of Medicine. We created cultured cell sheets fabricated from GFP

rat oral mucosal epithelial cells and transplanted it onto the eyes of a nude rat limbal stem cell deficiency model (Fig. 1). Oral mucosal biopsy specimens (2 mm radius) were excised from 4 green fluorescent protein (GFP) rats ("green rat CZ-004," SD TgN (act-EGFP) OsbCZ-004; Japan SCL, Inc., Shizuoka, Japan). Each rat weighed 200 g. Anesthesia was induced by intraperitoneal administration of ketamine hydrochloride (25 mg/kg) and xylazine hydrochloride (10 mg/kg). Biopsy specimens were incubated at 4°C for 4 h with Dispase II (Roche Diagnostics GmbH, Mannheim, Germany) and treated with trypsin/EDTA solution (Invitrogen, Carlsbad, NM) at room temperature for 20 min. Cell suspensions were cultured on temperature-responsive culture inserts (CellSeed Inc., Tokyo, Japan) at an initial density of 4×10^5 cells/23-mm insert along with mitomycin C (MMC)-treated NIH/3T3 cells that were separated by these cell culture inserts in the keratinocyte culture medium (KCM) (Dulbecco's modified Eagle's medium [DMEM]/F12 [3:1] supplemented with 10% fetal bovine serum [Japan Bio Serum, Hiroshima, Japan], 0.5% Insulin–Transferrin–Selenium [ITS; Invitrogen, Carlsbad, CA], 10 µM isoproterenol [Kowa, Tokyo, Japan], 2.0×10^{-9} M triiodothyronine [MP Biomedicals, Aurora, OH], 0.4 µg/mL hydrocortisone succinate [Wako, Osaka, Japan], and 10 ng/mL EGF [R&D Systems, Minneapolis, MN]) [6]. Five days later, oral epithelial cells achieved confluence. After an additional 5–7 days of culture, the resulting cell sheets were harvested by reducing the culture temperature to 20°C for 30 min.

Figure 1. Transplantation strategy of cultured cell sheets fabricated from GFP rat oral mucosal epithelial cells onto the eyes of a nude rat limbal stem cell deficiency model.
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2.2. Transplantation of cultured oral epithelial cell sheets

A limbal stem cell deficiency model was generated in one eye each of anesthetized nude rats (F344/NJcl-rnu/rnu) by excising all corneal epithelial and limbal tissues (N = 4). Three weeks after surgery, conjunctival scar tissue with some neovascularization covered the entire corneal surface and invaded into the stroma. Prior to cell sheet transplantation, the conjunctivalized ocular surface was surgically removed to re-expose the corneal stroma. Tissue-engineered culture cell sheets, fabricated ex vivo from GFP rat oral mucosal epithelial cells, were harvested and transplanted over the stromal bed. For healing protection, tarsorrhaphy was performed after transplantation. Antibiotics and steroids were topically applied postoperatively 3 times daily. The eyes were carefully observed using a slit lamp biomicroscope and a fluorescence stereomicroscope. Eight weeks after surgery, the rats were sacrificed with an overdose of anesthetic agent (pentobarbital), and their eyes were enucleated for histology examinations and CFA.

2.3. Immunofluorescence and histological examinations

Oral mucosal epithelium, tissue-engineered epithelial cell sheets, and reconstructed corneas were assessed using immunofluorescence examinations. Cryosections (thick, 10 µm) were treated with 5% bovine serum albumin (BSA) in 50 mM Tris-buffered saline (TBS; pH 7.2) containing 0.4% Triton X-100 at room temperature for 60 min. The sections were then incubated overnight at 4°C with primary antibodies diluted with 1% BSA in PBS containing 0.4% Triton X-100. Primary antibodies included mouse monoclonal anti-p63 (4A4; Santa Cruz Biotechnology Inc., Santa Cruz, CA), mouse monoclonal anti-K14 (CKB1; Abcam, Tokyo, Japan), rabbit polyclonal anti-CD31 (Abcam), and Alexa Fluor 495 or 555-labeled secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) were used. All sections were counterstained with Hoechst 33342 and observed under a fluorescence microscope (Axiovert 200; Carl Zeiss, Oberkochen, Germany). Transplanted corneal sections were also conventionally stained with hematoxylin and eosin (HE) and observed under a light microscope (BX50; Olympus, Tokyo, Japan).

2.4. CFA

We used CFA to assess if there were putative progenitor cell populations in the biopsied, cultured, and transplanted cells. Primary cells isolated from GFP rat oral epithelial tissues were seeded in untreated 6-well culture plates for CFAs. Secondary cells isolated from tissue-engineered cell sheets by trypsin digestion were also used for CFAs. Transplanted epithelial cells from reconstructed corneas were also used for CFAs as follows. Corneoscleral tissues were excised from enucleated eyes 8 weeks after transplantation. As assessed using the fluorescence stereomicroscope, all of the epithelium that covered a cornea expressed GFP. The central areas (diameter, 2 mm) and peripheral areas (width, 1 mm) of the corneas were excised and treated with Dispase II at 37°C for 1 h. Epithelial cells were then separated and treated with 0.25% trypsin/EDTA solution at 37°C for 20 min to create single cell suspensions. Cells at a density of 3×10^3 cells/well were used for CFAs for biopsied, cultured, and transplanted epithelial cells along with MMC-treated 3T3 feeder cells in 6-well culture plates. After 12 days in culture, the cells were fixed and stained with rhodamine B. The colony-forming efficiency (CFE) of primary, cultured, and transplanted cells was determined by dividing the number of colonies per well by the total number of seeded cells in each well (N = 4, duplicates used for each sample).

2.5. Statistical Analysis

Results are presented as mean ± SEs. Data were analyzed using t tests; $p<0.05$ was considered statistically significant. All statistical analyses were carried out using JMP version 9.0.3.

Results

To monitor the cell fates of transplanted cell sheets, we prepared epithelial cell sheets fabricated from GFP rats. Phase contrast microscopy showed that oral mucosal epithelial cells obtained from the GFP rats proliferated and became stratified after culture for 10 days (Fig. 2A). These cells showed tight, dense packing on culture inserts as well as a cobble stone-like cell morphology, and fluorescence microscopy showed that all of these cells were GFP positive (Fig. 2B). Epithelial cell sheets were also evaluated on sections. The epithelial cell sheets were well stratified with 2–3 layers of GFP-positive epithelial cells (Fig. 2C). Immunohisto-chemistry results showed that the basal cells of cultured epithelial cell sheets expressed p63, a putative epithelial stem cell marker (Fig, 2D). The mean (±SE) CFE values for cells from the primary oral mucosa and cultured cell sheets were 3.17±0.67% and 2.12±0.68%, respectively, and both primary oral mucosa and cultured cell sheets contained sufficient numbers of progenitor cells (Fig. 2E).

Eight weeks after transplantation, a slit lamp photograph showed that the surface of a cornea was completely covered with epithelium (Fig. 3A). HE staining of a transplanted corneal section showed that 3–4 cell layers of epithelium were reconstructed (Fig. 3B). Immunostaining results showed that a transplanted cornea expressed p63 in the basal layers and K14 in all epithelial layers (Fig. 3C, 3D, 3E, 3F). In the peripheral region obvious neovascularization was observed in slit lamp examination (Fig. 3A). The presence of neovascularization was confirmed by the immunohistochemistry of CD31 (Fig. 3G, 3H). The trans-planted cell sheet successfully reconstructed the corneal surface, and the entire cornea was covered by the GFP-positive multilay-ered epithelium (Fig, 3I). Cross-section analysis also demonstrated that the GFP-positive cells were on a reconstructed cornea (Fig. 3J).

To compare the distributions of putative epithelial stem/progenitor cells in the reconstructed corneas with those in the normal corneas, we used CFAs for epithelial cells harvested from both peripheral and central areas of the corneas in cell sheet-transplanted eyes (Fig. 3K). The reconstructed cornea contained colony-forming cells, and the mean (±SE) CFE values for the peripheral and central epithelial cells removed from the trans-planted corneas were 4.67±1.53% and 0.41±0.26%, respectively, which results were significantly different (p = 0.036; Fig. 3L). In normal rats, the mean CFE value for the peripheral cornea was significantly higher than that for the central cornea (2.78±0.53% vs. 0.73±0.50%, p = 0.024; Fig. 3L).

Discussion

The aim of this study was to examine the maintenance and distribution of epithelial stem/progenitor cells after corneal reconstruction using oral mucosal epithelial cell sheets in a rat model. Our findings indicate that cultivated oral mucosal epithelial cell sheet survives and contains putative epithelial stem/progenitor cells after transplantation. In addition, epithelial stem/progenitor cells are maintained abundantly in peripheral cornea, which shows a similar distribution pattern of stem cells to normal eyes.

When we performed autologous cell sheet transplantation in LSCD patients, it was difficult to distinguish whether the transplanted cell sheets survived, because these sheets were derived from the patients themselves. To resolve this problem, we used cultured cell sheets fabricated from GFP rats in this study. Using this method, the presence of GFP-positive cells after cell sheet transplantation in reconstructed corneas established that the transplanted cell sheets survived postoperatively.

We successfully generated the oral mucosal epithelial cell sheets from GPF rat. The epithelial cell sheets expressed p63 in the all of the basal layers and contained sufficient numbers of progenitor cells. Based on these results, we determined that these rat oral mucosal epithelial cell sheets were of sufficient quality for our subsequent transplant experiments [17].

After cell sheet transplantation, the entire cornea was covered by the GFP-positive cells, and they also expressed p63 in the basal layer and K14 in all epithelial layers. These findings suggested that the transplanted cell sheets survived and retained stem/progenitor cells for at least 8 weeks postoperatively. These results strongly support a hypothesis that stem/progenitors cells can survive after this treatment, which results in the long-term success of
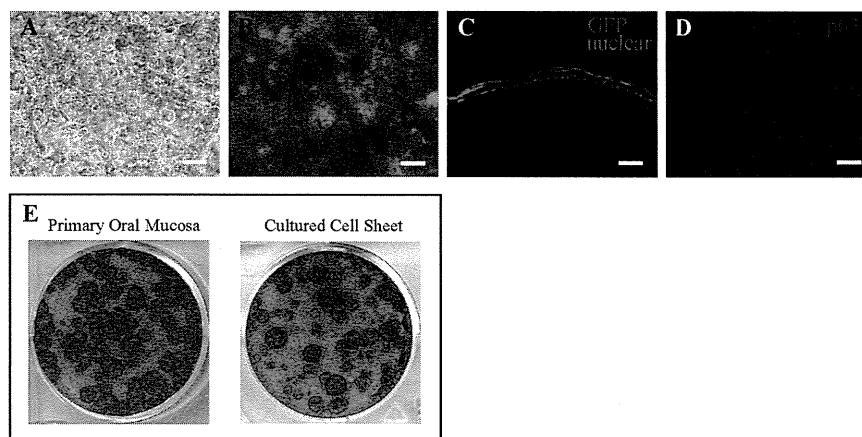
Figure 2. Successful generation of transplantable epithelial cell sheets from GFP rats. (A) Phase-contrast image of oral mucosal epithelial cells. (B) Fluorescence microscopy showing GFP-positive cells from a donor GFP rat. (C) Cross-section of a GFP-positive cell sheet. (D) Basal cells of cultured epithelial cell sheets express p63. (E) Both primary oral mucosa and cultured cell sheets contained sufficient numbers of progenitor cells. Scale bars = 200 μm (A, B) and 50 μm (C, D).
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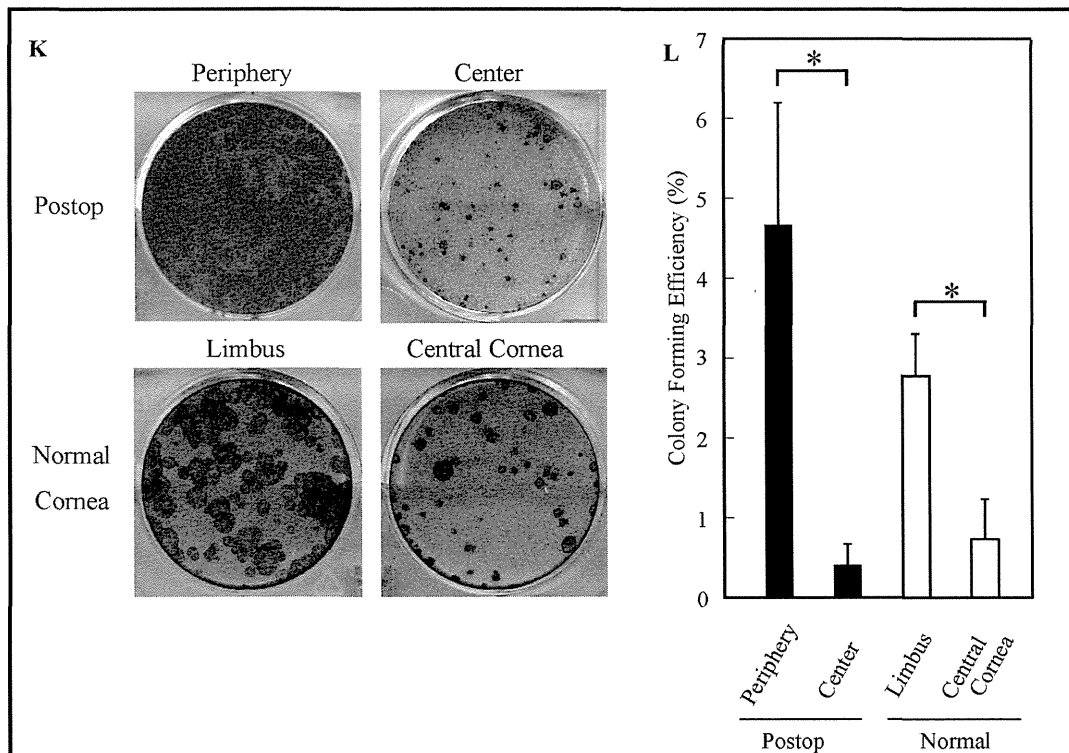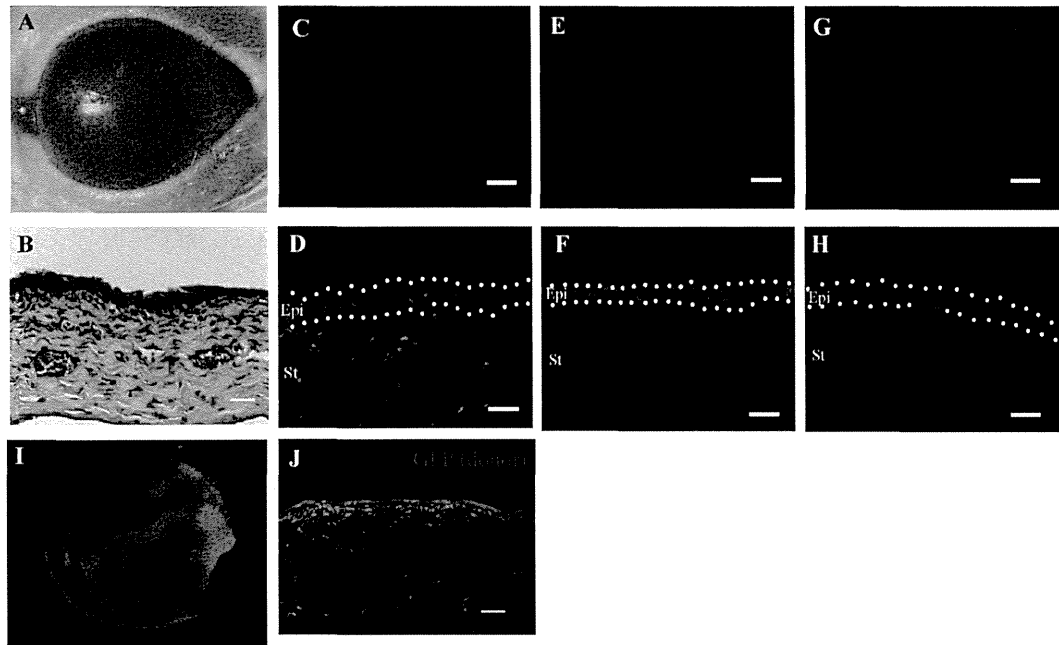
Figure 3. Eight weeks after transplanting cultured cell sheets prepared from GFP rats. (A) Slit lamp photograph of a reconstructed nude rat cornea. (B) HE staining showing that the reconstructed cornea was covered with 3–4 epithelial cell layers. (C, D) Immunostaining results showing that a reconstructed cornea still expressed p63 in the basal layers. (E, F) K14 was expressed in all epithelial layers after cell sheet transplantation. (G, H) The expression of CD 31 was observed in the basal epithelial layer and the superficial stroma in the peripheral cornea. (I) Corneal surface completely covered with GFP-positive epithelial cells. (J) Fluorescence microscopy showing that surviving GFP-positive epithelial cells were on the surface of a transplanted cornea. (K) CFAs for peripheral and central epithelial cells prepared from a postoperative eye and limbal and central epithelial cells prepared from a normal cornea. (L) The mean CFE values for the peripheral cells removed from the transplanted cornea was significantly greater than that for the central cell, which was also observed in a normal cornea (N = 4, *p<0.05). Epi, epithelium; St, stroma. Scale bars = 20 µm (C, D, E, F, G, H) and 25 µm (B, I).
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transplantation of cultured oral mucosal cell sheets in LSCD patients.

The CFE values for the peripheral epithelial cells removed from the reconstructed cornea were significantly higher than that for the central cells. This non-uniform pattern over the cornea indicates that, after transplantation, stem cell precursors are predominantly in the peripheral cornea compared with the central cornea. This suggests that stem cell progenitor maintenance is not cell autonomous, possibly owing to different microenvironments. It is interesting that this is also true in normal corneas [18].

The mechanism underlying the enrichment of progenitors in peripheral regions is not entirely clear. It may be due to the peripheral vascularization. The origin of the cell sheet is oral mucosa, a vascularized tissue, inducing neovascularization. We and others reported cultivated oral mucosal cell sheet contains highly level of angiogenic factors, such as basic Fibroblast growth factor or Thrombospondin 1, compared to cultivated corneal epithelial cell sheet [19,20]. In fact, peripheral neovascularization occurs frequently in human clinical application of oral mucosal cell sheet transplantation reported by us and others [7,12–14]. These neovascularization might contribute to maintain the stemness. For example, in the bone marrow, several reports have shown that vascular endothelial cells regulate hematopoietic stem/progenitor cells through the production of specific paracrine growth factors

[21–24]. Kobayashi et al. demonstrated that the endothelial cells modulates reconstitution of hematopoietic stem/progenitor cells through the modulation of angiocrine factors with Akt-mTOR-activated endothelial cells supporting the self-renewal and expansion of hematopoietic stem/progenitor cells [24]. Recent studies also demonstrated endothelial cells regulate stem/progenitor cell niche in the central nervous system, and adipose tissues [25,26]. Regarding the corneal epithelium, there is an indirect evidence of vascular niche that limbal niche cells have the angiogenesis potential and prevent corneal epithelial stem/progenitor cells differentiation [27].

In conclusion, we have shown that oral mucosal epithelial cell sheets can survive and contain putative epithelial stem/progenitor cells after transplantation. Because stem/progenitor cell maintenance in transplanted cell sheets is the key for a good prognosis, the results of our study are a first step toward understanding the behavior of these precursor cells in transplanted tissue.

Author Contributions

References

1. Schermer A, Galvin S, Sun TT (1986) Differentiation-related expression of a major 64 K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. J Cell Biol 103: 49–62.
2. Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM (1989) Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell 57: 201–209.
3. Thoft RA, Friend J (1983) The X, Y, Z hypothesis of corneal epithelial maintenance. Invest Ophthalmol Vis Sci 24: 1442–1443.
4. Tseng SC (1989) Concept and application of limbal stem cells. Eye (Lond) 3 (Pt 2): 141–157.
5. Tsai RJ, Sun TT, Tseng SC (1990) Comparison of limbal and conjunctival autograft transplantation in corneal surface reconstruction in rabbits. Ophthalmology 97: 446–455.
6. Nishida K, Yamato M, Hayashida Y, Watanabe K, Maeda N, et al. (2004) Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded ex vivo on a temperature-responsive cell culture surface. Transplantation 77: 379–385.
7. Nishida K, Yamato M, Hayashida Y, Watanabe K, Yamamoto K, et al. (2004) Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. N Engl J Med 351: 1187–1196.
8. Nakamura T, Inatomi T, Sotozono C, Amemiya T, Kanamura N, et al. (2004) Transplantation of cultivated autologous oral mucosal epithelial cells in patients with severe ocular surface disorders. Br J Ophthalmol 88: 1280–1284.
9. Samson CM, Nduaguba C, Baltatzis S, Foster CS (2002) Limbal stem cell transplantation in chronic inflammatory eye disease. Ophthalmology 109: 862–868.
10. Ilari L, Daya SM (2002) Long-term outcomes of keratolimbal allograft for the treatment of severe ocular surface disorders. Ophthalmology 109: 1278–1284.
11. Santos MS, Gomes JA, Hofling-Lima AL, Rizzo LV, Romano AC, et al. (2005) Survival analysis of conjunctival limbal grafts and amniotic membrane transplantation in eyes with total limbal stem cell deficiency. Am J Ophthalmol 140: 223–230.
12. Inatomi T, Nakamura T, Koizumi N, Sotozono C, Yokoi N, et al. (2006) Midterm results on ocular surface reconstruction using cultivated autologous oral mucosal epithelial transplantation. Am J Ophthalmol 141: 267–275.
13. Nakamura T, Takeda K, Inatomi T, Sotozono C, Kinoshita S (2011) Long-term results of autologous cultivated oral mucosal epithelial transplantation in the scar phase of severe ocular surface disorders. Br J Ophthalmol 95: 942–946.

14. Satake Y, Higa K, Tsubota K, Shimazaki J (2011) Long-term outcome of cultivated oral mucosal epithelial sheet transplantation in treatment of total limbal stem cell deficiency. Ophthalmology 118: 1524–1530.
15. Nakamura T, Inatomi T, Cooper LJ, Rigby H, Fullwood NJ, et al. (2007) Phenotypic investigation of human eyes with transplanted autologous cultivated oral mucosal epithelial sheets for severe ocular surface diseases. Ophthalmology 114: 1080–1088.
16. Chen HC, Chen HL, Lai JY, Chen CC, Tsai YJ, et al. (2009) Persistence of transplanted oral mucosal epithelial cells in human cornea. Invest Ophthalmol Vis Sci 50: 4660–4668.
17. Pellegrini G, Dellambra E, Golisano O, Martinelli E, Fantozzi I, et al. (2001) p63 identifies keratinocyte stem cells. Proc Natl Acad Sci U S A 98: 3156–3161.
18. Chen W, Hara K, Tian Q, Zhao K, Yoshitomi T (2007) Existence of small slow-cycling Langerhans cells in the limbal basal epithelium that express ABCG2. Exp Eye Res 84: 626–634.
19. Kanayama S, Nishida K, Yamato M, Hayashi R, Sugiyama H, et al. (2007) Analysis of angiogenesis induced by cultured corneal and oral mucosal epithelial cell sheets in vitro. Exp Eye Res 85: 772–781.
20. Sekiyama E, Nakamura T, Kawasaki S, Sogabe H, Kinoshita S (2006) Different expression of angiogenesis-related factors between human cultivated corneal and oral epithelial sheets. Exp Eye Res 83: 741–746.
21. Hooper AT, Butler JM, Nolan DJ, Kranz A, Iida K, et al. (2009) Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. Cell Stem Cell 4: 263–274.
22. Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, et al. (2002) Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell 109: 625–637.
23. Lane SW, Scadden DT, Gilliland DG (2009) The leukemic stem cell niche: current concepts and therapeutic opportunities. Blood 114: 1150–1157.
24. Kobayashi H, Butler JM, O'Donnell R, Kobayashi M, Ding BS, et al. (2010) Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. Nat Cell Biol 12: 1046–1056.
25. Shen Q, Goderie SK, Jin L, Karanth N, Sun Y, et al. (2004) Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. Science 304: 1338–1340.
26. Tang W, Zeve D, Suh JM, Bosnakovski D, Kyba M, et al. (2008) White fat progenitor cells reside in the adipose vasculature. Science 322: 583–586.
27. Li GG, Chen SY, Xie HT, Zhu YT, Tseng SC (2012) Angiogenesis potential of human limbal stromal niche cells. Invest Ophthalmol Vis Sci 53: 3357–3367.

Translational Research on Ocular Surface Reconstruction Using Oral Mucosal Epithelial Cell Sheets

Yoshinori Oie, MD, PhD, and Kohji Nishida, MD, PhD

Abstract: Ocular surface reconstruction using autologous oral mucosal epithelial cell sheets has drastically changed the treatment of limbal stem-cell deficiency. The morphological and functional characteristics of oral mucosal epithelial cell sheets are similar to those of normal corneal epithelium. Ocular surface reconstruction can prevent potential problems associated with limbal transplantation, including immune rejection and donor tissue shortages. Thus far, ocular reconstruction using epithelial cell sheets has been limited to clinical research. Although the effectiveness and safety of this surgical approach have been confirmed to some extent, efforts to make its use more widespread are required. "Translational research" refers to the process of developing a new treatment based on basic research findings with useful practical applications in the field of health care. Medical centers for translational research are required to promote translational research in academic institutes. The Pharmaceutical Affairs Law was revised to promote technologies in the field of regenerative medicine in Japan. This article reviews translational research of ocular surface reconstruction using oral mucosal epithelial cell sheets.

Key Words: ocular surface reconstruction, oral mucosal epithelial cell sheets, translational research

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OCULAR SURFACE EPITHELIUM

The ocular surface is composed of 3 types of epithelium: corneal epithelium, limbal epithelium, and conjunctival epithelium (Fig. 1), all of which are classified as nonkeratinized stratified squamous epithelia.

The corneal epithelium is a transparent epithelial layer that expresses the specific markers keratins 3 and 12.[1,2] The corneal epithelium exerts a very strong barrier function owing to the presence of tight junctions, which prevent fluid penetration into the cornea, and membrane-associated mucin, which prevents the entry of pathogens.[3–5]

The limbus is a transitional zone between the cornea and the conjunctiva. The limbal epithelium is structurally similar to the corneal epithelium; however, one important difference between these tissues is the larger numbers of melanocytes and Langerhans cells in the limbal epithelium. Additionally, there is evidence supporting the limbal localization of stem cells. For example, compared with corneal epithelial cells, limbal epithelial cells have a lower level of expression of cell differentiation markers, such as keratins 3 and 12,[1] and they have a higher level of expression of stem cell markers, including p63, ATP-binding cassette subfamily G member 2 (ABCG2), N-cadherin, K19, nerve growth factor receptors (eg, TrkA), and integrin a6.[6–10] Limbal basal cells also contain label-retaining cells[11] and exhibit higher proliferative activity than central corneal cells.[12,13] Limbal transplantation can be performed to reconstruct the ocular surface of patients with limbal stem-cell deficiency (LSCD).[14]

The conjunctival epithelium is a mucous membrane that covers the inner surfaces of the upper and lower eyelids and the outer surface of the eyeball. Conjunctival epithelial cells express keratins 5 and 14, which are generally expressed in mucous epithelia. The conjunctival epithelium is vascularized and less transparent than the corneal epithelium and contains goblet cells, which secrete mucin (MUC5AC) into the tear film.[3]

OCULAR SURFACE RECONSTRUCTION WITH CULTIVATED LIMBAL EPITHELIAL CELL TRANSPLANTATION

LSCD is caused by the loss of limbal stem cells.[15,16] In patients with LSCD, the cornea is covered by vascularized conjunctival epithelium, which causes visual impairment. The causes of LSCD can be classified into 4 categories: (1) congenital diseases resulting in stem cell aplasia (eg, aniridia or sclerocornea), (2) external causes (eg, thermal, alkali, or acid burns), (3) internal diseases such as Stevens–Johnson syndrome and ocular cicatricial pemphigoid, and (4) unknown causes (idiopathic LSCD).

For ocular surface reconstruction, the entire ocular surface environment should be considered, including the eyelid, tear film, fornix, bulbar conjunctiva, and palpebral conjunctiva, as well as the corneal surface. The presence of an
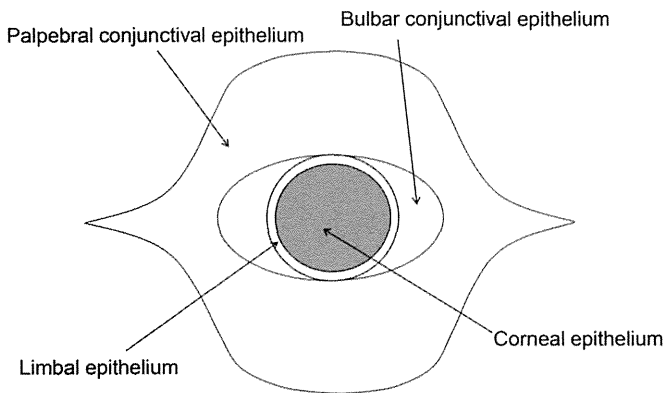
FIGURE 1. Ocular surface epithelium. The ocular surface consists of 3 types of epithelium: corneal epithelium, limbal epithelium, and conjunctival epithelium.

eyelid abnormality, such as entropion or ectropion, may cause corneal erosion or lagophthalmos with evaporation of the tear film, which can lead to graft failure. Therefore, lid abnormalities should be treated before ocular surface reconstruction. Tear function is reported to significantly influence the surgical outcome after a keratolimbal allograft in patients with Stevens–Johnson syndrome.[17] Therefore, a Schirmer test should be performed preoperatively, and if the value is low, artificial tears eye drops should be administered.

Limbal allograft transplantation has been performed for patients with LSCD.[18,19] However, the shortage of donor tissue and the potential for graft rejection led to the development of cultivated limbal epithelial transplantation (CLET). Pellegrini et al[20] reported the first 2 cases of CLET in 1997. They used cultured limbal stem cells to treat 2 patients with alkali burns and showed that reconstruction using autologous cultured corneal epithelium restored the corneal surface for more than 2 years.

Since the report of Pellegrini et al, many investigators have used cultivated limbal epithelial cells to treat patients with

LSCD and have reported positive results.[21–25] Rama et al[26] reported long-term corneal regeneration using autologous cultivated limbal stem cells, demonstrating that long-term restoration and renewal of the corneal epithelium was achieved in 76.6% of 107 eyes with LSCD caused by ocular burns. The success rate of ocular surface reconstruction was significantly associated with the percentage of p63-bright holoclone-forming stem cells in the culture. No severe adverse events were observed. These results demonstrated the effectiveness and safety of CLET as well as the importance of a substantial stem cell population within the cultured cells for clinical success.

OCULAR SURFACE RECONSTRUCTION USING CULTURED ORAL MUCOSAL EPITHELIUM

Ocular surface reconstruction with cultivated limbal epithelium has been reported to be effective and safe. However, this treatment can be applied only to patients with unilateral LSCD; patients with bilateral LSCD do not have a cell source for CLET. Therefore, ocular surface reconstruction using oral mucosal epithelial cell sheets was developed for patients with bilateral LSCD[27,28] (Fig. 2). To produce cell sheets, we use a temperature-responsive culture surface that becomes hydrophobic at temperatures greater than 32°C but is hydrophilic at temperatures below 32°C.[29] Using this approach, an epithelial cell sheet cultured on this surface can be harvested by simply reducing the temperature to 20°C for 30 minutes.[30] Importantly, cell–cell junctions and the extracellular matrix on the basal side of the sheet remain intact after harvest. Furthermore, basal cells in the cell sheets are positive for p63, a putative stem cell marker, suggesting that the cell sheets can maintain the corneal epithelium over the long-term. In addition, harvested cell sheets can be manipulated without the use of carriers such as an amniotic membrane or fibrin. The morphological and functional characteristics of oral mucosal epithelial cell sheets are similar to those of the normal corneal epithelium.[31] There have been several reports on the long-term outcomes of cultivated oral mucosal
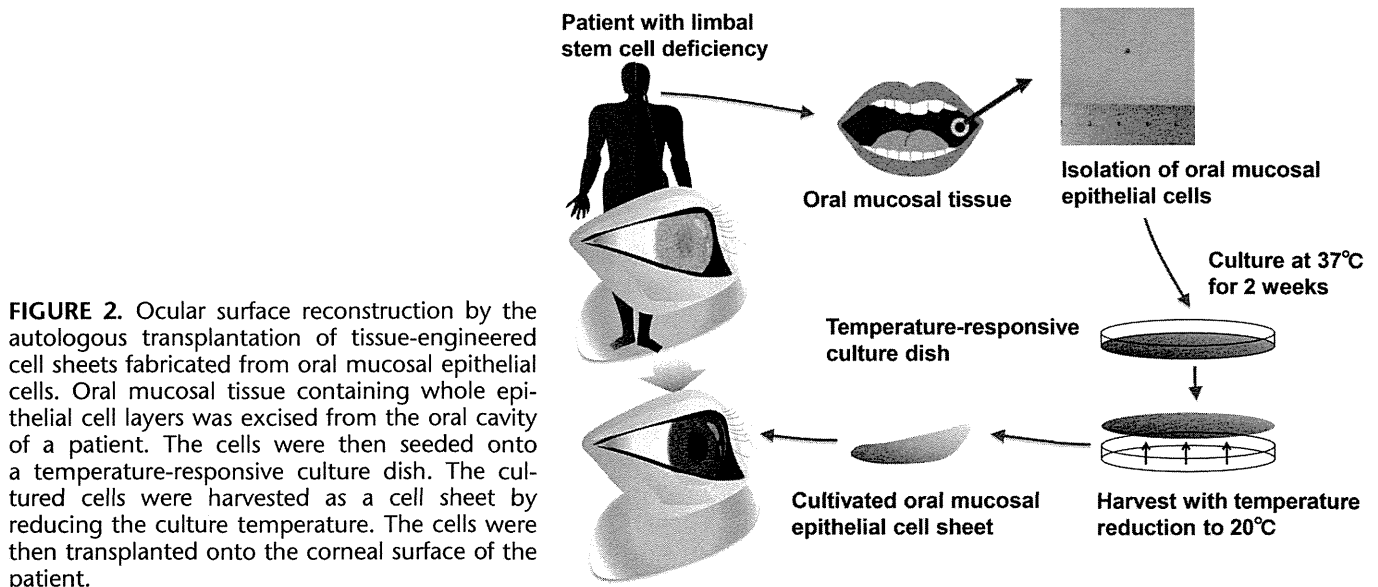
FIGURE 2. Ocular surface reconstruction by the autologous transplantation of tissue-engineered cell sheets fabricated from oral mucosal epithelial cells. Oral mucosal tissue containing whole epithelial cell layers was excised from the oral cavity of a patient. The cells were then seeded onto a temperature-responsive culture dish. The cultured cells were harvested as a cell sheet by reducing the culture temperature. The cells were then transplanted onto the corneal surface of the patient.

epithelial transplantation. Nakamura et al reported results of 19 eyes with a follow-up period of at least 36 months.[32] They showed that postoperative conjunctivalization and symblepharon were significantly inhibited. Visual acuity recovered in 10 of 19 eyes at postoperative month 36. Adverse events included persistent epithelial defects (37%), ocular hypertension (16%), and corneal infection (5%). Satake et al reported results of 40 eyes with a mean follow-up period of 25.5 months.[33] They showed that corneal surface stability was obtained in 64.8% of patients at 1 year, 59.0% at 2 years, and 53.1% at 3 years. Complications included stromal melting or perforation in 20% of patients, infectious keratitis in 5%, and glaucoma in 25%. Overall, cultivated oral mucosal epithelial transplantation has been reported to be effective and safe.

Although neovascularization occurs in some patients, ocular surface reconstruction with oral mucosal epithelial cell sheets has shown preferable results, even during long-term follow-up (Fig. 3).

MULTICENTER CLINICAL RESEARCH USING ORAL MUCOSAL EPITHELIAL CELL SHEETS

To conduct multicenter clinical research using oral mucosal epithelial sheets for ocular reconstruction, techniques for the transportation of oral mucosal epithelial cell sheets have been developed.[34] We created a transportation container that performs the basic functions of maintaining interior temperature, air pressure, and sterility (Fig. 4). The temperature and air pressure in the container are monitored by a recorder. Human oral mucosal epithelial cells obtained from 2 healthy volunteers were cultured on temperature-responsive culture dishes, and the epithelial cell sheets were transported by airplane between Osaka University and Tohoku University using this cell transportation container. During transportation, the temperature inside the container was maintained above 32°C, and variations in the air pressure did not exceed 10 hPa. The cell sheets, which were well stratified, were successfully harvested both before and 12 hours after transportation. Immunohistochemical analysis confirmed that the expression patterns of keratin 3/76, p63, ZO-1, and MUC16 were equivalent before and after transportation. However, the expression of ZO-1 in the cell sheet after transportation was slightly lower than that before transportation. The cell viability was 72.0% before transportation and 77.3% after transportation,

as determined by flow cytometric analysis. The epithelial purity was 94.6% before transportation and 87.9% after transportation. The results of sterility tests and screens for endotoxin and mycoplasma were negative for all the cell sheets. Thus, the newly developed transportation technique for air travel could be applied to promote standardization and widespread adoption of regenerative medicine.

We have begun a multicenter clinical research study using the newly developed transportation technique with Osaka University, Tohoku University, Tokyo University, and Ehime University (Fig. 5). For clinical research at Osaka University and Tohoku University, which have Good Manufacturing Practice facilities for the production of cells used for regenerative medicine, the cell sheets are fabricated at their respective cell processing centers. For clinical research at Tokyo University and Ehime University, the cell sheets are fabricated at Osaka University and then transported using the newly developed transportation system. If this research is successful, it will represent a proof of concept for this treatment.

TRANSLATIONAL RESEARCH FOR THE CORNEAL EPITHELIUM

Oral mucosal epithelial cell sheet transplantation has been performed in clinical studies, and its effectiveness and safety have been confirmed to some extent. However, because it is not ethical to continue this treatment as a clinical study, efforts should be made to promote oral mucosal epithelial sheet transplantation as a general and widely available treatment. Translational research refers to the process of developing a new treatment based on basic research findings with useful practical applications in the field of health care (Fig. 6). The steps involved in translational research include basic research, nonclinical research, clinical research, advanced medical care, and general treatment. General medical treatment means that all patients have access to the treatment, regardless of their location.

Basic research, the starting point for translational research, is conducted to evaluate whether a novel hypothesis is true. Additionally, novel ideas can be generated from basic research. Nonclinical research, the next phase of translational research, is conducted before clinical studies in humans. The primary aim of nonclinical research is to collect data through

FIGURE 3. Slit-lamp photographs of patients before and after cultivated oral mucosal epithelial cell transplantation. A, The left eye has a total LSCD. The visual acuity was 20/2000. B, One year postoperatively, the corneal epithelial clarity was well maintained and the visual acuity was 20/33. Reprinted from Nishida et al[27] with permission from the Massachusetts Medical Society.
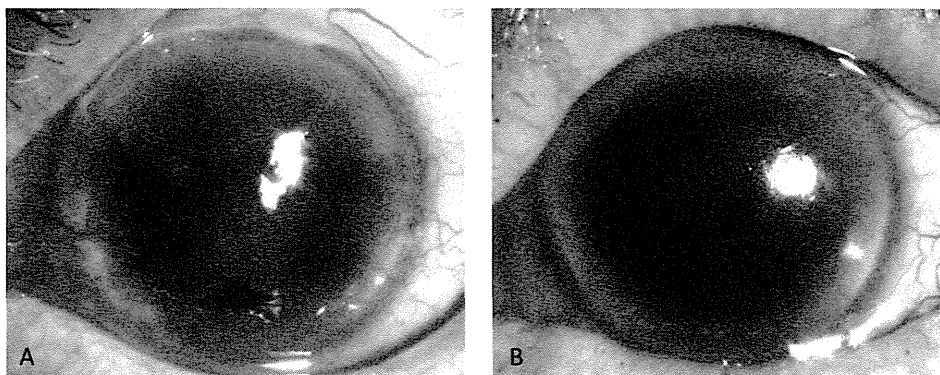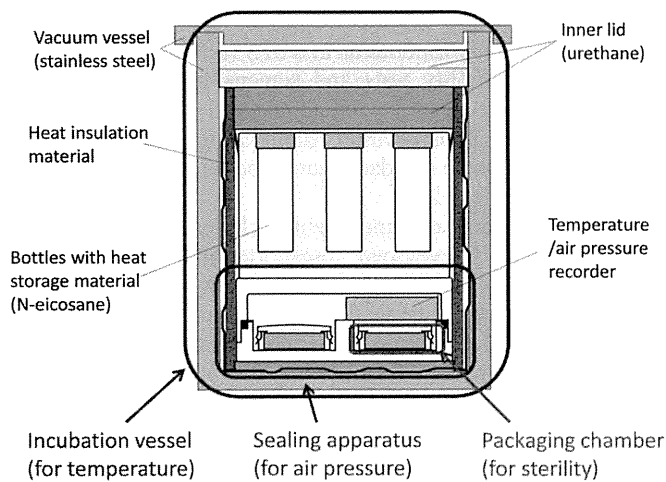
FIGURE 4. Cross-sectional view of newly developed transportation container for cultivated epithelial cell sheets. The transportation container consists of 3 parts: incubation vessel for maintenance of inner temperature, sealing apparatus for inner air pressure, and packaging chamber for sterility.

interactive testing and to assess product safety. The effectiveness and safety of a novel treatment should be confirmed by performing experiments using an animal disease model. Additionally, a novel therapy in the field of regenerative medicine should be evaluated for tumorigenicity before the treatment is administered to human subjects, especially if the treatment involves pluripotent stem cells, such as embryonic or induced pluripotent stem cells.[35] Newly developed candidate drugs may be evaluated using animal experiments to assess a drug's pharmacodynamic (effects of drug on the body), pharmacokinetic (effects of the body on the drug), and toxicity profiles. These data facilitate the identification of a safe staring dose of the drug for clinical studies. The clinical research stage of translational research involves the treatment of human subjects. The purpose of clinical research is to answer specific questions about biomedical behavioral interventions and to generate safety and efficacy data. Approval by an ethics committee is required before clinical research may commence. In Japan, clinical studies in the field of regenerative medicine must be approved by an institutional review board, and the research plan must adhere to guidelines for clinical research using human stem cells. Thus, a clinical research plan is evaluated twice before the study may commence. The following documents must be prepared before

clinical research is initiated: a clinical protocol, a product master formula, an investigator's brochure, standard operating procedures for the fabrication of cell sheets, and a case report form. A team consisting of a medical doctor, a medical writer, a specialist in the Pharmaceutical Affairs Law, a biostatistician, and a data manager should be assembled to prepare these documents. Furthermore, partnerships between government, industry, and academic institutions are important for the advancement of translational research.[36]

## SUPPORTING SYSTEMS TO EXPEDITE TRANSLATIONAL RESEARCH IN ACADEMIC INSTITUTES

When researchers conduct translational research, they have to undertake many investigational and legislative processes. However, researchers in academic institutes are not trained for the steps that often involve specialized paperwork. Therefore, a supporting system is necessary to expedite translational research in academic institutes.

Osaka University Hospital has a Department of Medical Innovation, which comprises 2 centers, the Medical Center for Translational and Clinical Research (MTR) and the Data Coordinating Center. The MTR provides centralized support for translational research, with the goal of developing practical applications for new ideas in medical technology that originate in academia. The MTR also supports the practical development of new pharmaceutical products and medical devices through clinical trials. The Data Coordinating Center provides comprehensive support to both intervention trials and analytic research by performing independent data management and statistical analyses. Through their organic links, both centers provide Osaka University Hospital with integrated and efficient support, enabling it to fulfill its obligation to research and develop advanced medical technologies as an advanced treatment hospital.

The Department of Medical Innovation provides comprehensive contract research consulting and support services, including cell processing center-related activities (usage of cell culture facilities and sterility testing), clinical study support activities (document creation, coordinator activities, monitoring activities, and creation of clinical study reports), nonclinical study activities (tumorigenicity studies in nude or NOG mice and soft agar colony formation studies), registration activities (creation of case report forms and case registration activities), data management activities (database

FIGURE 5. Multicenter clinical research using the cell sheet transportation system. Multicenter clinical research is conducted at 4 universities. Oral surgeries for mucosal tissue and cell sheet transplantation are performed at the Institute for Surgery. The cell sheets are cultured at the cell processing center at the Institute for Cell Culture. The tissues and cell sheets are transported between institutes 1 and 2.
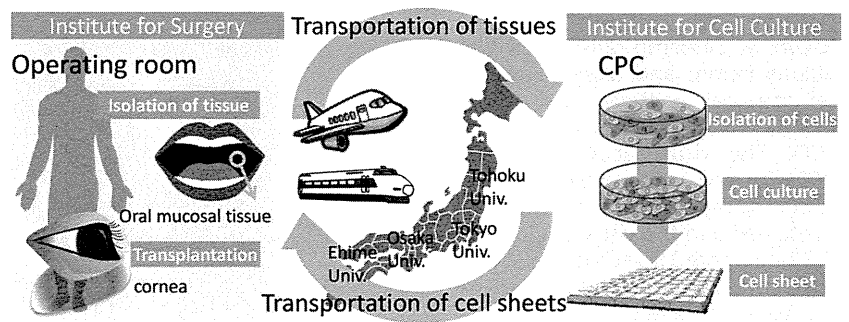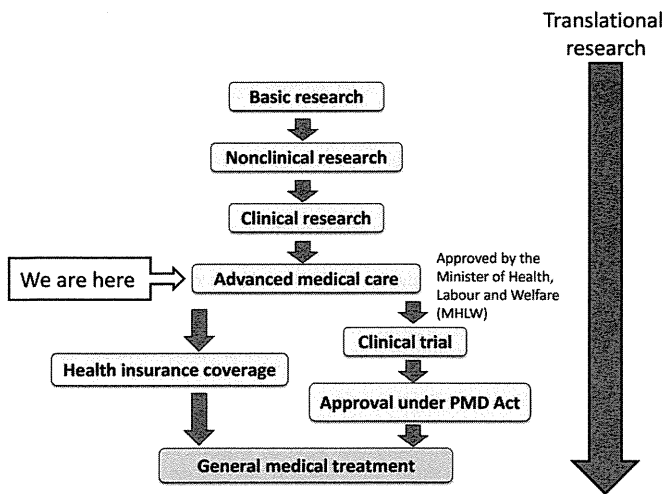
FIGURE 6. Translational research. The steps from basic research to general medical treatment are referred to as "translational research."

creation and management), and statistical analysis activities (statistical analysis planning support and statistical analyses). These activities help researchers to promote translational research at Osaka University Hospital.

ADVANCED MEDICAL SERVICE AND GENERAL MEDICAL TREATMENT

Advanced medical care refers to any medical service provided in the absence of health insurance coverage that involves the use of unapproved drugs or medical devices or the off-label use of approved drugs or medical devices. Advanced medical care is approved by the Minister of Health, Labour and Welfare in Japan. The features of advanced medical care include evaluation for future health insurance coverage and exceptional approval of mixed treatment of health insurance and non-health insurance. Advanced medical care procedures are categorized as either A or B based on the level of invasiveness. Category A indicates a minimally invasive procedure, whereas category B indicates a more invasive procedure.

Examples of advanced medical care in the field of ophthalmology include cataract surgery with multifocal intraocular lenses, the genetic analysis of patients with corneal dystrophy, and amniotic membrane transplantation for patients with severe ocular surface diseases. Additionally, our technique, the transplantation of cultivated autologous oral mucosal epithelial cell sheets, was approved as an advanced medical care procedure on December 28, 2012. This approval means that this surgery is close to becoming a general medical service.

The Regenerative Medicine Promotion Law and the Act on the Safety of Regenerative Medicine were recently established in Japan. Additionally, the Pharmaceutical Affairs Law was revised as the Pharmaceuticals, Medical Devices, and Other Therapeutic Products Act (PMD Act), which introduced early approval for regenerative and cellular
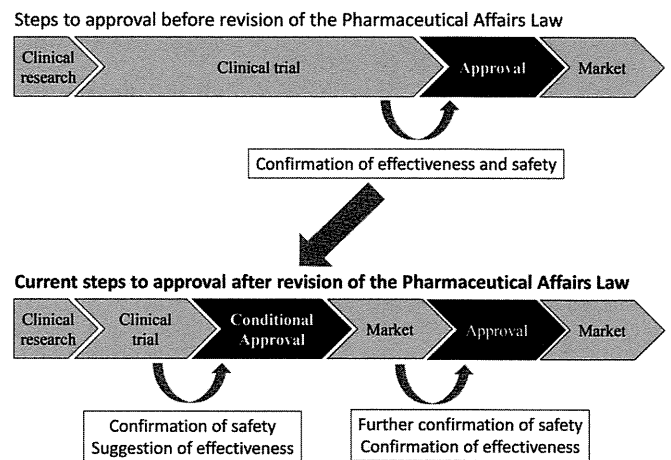
FIGURE 7. Revision of the Pharmaceutical Affairs Law has enabled the early approval of regenerative and cellular therapeutic methods. Although confirmation of a product's effectiveness and safety were necessary for approval before the revision, confirmation of safety and the suggestion of effectiveness are now required for conditional approval.

therapeutic products (Fig. 7). Before this revision, confirmation of a product's effectiveness and safety was necessary for its approval. However, the confirmation of safety and the suggestion of effectiveness are now required for conditional approval. These changes will increase the number of approved regenerative medicine products and will promote the standardization of regenerative medicine.

There are 2 ways for a new treatment to become widely available as a general medical treatment: coverage by health insurance or approval as a medical device under the PMD Act. A treatment that receives approval as a regenerative and cellular therapeutic product under the PMD Act will be more accessible to patients because all hospitals can purchase commercially available products.

CONCLUSIONS

Many researchers have been engaged in the development of regenerative medicine treatments for the cornea. We believe that sufficient data regarding basic and clinical research using cultivated oral mucosal epithelial cell sheets has already been published. The final goal of corneal translational research is to develop therapies as general treatments that are accessible to all patients. The approval of a novel therapy by the PMD act would be ideal because it would ensure access to the treatment for all patients regardless of their location. We are struggling to make oral mucosal epithelial cell sheet transplantation a widely available general medical treatment, and we hope that many patients will benefit from it in the near future.

REFERENCES
1. Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol.* 1986;103:49–62.