Persistence of Transplanted Oral Mucosal Epithelial Cells in Human Cornea

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PURPOSE. To determine the expression of differentiation and progenitor cell markers in corneal tissues that previously underwent autologous cultivated oral mucosal epithelial transplantation (COMET).

METHODS. Four eyes from three alkaline-injured patients and one thermally injured patient underwent COMET to promote re-epithelialization or corneal reconstruction. Between 10 and 22 months (mean, 14.2 ± 5.5 months [SD]) after COMET, the corneal tissues were obtained after penetrating keratoplasty (n = 1) or autologous limbal transplantation (n = 3). Immunofluorescent microscopy for keratin (K3, -12, -4, -13, and -8; connectin [Cx43]; MUC5AC; laminin-5; pan-p63; ABCG2; and p75 was performed in those specimens as well as in the oral mucosa and cultivated oral mucosal epithelial cells (OMECs).

RESULTS. All four specimens were unanimously positive for K3, -4, and -13 but negative for K8 and MUC5AC, suggesting that the keratinocytes were oral mucosa-derived. However, peripheral K12 staining was positive only in patient 2, suggesting a mixed oral and corneal epithelium in that case. Cx43 staining in the basal epithelium was negative in patients 1, 2, and 3, but was positive in patient 4. Small, compact keratinocytes in the basal epithelium preferentially expressed pan-p63, ABCG2, and p75. Although the staining of pan-p63 and ABCG2 tended to be more than one layer, signal for p75 was consistently localized only to the basal layer.

CONCLUSIONS. The study demonstrated the persistence of transplanted OMECs in human corneas. In addition, small, compact cells in the basal epithelium preferentially expressed the keratinocyte stem/progenitor cell markers, which may be indicative of the engraftment of the progenitor cells after transplantation. (Invest Ophthalmol Vis Sci. 2009;50: 4660–4668) DOI:10.1167/iovs.09-3377

Since the first report by Pellegrini et al.1 in 1997, cultivated corneal epithelial transplantation (CCET) has become a promising therapy for severe ocular surface diseases (OSDs). This tissue-engineering technique utilizes carriers such as amniotic membrane (AM)1–5 or fibrin gel6 as carriers to deliver the cultivated keratinocytes. Because the survival rate of allogeneic limbal stem cell transplantation is low even after immunosuppression17–19 in patients with bilateral limbal stem cell deficiency (LSCD), nonocular self-mucosal epithelium has been considered an alternative source of keratinocytes.11 Since 2004, there have been reports regarding cultivated autologous oral mucosal epithelial transplantation (COMET) for human OSDs, which have shown satisfactory results.12–16 Recently, our team has been conducting a clinical trial of COMET. We found that especially in acute chemical burn, COMET can promote re-epithelialization and stabilize the corneal surface, thereby dramatically reducing the hospitalization time. However, unlike CCET, corneas reconstructed by COMET often show peripheral neovascularization (NV), which tends to be more severe in acute burns. Given that corneal NV associated with inflammation is a key factor favoring conjunctivalization,17,18 it remains to be answered whether in the presence of prominent corneal NV, the conjunctival epithelium will eventually replace the transplanted oral mucosal epithelial cells (OMECs).

Nakamura et al.19,20 have reported the phenotypic findings on successful and failed COMET. In this study, we further demonstrated that transplanted OMECs survived in vascularized corneas long after transplantation, and together with the expression of putative stem/progenitor cell markers, supported the plausibility of engraftment of oral mucosal epithelial stem/progenitor cells in the cornea.

MATERIALS AND METHODS

Subjects

The COMET clinical trial study was first approved by the Institutional Review Board of Chang Gung Memorial Hospital in Taoyuan (trial no. 93-292A) and then was approved by and under the supervision of Department of Health of Taiwan as a phase I clinical study (trial no.: 0950206914). Informed consent was obtained from all patients, in accordance with the tenets of the Declaration of Helsinki. Written consent was obtained not only before surgery, but also when using the patient’s tissue for study. Four eyes of four patients with total LSCD caused by chemical/thermal injury were included in the study. Among them, COMET was performed to reconstruct the corneal surface after a chronic thermal burn (patient 1) or to promote re-epithelialization in acute (patients 2 and 3) and chronic (patient 4) alkaline burn with persistent epithelial defect. Demographic data of the patients are summarized in Table 1.

Disclosure: H.-C.J. Chen, None; H.-L. Chen, None; J.-Y. Lai, None; C.-C. Chen, None; Y.-J. Tsai, None; M.-T. Kuo, None; P.-H. Chu, None; C.-C. Sun, None; J.-K. Chen, None; D.-H. K. Ma, None

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TABLE 1. Demographic Data and Clinical Information of Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Initial VA</th>
<th>Pre-COMET Surgeries</th>
<th>Post-COMET Surgeries</th>
<th>Interval* (mo)</th>
<th>Post-OP BCVA</th>
<th>Follow-up (mo)</th>
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<tr>
<td>1</td>
<td>27</td>
<td>Male</td>
<td>Chronic thermal burn</td>
<td>HM</td>
<td>AMD, AMT</td>
<td>PKP + ECCE</td>
<td>9</td>
<td>20/200</td>
<td>35</td>
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<tr>
<td>2</td>
<td>18</td>
<td>Male</td>
<td>Acute alkaline burn</td>
<td>CF/60 cm</td>
<td>AMD</td>
<td>CLAU</td>
<td>22</td>
<td>20/40</td>
<td>34</td>
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<tr>
<td>3</td>
<td>25</td>
<td>Male</td>
<td>Acute alkaline burn</td>
<td>CF/20 cm</td>
<td>AMD, AMT, tenonoplast</td>
<td>CLAU, ECCE + PCIOl</td>
<td>14</td>
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<td>4</td>
<td>56</td>
<td>Male</td>
<td>Chronic alkaline burn</td>
<td>HM</td>
<td></td>
<td>CLAU, PKP</td>
<td>12</td>
<td>20/400</td>
<td>27</td>
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</table>

HM, hand movement; CF, counting fingers; AMD, amniotic membrane dressing; AMT, amniotic membrane transplantation; ECCE, extracapsular cataract extraction; PC-IOL, posterior chamber intraocular lens; BCVA, best corrected visual acuity.

* Interval between COMET and subsequent surgeries.

Cultivation and Procurement of OMECs, COMET, and Subsequent Surgeries

The protocol of cultivating OMECs was similar to that described by Nakamura et al., including the use of de-epithelialized AM as a carrier, coculture with NIH/3T3 fibroblasts, and use of SHEM with 5% fetal calf serum as the culture medium. However, unlike the previous report, a larger area of oral mucosal tissue (6 x 6 mm or larger) was harvested, and the culture was not air lifted. After the fibrovascular pannus and Tenon’s tissue were removed and the subconjunctival space was soaked in topical mitomycin C, the graft was sutured with interrupted 10-0 Vicryl suture, and a therapeutic contact lens was placed to prevent contact with the graft. One day after surgery, the graft was soaked in topical mitomycin C, the graft was sutured with an interrupted 10-0 Vicryl suture, and a therapeutic contact lens was placed to prevent contact with the graft. On the 5th post-operative day, sections were washed thrice in Tris-buffered saline with Tween 20 minutes. Nonspecific reactions were minimized by incubating the sections at room temperature with sterilized PBS containing 2.5% bovine serum albumin for 30 minutes. The slides were then incubated at 4°C overnight with the appropriate primary antibodies (Table 2), which included the epithelial differentiation markers K3, -4, -8, -12, and -13; the goblet cell mucin MUC5AC; and the gap junction protein connexin (Cx)43. An antibody to laminin-5 was used to detect basement membrane component, and the epithelial stem/progenitor cell markers p63, ABCG2, and p75 were used to localize putative stem/progenitor cells in the tissue. Sections incubated with irrelevant mouse or goat immunoglobulin G were used as negative controls. After the sections were washed three times in Tris-buffered saline containing 0.5% Tween 20 for 5 minutes, they were incubated at room temperature for 1 hour with the appropriate secondary antibodies, including cyanine-3 (Cy3)-conjugated donkey anti-mouse IgG (for p63 and ABCG2), fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat IgG (for K12 and p75), or FITC-conjugated donkey anti-mouse IgG (for Cx43; K3, -4, -8, and -13; Ln-5; and MUC5AC). Finally, sections were washed thrice in Tris-buffered saline with Tween 20 for 5 minutes and covered with anti-fade mounting medium containing propidium iodide (PI) or DAPI Laser confocal microscopy (TCS SP2-MP system; Leica, Heidelberg, Germany) was performed with filters for FITC (excitation 488 nm, emission 500–535 nm), DAPI (excitation 359 nm, emission 450–460 nm), and PI (excitation 514 nm, emission 595–633 nm). The image was averaged from 14 scans within a thickness of 5 to 7 μm.

Immunohistochemistry and Confocal Laser Scanning Microscopy

Freshly removed human corneas were embedded in OCT compound and then flash frozen in liquid nitrogen. Immunofluorescent staining was modified from our previous report. Briefly, frozen sections were rinsed with PBS and then fixed with 100% methanol at 4°C for 10 minutes. Nonspecific reactions were minimized by incubating the sections at room temperature with sterilized PBS containing 5% bovine serum albumin for 30 minutes. The slides were then incubated at 4°C overnight with the appropriate primary antibodies (Table 2), which included the epithelial differentiation markers K3, -4, -8, -12, and -13; the goblet cell mucin MUC5AC; and the gap junction protein connexin (Cx)43. An antibody to laminin-5 was used to detect basement membrane component, and the epithelial stem/progenitor cell markers p63, ABCG2, and p75 were used to localize putative stem/progenitor cells in the tissue. Sections incubated with irrelevant mouse or goat immunoglobulin G were used as negative controls. After the sections were washed three times in Tris-buffered saline containing 0.5% Tween 20 for 5 minutes, they were incubated at room temperature for 1 hour with the appropriate secondary antibodies, including cyanine-3 (Cy3)-conjugated donkey anti-mouse IgG (for p63 and ABCG2), fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat IgG (for K12 and p75), or FITC-conjugated donkey anti-mouse IgG (for Cx43; K3, -4, -8, and -13; Ln-5; and MUC5AC). Finally, sections were washed thrice in Tris-buffered saline with Tween 20 for 5 minutes and covered with anti-fade mounting medium containing propidium iodide (PI) or DAPI Laser confocal microscopy (TCS SP2-MP system; Leica, Heidelberg, Germany) was performed with filters for FITC (excitation 488 nm, emission 500–535 nm), DAPI (excitation 359 nm, emission 450–460 nm), and PI (excitation 514 nm, emission 595–633 nm). The image was averaged from 14 scans within a thickness of 5 to 7 μm.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Category</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-3</td>
<td>Mouse monoclonal</td>
<td>×100</td>
<td>Chemicon, Temecula, CA</td>
</tr>
<tr>
<td>K-12</td>
<td>Goat polyclonal</td>
<td>×50</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>K-4</td>
<td>Mouse monoclonal</td>
<td>×100</td>
<td>AbCam, Cambridge, UK</td>
</tr>
<tr>
<td>K-13</td>
<td>Mouse monoclonal</td>
<td>×100</td>
<td>AbCam</td>
</tr>
<tr>
<td>K-8</td>
<td>Mouse monoclonal</td>
<td>×50</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Cx43</td>
<td>Mouse monoclonal</td>
<td>×50</td>
<td>Chemicon</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>Mouse monoclonal</td>
<td>×100</td>
<td>Novocastra, Newcastle-upon-Tyne, UK</td>
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<td>Laminin 5</td>
<td>Mouse monoclonal</td>
<td>×50</td>
<td>Santa Cruz Biotechnology</td>
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<tr>
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<td>Mouse monoclonal</td>
<td>×50</td>
<td>Chemicon</td>
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<tr>
<td>ABCG2</td>
<td>Mouse monoclonal</td>
<td>×50</td>
<td>Calbiochem, San Diego, CA</td>
</tr>
<tr>
<td>p75</td>
<td>Goat polyclonal</td>
<td>×100</td>
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months after surgery (F). Perilimbal scleral necrosis and total corneal epithelial defect persisted in patient 3 despite conjunctivovonoplasry, AMT, and AMD, 3 months after alkaline burn (G). Fourteen months after COMET, the cornea became clearer, but superficial blood vessels were still present in the central cornea (H). BLR was performed to reconstruct the inferior limbus, with the photograph taken 9 months later (I). Patient 4, with a chronic alkaline burn injury, presented with persistent epithelial defect and dense stromal infiltration over the inferior cornea despite multiple AMTs, CLAU, and PKP (J). To promote re-epithelialization, the patient underwent COMET and release of symblepharon. Ten months after surgery, the cornea was covered by a smooth epithelium without epithelial defect; however, prominent corneal NV was still observed (K). The patient then underwent repeated CLAU followed by PKP. Twenty months after COMET, the cornea was stable and clear (L).

**RESULTS**

**Surgical Outcome**

Although after surgery the corneal surface became stabilized and residual stromal opacity and mild-to-profound corneal NV were evident. Therefore, CLAU (patients 2, 3, and 4) or PKP (patients 1 and 4) was performed to further improve vision (Fig. 1). Clinical information, including the interval between COMET and the subsequent surgeries (PKP and CLAU), follow-up duration, and best corrected visual acuity, are shown in Table 1.

**Histology**

Normal oral mucosa is made up of multiple layers of stratified squamous epithelium that forms papillary structures in deep layers, and the underlying connective tissue is composed of lamina propria and submucosa (Fig. 2A). Keratinocytes in the basal layer tended to be smaller and more compact (Fig. 2A, inset). After an average of 12 days in submerged culture, the cells grew into a confluent sheet that was mostly two to five layers in thickness. Unlike previous reports,12,13,15 the nuclei were elongated (Fig. 2D). The BrdU label–retaining cells were entirely located in the basal layer (Fig. 2E). The corneal button obtained after PKP or CLAU showed 5 to 10 stratified epithelial layers oriented similarly to normal corneal epithelial cells. However, there was no epithelial papillary structure observed. In some areas in patients 1, 2, and 4, epithelial stratification increased to 8 to 12 layers (Figs. 2B, 2F, 2G). The basal keratinocytes in patients 1, 2, and 3 were smaller and more compact, similar to those found in the papilla of normal oral mucosa. In contrast, superficial keratinocytes of patient 2 were only loosely organized, resembling parakeratinization (F).
mucosa. In contrast, superficial keratinocytes of patient 2 were only loosely organized in a parakeratinized pattern (Fig. 2F). The AM substrate was barely visible beneath the epithelium with some inflammatory cells. In addition, superficial peripheral NV was occasionally noted just under the AM in the anterior stroma (not shown).

**Immuonoconfocal Microscopy**

**Expression of Epithelial Differentiation Markers.** Type 3 keratin was expressed in full-thickness corneal epithelium and suprabasal limbal epithelium (not shown). It was also expressed by the suprabasal oral mucosal epithelium (Fig. 3A). Of interest, full-thickness K3 staining was observed in cultivated keratinocytes (Fig. 3D) as well as in all corneal specimens (Figs. 3B, 3C, 3E, 3F). Keratin 12, a more specific marker for differentiated corneal epithelium was seen in the full thickness of normal corneal and suprabasal limbal epithelium (Figs. 4A, 4D), but was not seen in patients 1, 3 and 4 (Figs. 4B, 4E, 4F). Of interest, the left peripheral portion of patient 2 showed full-thickness K12 staining (Fig. 4C), whereas K13 staining in the adjacent OMECs was positive in the suprabasal area (Fig. 4A, inset). This finding implies a mixture of oral mucosal and corneal epithelium in the cornea of patient 2. K4 is the keratin paired with K13 and can be expressed by the suprabasal epithelia of normal oral mucosa (Supplementary Fig. S1A; all supplementary figures are online at http://www.iovs.org/cgi/content/full/50/10/4660/DC1), normal cornea (Supplementary Fig. S1B), and corneal specimens from patients 1 and 2 (Supplementary Figs. S1E, S1F), but it was expressed only in the superficial layers of normal conjunctiva (Supplementary Fig. S1C) and cultivated OMECs (Supplementary...
Fig. S1D). K13 is a mucosa-specific keratin, and its expression was constantly suprabasal all in normal oral mucosa (Supplementary Fig. S2A), cultivated OMECs (Supplementary Fig. S2D), and all corneal specimens (Supplementary Figs. S2B, S2C, S2E, S2F). Supplementary Figures S2B and S2E clearly show that the basal small-cell zone was K3 negative. K8 staining was positive both in the normal corneal (Fig. 5A) and conjunctival epithelium (Fig. 5B), and also in the removed pannus of patients 3 (not shown) and 4 (Fig. 5C) during COMET. Of note, its expression was consistently negative in the normal oral mucosa (Fig. 5D), cultivated OMECs (not shown), and all corneal specimens (Figs. 5E, 5F).

Connexin 43 is a gap junction protein that is preferentially expressed by differentiated keratinocytes. Cx43 was observed in the suprabasal layer of the oral mucosa (Fig. 6A) and was only sparsely expressed in the cultivated OMECs (Fig. 6D). This observation implies that the submerged keratinocytes were less differentiated. In the cornea, Cx43 was negative in the basal small-cell zone in patients 1, 2, and 3 (Figs. 6B, 6E, 6C), but full-thickness Cx43 staining was seen only in patient 4 (Fig. 6F). Overall, although the epithelium varied in thickness, the expression pattern of individual keratins was homogenous, except for K12 in patient 2 (Fig. 4C).

MUC5AC, a goblet cell–specific mucin, was expressed in the removed pannus of patient 4 during COMET (Supplementary Fig. S3D), but all the other specimens, including the oral mucosa (S3A), cultivated OMECs (not shown) and the four corneal specimens (Supplementary Figs. S3B, S3C, S3E, S3F) were universally negative. Because histologically we cannot find an obvious AM-like structure, laminin-5 staining was performed and confirmed that all the transplanted epithelia were

**FIGURE 5.** Immunoconfocal microscopy for K8 (green) expressed by normal cornea (A), conjunctiva (B), and the pannus removed from patient 4 while undergoing COMET (C). K8 staining was consistently negative in normal oral mucosa (D) and also in the corneal specimens from patients 1 and 2 (E, F). Cell nuclei were counterstained with PI (red).

**FIGURE 6.** Immunoconfocal microscopy for Cx43 (green) expressed by normal oral mucosa (A), cultivated OMECs (D), and corneal tissues from patients 1 (B), 2 (E), 3 (C), and 4 (F). Cell nuclei were counterstained with PI (red). Cx43 staining was suprabasal in the oral mucosa (A) but was only sparsely expressed in the cultivated OMECs (D), suggesting that the submerged keratinocytes were less differentiated. In the cornea, Cx43 staining was negative in the basal small-cell zone in patients 1 (B), 2 (E), and 3 (C), but full-thickness Cx43 was noted in patient 4 (F).
laid over an intact basement membrane (Supplementary Figs. S4B, S4C, S4E, S4F).

Expression of Keratinocyte Progenitor Cell Markers

Using A4A monoclonal antibody which reacts to all isoforms of p63, the nuclear pan-p63 signals were found in the basal keratinocytes lining the papilla and ridge of oral mucosa (Supplementary Fig. S5A). The signals were also found in the basal layer of cultivated OMECs (Supplementary Fig. S5D). However, pan-p63 signal in the four corneal specimen may not be limited to the basal layer, since in areas with more stratification, two to five layers of p63-positive keratinocytes were noted in all the patients (Supplementary Figs. S5B, S5C, S5E, S5F). Although cytoplasmic ABCG2 staining was mainly limited to the basal layer in normal oral mucosa (Supplementary Fig. S6A), and was most prominent in the basal small-cell zone in the cornea, its expression too was not limited only to the basal layer (Supplementary Figs. S6B, S6C, S6E). In some areas of cell culture (Supplementary Fig. S6D), the staining could even be full-thickness. Finally, the newly proposed OMECs stem/progenitor cell marker p75 was expressed solely in the basal layer of ridge and papilla of oral mucosa (Fig. 7A). Cytoplasmic p75 was also uniquely positive in the basal layer of cultivated OMECs (Fig. 7D). Unlike p63 and ABCG2, the expression of p75 was strictly limited to the basal layer in the epithelium of patients 1, 2, and 3 (Figs. 7B, 7C, 7E). However, p75 was negative in the specimen from patient 4 (Fig. 7F). The immunostaining patterns of all markers are summarized in Table 3.

DISCUSSION

CCET and COMET have both been reported to be successful in reconstructing corneas with severe LSCD. In our study, we found that COMET was very useful in promoting re-epithelialization and decreasing inflammation in corneas with severe burns. Although after surgery the antiangiogenic activity of COMET is inferior, the procedure nevertheless stabilizes the cornea, and facilitates future autologous limbal transplantation if the other eye is intact. For successful cultivated keratinocyte transplantation, it is essential to include progenitor/stem cells in the cultures if long-term graft survival is

Table 3. Qualitative Immunohistochemical Localization of Keratinocyte Differentiation-Related Markers

<table>
<thead>
<tr>
<th>Markers</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Normal Conjunctiva</th>
<th>Oral Mucosa</th>
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<tbody>
<tr>
<td>K-3</td>
<td>+</td>
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+, Full-thickness positive; −, full-thickness negative; +/−, positive in the superficial layers; −/+, positive in the basal layer; NA, not available.

* Only in the left peripheral portion.

† More exactly in the basement membrane zone.
‡ More than two basal layers.
anticipated. However, previously there have been only few studies reporting the fate of ex vivo expanded keratinocyte progenitor cells after transplantation.24 Nakamura et al.19 published the histologic findings of cornneas after COMET. They found that transplanted OMECs survive in clinically successful grafts, which are K3 positive and MUC5AC negative; however, in clinically failed grafts, neighboring conjunctival epithelial cells invade the corneal surface and are K3 negative and MUC5AC positive, along with many blood vessels and inflammatory cell infiltration. Because the authors did not mention the expression of progenitor/stem cell markers in the transplanted OMECs, a panel of differentiation- and progenitor-associated markers were used in our study in hope of answering the important question of whether the progenitor cells of OMECs can survive after transplantation in an ectopic site such as the cornea.

The differentiation patterns of oral mucosa vary from fully keratinized epidermis-like epithelium in the hard palate to nonkeratinized epithelium covering the buccal mucosa.25 Surface cells in fully keratinized oral mucosa are arranged neatly, with closely approximated and slightly overlapping borders, whereas those of nonkeratinized oral mucosa are arranged unevenly and are widely separated by intercellular clefs.26 Cells with the highest potential for cell division have been supposed to reside in the deepest portions of the epithelium (i.e., at the base of the epithelial ridges27), as is the case in the oral mucosal epithelium.28 Hosoya et al.29 reported that after chasing, BrdU-positive basal keratinocytes were localized solely within the basic layer of the oral mucosal epithelium facing the tips of dermal papillae, indicating that epithelial stem cells of oral mucosa may be localized in the basic layer of the epithelium facing the tips of the dermal papillae. These basal cells were small sized and compact and were not only seen in the oral mucosa (Figs. 2A, inset); interestingly, this pattern was also seen in the cornneas after COMET (Figs. 2B, 2C, 2F). Previously, Grueterich et al.30 reported that labilum cultures on intact AM retained a limbal epithelial phenotype, whereas those on denuded AM differentiated into a corneal phenotype, regardless of the addition of 3T3 fibroblasts. Our results clearly showed that denuded AM sustained a portion of highly proliferative keratinocytes, which later differentiated into a cornnea-like epithelium in vitro. However, in this study we did not observe the AM-like acellular matrix beneath the epithelium in all four patients. Nevertheless, it will be interesting to know whether progenitor cells can also be preserved in the AM-free culture system, such as a temperature-responsive culture dish.42 In addition, whether the cultures’ remaining submerged during cultivation helped to preserve the progenitor cells remains to be elucidated.

Nonkeratinized buccal and soft palate mucosa express K4/13 in the superficial and intermediate layer, and K5/K14 and K19 in the basic layer,31 whereas K5/12 are markers for differentiated corneal epithelial cells.32 Although the supra-basal epithelial cells of oral mucosal tissue were positive for K3, consistent with previous reports,41,47,49,50 in this study K3 staining was seen in the whole layer of epithelium in all the specimens including the cells in culture. On the contrary, the staining patterns of K4 and K13 were similar among oral mucosa, cell culture, and cornneas after COMET, in that the basic layer was negative for staining. Because K3 was expressed by both OMECs and corneal epithelial cells, and K4/ K13 by both OMECs and conjunctival epithelial cells, we used K13-positive and K8- and MUC5AC-negative staining to identify OMECs in the cornea. Normally expressed by simple epithelium, keratin 8 is expressed by both normal corneal and conjunctival epithelium41–45; however, normal OMECs do not express K8 unless they are undergoing malignant transformation.50 All four cornneas expressed K13, suggesting that they were either OMECs or conjunctival epithelium; however, because none of them expressed either K8 or goblet cell–derived mucin MUC5AC, the epithelium was most likely OMEC in origin, overlaying an intact basement membrane, as evidenced by a homogenous LN-5 staining.

At the time of the second surgery, despite the presence of corneal NV, owing to the persistence of OMECs, the conjunctival epithelium was prevented from invading the cornea, as evidenced by the lack of subconjunctival fibrous tissue invasion. However, our data cannot rule out the possibility of conjunctival epithelium ingrowth into the peripheral cornea, because histologically we were examining mainly the central part of the cornea. Using impression cytology, Satake et al.38 reported that over time in some cases the transplanted OMECs were gradually replaced by (or mixed with) host conjunctival cells. Because impression cytology, which was not performed in this study, can reveal topographic distribution of surface epithelia and is repeatable, it is recommended for long-term follow-up of surface change after COMET. On the other hand, negative K12 staining was observed in three of the patients, but mixed K13 and peripheral K12 staining were seen in patient 2 (Fig. 4A, inset), suggesting the coexistence of transplanted OMECs and corneal epithelium in that patient.

β1-integrin and pan-p63 have been used to characterize the progenitor cells of OMECs.12,39–41 However, these markers are neither specific enough, nor are they tissue-specific. In this study, pan-p63, ABCG2, and p75 were used to better localize the putative keratinocyte progenitor cells in the graft. We found that signals of pan-p63 and ABCG2 were found in the deep epithelial layers of the graft. The staining for pan-p63 and ABCG2 in the small, compact basal epithelial cells in patient 1, 2, and 3 were prominent; however, ABCG2 signal was weak in patient 4’s cornea (Supplementary Fig. S6E). Although ABCG2 was proposed to be a marker for corneal epithelial progenitor cells,22 in this study neither the pattern of pan-p63 expression nor that of ABCG2 was compatible with previous BrdU labeling results, in that the label-retaining cells were located only in the basal epithelial layer of oral mucosa and cell culture. At this moment we do not know whether ΔNp63 is a suitable stem cell marker for OMECs,43 as we were unable to obtain reliable signals with commercially available antibodies.

Recently, Nakamura et al.20 proposed that p75, the lower affinity NGF receptor, is a potential marker of oral keratinocyte stem/progenitor cells. They found that p75 was exclusively expressed in the basal cell layer of both the tips of the papillae and the deep rete ridges. The p75-positive cells were smaller, did not actively cycle in vivo, and possessed higher in vitro proliferative capacity and clonal expansion potential. Our observation confirmed that the expression of p75 was strictly confined to the basal layer, no matter whether in the oral mucosa, in culture, or in the corneal specimens. Basal p75-positive OMECs was detected in patients 1, 2, and 3 at 9, 22, and 14 months after surgery (Figs. 7B, 7E, 7C). These data, although it is still too early to assert that stem cells of OMECs persisted in the cornea after transplantation, nevertheless imply that long-term engrainment of less-differentiated progenitor cells of keratinocytes, with high proliferative potential, is possible after COMET.

Because corneal epithelial progenitor cells also express p63,44 p75,20,45 and ABCG2,46 the source of the progenitor-like cells in the specimens needs clarification. It may be that the p63- or ABCG2-positive basal cells actually stem from residual LSCs not destroyed during the initial burn injury, and these stem cells probably hibernate in ectopic sites47 and are revived only after COMET. However, the expression of K13 but not K12 in the epithelium suggests that the epithelium was not corneal in origin, and the epithelium was also negative for K8 and goblet cell–specific mucin MUC5AC. Therefore, it is
more likely that the progenitor-like cells from OMECs were isolated from oral mucosal tissue, cultivated, and preserved on AM just like the limbal epithelial cells,\textsuperscript{48} then were relocated to the cornea. In patient 2 with mixed corneal keratinocytes and OMECs, it is less likely that the transplanted oral epithelium can transdifferentiate to corneal epithelium, more likely ectopic K12(+) cells in the conjunctiva had migrated into the cornea, as mentioned earlier in the article.\textsuperscript{47}

The degree of epithelial stratification of the cornea varied among our patients. In patient 2, the epithelium varied from 4 to 5 to \(>12\) layers. Such thickened, parakeratotic area resembles gingival oral mucosa. Although suspension culture of OMECs on AM never showed such a picture, previous studies have reported that buccal epithelial cells in explant culture\textsuperscript{49} or cultivation on the skin-derived substrates\textsuperscript{50} undergo a pattern of growth and differentiation that mimics parakeratinization in vivo. Despite the difference in stratification, the K3, -4, and -13-positive and K8-negative staining of the epithelium was homogenous in all the specimens, suggesting that epithelial stratification did not influence the pattern of keratin expression. However, the degree of epithelial differentiation of OMECs was variable among our patients. The basal epithelial cells of patients 1, 2, and 3 were p75-positive and Cx43-negative, which was most characteristic of progenitors of OMECs; however, the basal epithelium in patient 4 was p75-negative and Cx43-positive. Nevertheless, even in patient 4, the pan-p63 and ABCG2 staining were still positive, suggesting that the basal epithelial cells in patient 4 may still contain a remarkable proliferative potential (more likely transient amplifying cells), as the specimen was harvested 1 year after COMET.

In summary, in this study we have demonstrated the long-term existence of cultivated OMECs in the cornea after COMET. Small, compact cells in the basal epithelium are very likely to contain the keratinocyte stem/progenitor cells, which were best characterized by K18-negative but p75-positive staining. Our study thus justifies the rationale for the clinical use of cultivated keratinocyte transplantation. However, whether the AM substrate is the key in preserving the progenitor cells and what mechanism regulates keratinocyte differentiation after transplantation must be further investigated.

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**References**


