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ORIGINAL ARTICLE

Markers for Distinguishing Cultured Human Corneal Endothelial Cells from Corneal Stromal Myofibroblasts

Masahiro Yamaguchi[1,2], Nobuyuki Shima[1,3], Miwa Kimoto[1], Nobuyuki Ebihara[2,4], Akira Murakami[2] and Satoru Yamagami[1,5]

[1]Corneal Regeneration Research Team, Foundation for Biomedical Research and Innovation, Kobe, Japan, [2]Department of Ophthalmology, Juntendo University School of Medicine, Tokyo, Japan, [3]Department of Ophthalmology, University of Tokyo Graduate School of Medicine, Tokyo, Japan, [4]Department of Ophthalmology, Juntendo University Urayasu Hospital, Chiba, Japan and [5]Corneal Transplantation Section, University of Tokyo Graduate School of Medicine, Tokyo, Japan

ABSTRACT

Purpose: Eliminating contamination by corneal stromal cells is critical when preparing cultured human corneal endothelial cells (CECs) transplantation. We investigated markers for the purification of cultured human CECs and markers for excluding cultured human corneal stromal myofibroblasts (CSMFs) from cultured human CECs.

Materials and methods: CECs and CSMFs were obtained from human donor corneas by culturing separately in serum-containing medium. Candidate markers of CECs and CSMFs were screened with microarray analysis in the fourth passaged CECs and CSMFs. Then, selected factors were evaluated in reverse transcription polymerase chain reaction (RT-PCR), western blot, immunocytochemistry, and flow cytometry to investigate differential markers for each cell.

Results: Among the genes identified by microarray analysis, cultured human CECs, but not CSMFs, expressed integrin alpha 3 (ITGA3 and CD49c) protein according to immunocytochemistry and western blotting. Iroquois homeobox 2 (*IRX2*) gene was a marker that distinguished CSMFs from cultured human CECs by RT-PCR. The *IRX2* gene can be used as a marker of CSMFs contaminating cultured CECs.

Conclusion: These molecules could be important markers for the production of highly purified cultured CECs for regenerative medicine.

Keywords: Corneal endothelial cell, differential marker, keratocyte, myofibroblast, regenerative medicine

INTRODUCTION

The corneal endothelial cells (CECs) on Descemet's membrane play a critical role in maintaining corneal hydration and transparency. Full-thickness corneal transplantation and Descemet's stripping with endothelial keratoplasty (DSEK)[1–3] are effective treatments for reduced visual acuity associated with CEC loss, but there is a worldwide donor shortage. Regenerative medicine using cultured CECs[4–8] is an attractive method to compensate for this lack of donors and could become clinically available in the future. Although human CECs exist in a non-replicative state *in vivo*,[9–12] adults have some precursor cells that are largely committed to the CEC lineage[13,14] and display proliferative capacity *in vitro*,[15–18] with some efficient culture methods having been reported.[19,20] We have also established a highly efficient human

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Correspondence: Nobuyuki Shima, Department of Ophthalmology, University of Tokyo Graduate School of Medicine, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-8655, Japan. Tel: +81 3 5800 9085. Fax: +81 3 3817 0798. E-mail: noshima-tky@umin.ac.jp

CEC culture technique that minimizes the risk of bovine spongiform encephalopathy.[21,22]

Cell transplantation in regenerative medicine requires highly purified cells with no contamination of the other cells.[23] For cultivation of CECs, cells are initially stripped off the donor cornea together with Descemet's membrane. To obtain highly pure CECs, careful removal of the stromal tissue on Descemet's membrane is necessary, because this tissue contains corneal stromal cells. When corneal stromal cells (quiescent keratocytes) are cultured in the presence of bovine serum, they are activated by serum and other growth factors.[24–26] Their morphology then change from dendritic to a more spreading fibroblastic phenotype. Such serum-activated transformed keratocytes are called corneal stromal myofibroblasts (CSMFs), which totally express smooth muscle actin (α-SMA). Because CSMFs grow more rapidly than CECs in the culture condition with bovine serum, contamination of CSMFs has been a problem for CEC culture. Selective L-valine free culture medium reducing growth of CSFMs has been developed,[27] but the culture medium also suppresses growth of CECs. Thus, to reduce the risk of aberrant growth of CSFMs, cultured CECs for clinical use should be highly purified, therefore, a quality control method to distinguish CECs and CSFMs is necessary.

Cultured CECs show fibroblastic morphology during exponential growth stage but show polygonal form after confluent stage. Therefore, it is difficult to distinguish CECs and CSMFs from morphological findings only. Although some candidate genes like type VIII collagen[28] were reported to only be expressed by CECs, our preliminary experiments have shown that both CECs and CSMFs express such genetic markers (Supplemental Table 1). We also found that both cultured CECs and CSMFs express functional marker proteins for CECs such as zonula occludens 1 (barrier function) and sodium potassium ATPase (pump function) (Supplemental Table 1). Moreover, we compared expression levels of other known stromal CD markers (CDs: 14, 29, 31, 34, 44, 45, 73, 90, 105, 165) between CECs and CSMFs and found that these markers did not work as differential markers for CECs and CSMFs (Kimoto unpublished observation). Since CSMFs highly express α-SMA, we expected α-SMA could be a distinguishable gene, but we found that both tissue and cultured CECs expressed subtle levels of *α-SMA* gene (data not shown). During the preparation of this manuscript, new markers for CECs distinguishable from CSMFs such as glypican-4 and CD200 are reported.[29] These markers are useful for quality control for the purity of CECs. However, new definitive markers that can exclude CSMF contamination are still required for establishing a quality control method for impurity of CECs.

In this study, following microarray analysis of 40,000 of genes with cultured CECs and CSMFs to select genes that were highly expressed by each cell type (Supplemental Table 1), markers of CEC purity, which possibly express cell surface proteins on CECs, were identified by a combination of immunocytochemistry and western blotting. Moreover, definitive markers of contamination by CSMFs were determined by the reverse transcription polymerase chain reaction (RT-PCR) to prevent possible CSMFs contamination completely.

MATERIALS AND METHODS

This study was conducted in accordance with the Declaration of Helsinki. Donor corneas (ages: 48, 55, 62, and 65) were obtained from the Northwest Lions Foundation or the Rocky Mountain Lions Eye Bank. CECs and CSMFs were separated, cultured, and passaged for immunocytochemistry, western blotting, and RT-PCR. All examinations were repeated and were conducted on at least three different cell sources unless otherwise noted.

Cell Culture

Primary culture of CECs was performed as described elsewhere.[21] Briefly, cells on Descemet's membrane were stripped off the cornea, cut into small pieces, and then digested with 2 mg/mL collagenase A (Sigma-Aldrich, St. Louis, MO). The cells thus obtained were washed by centrifugation, incubated with 0.05% trypsin/EDTA (Gibco BRL, Grand Island, NY), washed again, and cultured on atelocollagen-coated dishes in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY) with 10% fetal bovine serum and antibiotics (Gibco BRL, Grand Island, NY) containing 2 ng/mL basic fibroblast growth factor (bFGF; Sigma, St. Louis, MO) in the presence of 0.3 mM L-ascorbic acid 2-phosphate (Wako, Osaka, Japan). When the proliferating cells had reached a sufficient density, passaging was done at ratios ranging from 1:1 to 1:4. To isolate corneal stroma cells, i.e. keratocytes, the CECs were peeled off in a sheet and the epithelium was scraped away with spatula. Then the corneal stroma was cut into small pieces (1–2 mm in diameter) that were incubated overnight at 37 °C in serum-free basal medium containing 0.02% collagenase (Sigma-Aldrich, St. Louis, MO). After the cells were washed three times with phosphate-buffered saline (PBS), a single-cell suspension was obtained by trituration with a pipette. Isolated keratocytes were cultured in DMEM with 15% FBS and passaged three times, after which the keratocytes were transformed into spindle-shaped CSMFs.

cDNA Microarray Analysis

The fourth passaged CSMFs and CECs were separately cultured on 10 cm dish for 2 weeks. All samples were tripsinized and collected to 15 mL tube followed by centrifuge (500 *g*, 5 min). Cell pellets were frozen by nitrogen and transferred to Takara Bio Inc. (Shiga, Japan) for outsourcing cDNA microarray analysis. Briefly, all samples were transferred into lysis buffer (Qiagen, Valencia, CA). Total RNA was then isolated from the samples using silica-membrane columns (RNeasy Mini Kit; Qiagen, Valencia, CA) according to the instructions of the manufacturer. RNA quality and quantity were checked (Bioanalyzer Nano Chips; Agilent Technologies, Santa Clara, CA). Two hundred nanograms of total RNA were used to prepare labeled cDNA (Agilent Expression Array Kit; Agilent Technologies, Santa Clara, CA). Reactions from all samples yielded sufficient cDNA for subsequent microarray analysis. cDNA samples from each three CEC and CSMF were hybridized (Gene expression hybridization kit, Agilent Technologies, Santa Clara, CA). Microarrays were scanned, the size and the morphology of each spot were examined, and low-quality spots were masked. Only scans with more than 99% appropriate spots were further analyzed. Spots were gridded and assigned, and the intensity values were calculated according to the standard protocol of the manufacturer. The resultant data were then normalized on per chip, and subsequently on per gene, basis. Normalized data were imported in a data analysis software package (GeneSpring GX10; Agilent Technologies, Santa Clara, CA).

Immunocytochemistry

CSMFs and CECs from the fourth passage were cultured on glass slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA) and fixed in 4% paraformaldehyde or methanol for 10 min. After rinsing with 0.1% Triton X-100/PBS, incubation was done for 30 min in PBS with 1% bovine serum albumin in 0.1% Triton X-100 to prevent non-specific staining. After rinsing twice with PBS for 5 min each time, incubation was done for 1 h at room temperature with the following antibodies: mouse anti-integrin α_3 (ITGA3, 1:100, Millipore, Bedford, MA), rabbit anti-regulator of G-protein signaling 5 (RGS5, 1:100, Imagenex Technology Corp., Port Coquitlam, Canada), rabbit anti-prostate-specific gene with homology to a G protein-coupled receptor (PSGR, 1:100, MBL, Nagoya, Japan), and mouse anti-potassium voltage-gated channel, Isk-related family, member 1 (KCNE1, 1:100, Abcam, Cambridge, MA) antibodies. Mouse IgG and rabbit polyclonal IgG were used as the controls. After three washes with PBS (−), incubation was performed at room temperature for 30 min with fluorescein-labeled goat anti-mouse and rabbit IgG (Alexa Fluor 488, 1:200; Molecular Probes, Eugene, OR). After another three washes, the sections were mounted with anti-fading mounting medium containing propidium iodide (PI) to simultaneously counterstain nuclear DNA. Images were obtained with a fluorescence microscope (Nikon, Tokyo, Japan) and were saved to a personal computer.

Western Blot Analysis

CSMFs and CECs from the fourth passage were collected in PBS and lysed in lysis buffer (CellLytic MT; Sigma, St. Louis, MO). Approximately 200 μg of protein/10 μL was subjected to 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Billerica, MA), and treated with PBS containing 5% skimmed milk for 30 min. The membranes were then reacted overnight at 4 °C with anti-ITGA3, RGS5, PSGR, and KCNE1 antibody (1.0 μg/mL) in PBS supplemented with 1% skimmed milk. After washing in PBS and treatment for 1 h with a peroxidase-conjugated secondary anti-goat immunoglobulin antibody diluted in PBS containing 1% skim milk, the reaction products were visualized by using an enhanced chemiluminescence kit (Amersham Bioscience, Little Chalfont, Buckinghamshire, UK).

RT-PCR

Total RNA was extracted from CSMFs or CECs with Trizol reagent (Life Technologies, Rockville, MD). The quantity of RNA was determined by measurement of the optical density at 260 nm. The cDNA was synthesized from 1 μg of total RNA using Oligo (dT) primers and SuperScript reverse transcriptase (Life Technologies, Rockville, MD). Then cDNA samples were subjected to PCR using specific primers for human iroquois homeobox gene (IRX2), human chemokine (C-X-C motif) receptor 7 (CXCR7), integrin α8 (ITGA8), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal standard for normalization. The amplification conditions were 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s. PCR was performed in a 50 μL reaction volume containing 1 μL of 10 mmol/L dNTPs, 5 μL of 10− buffer, 1 μL of Taq polymerase, and 2 μL of 10 mmol/L sense and antisense primers (Table 1). Normalized samples were amplified in the linear range that was established by using serial dilutions of cDNA and varying the number of cycles. Amplified products were separated by electrophoresis on 3% agarose gel and then were stained with ethidium bromide.

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TABLE 1 Primers used for RT-PCR.

Products	Sequence (5′–3′)	Base pair
IRX2	F: TGGTCCACAGGTAGGTGTCACAA R: GGCACAAGATGTGGTCTGGATTTA	135
ITGA8	F: ACCAAGCCTTCCCAAGTTCTG R: CAAATGCACCCACAATCAAATCT	130
CXCR7	F: AAACAGGGCTCACCAAGCTCA R: ATAGGGCCCATCACCCTGTTC	141
GAPDH	F: GCACCGTCAAGGCTGAGAAC R: TGGTGAAGACGCCAGTGGA	120

IRX2, iroquois homeobox 2; *ITGA8*, integrin alpha 8; *CXCR7*, chemokine (C-X-C motif) receptor 7.

RESULTS

Markers for the Purification of Cultured Human CECs

Protein expression was studied by immunocytochemistry in cultured CECs, with the target proteins being selected by microarray analysis and comparison with cultured CSMFs (Supplemental Table 1). Strong expression of ITGA3 was observed on the cell membranes of cultured CECs (Figure 1A), while no expression was detected on cultured CSMFs (Figure 1F) and cultured CECs stained with non-immunized isotype control antibody (data not shown). RGS5 was expressed in the nuclei of both CECs and CSMFs (Figure 1B and G). PSGR (Figure 1C and H) and KCNE1 (Figure 1D and I) were not detected in either cultured CECs or CSMFs, respectively.

Western blotting analysis showed that ITGA3 was expressed (estimated product size: 150 kD) in cultured CECs, but not cultured CSMFs (Figure 2A). RGS5 was detected in both cultured CECs and CSMFs (Figure 2B), consistent with the results of immunocytochemistry, indicating that RGS5 could not be used as a marker to distinguish between CECs and CSMFs. No positive band for PSGR was detected in cultured CECs or CSMFs (Figure 2C). KCNE1 was detected in both cultured CECs and CSMFs (Figure 2D), conflicting with the results of immunocytochemistry, suggesting that the KCNE1 antibody was not suitable for immunostaining. These gene transcription and protein expression studies revealed that ITGA3 was a marker for distinguishing cultured CECs from cultured CSMFs.

Markers for Excluding Cultured Human CSMFs from CECs

To ensure the high purity of cultured CECs, contamination by CSMFs derived from corneal stromal cells should be completely eliminated. Therefore, specific markers for CSMFs were identified to detect CSMFs contaminating cultured CEC. Based on the results of microarray analysis (Supplemental Table 1), the expression profiles of the IRX2, CXCR7, and ITGA8 genes were further investigated in CECs and CSMFs from the fourth passage by RT-PCR analysis. The results showed that IRX2 mRNA was only detected in CSMFs, while ITGA8 and CXCR7 mRNAs were detected in both CECs and CSMFs, although expression of ITGA8 by CECs was very low (Figure 3). Further experiments using different lots of cells confirmed that IRX2 was not detected in CECs under any cycling conditions, while ITGA8 was detected in some lots of CECs (data not shown). These results indicated that there was no detectable contamination of CSMFs in cultured CECs and subtle ITGA8 expression was derived from CECs but not from contaminated CSMFs. Based on these findings, we excluded ITGA8 as a potential contamination marker, and selected IRX2 as a possible marker for CSMFs contaminating cultured CECs.

DISCUSSION

We demonstrated that ITGA3 protein can be used as a purification marker for cultured human CECs, while *IRX2* gene expression can identify contamination of cultured human CECs by CSMFs on RT-PCR. Thus, these markers can provide a new quality control method for producing cultured CECs of high purity for regenerative medicine. Although *ITGA3* gene expression was detected in CSMFs by microarray analysis, methods of protein analysis such as immunohistochemistry and western blotting found no ITGA3 protein expression in these cells. This discrepancy may be explained by differences of expression time course, stability, and post-translational modification of each mRNA and protein, or protein expression levels in CSFMs are lower than the detection limit of ITGA3 antibody used in the present study. In either case, the present results demonstrated that anti-ITGA3 antibody is a useful commercially available tool for distinguishing CECs from CSMFs. ITGA3 is an essential molecule for spreading of cells on the

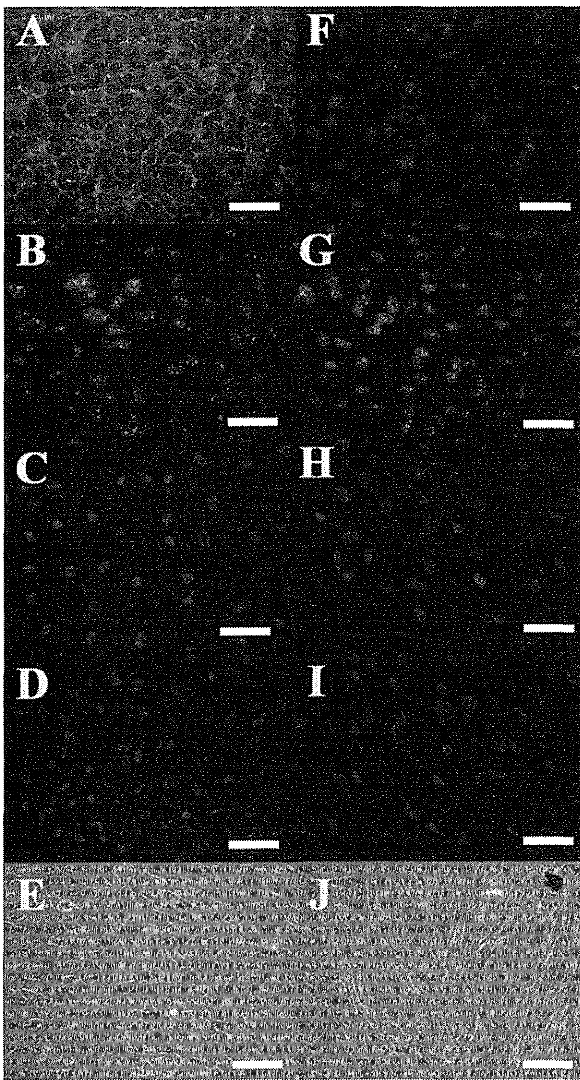

FIGURE 1 Immunocytochemistry of candidate markers for cultured human CECs. ITGA3 is expressed on the surface of CECs (A). No CSMFs express ITGA3 (F). RGS5 is expressed in the nuclei of both CECs (B) and CSMFs (G). No expression in CECs and CSMFs was found in PSGR (C, H) and KCNE1 (D, I), respectively. Immuno-staining of target protein is green and nuclear staining with propidium iodide (PI) is red. Morphological shape of CECs (E) and CSMFs (J) are shown in bright field image. Similar findings were obtained with repeated experiments and three different cell sources. Scale bars = 50 μm. CECs, corneal endothelial cells; CSMFs, corneal stromal myofibroblasts; ITGA3, integrin alpha3; RGS5, regulator of G-protein signaling 5; PSGR, prostate specific G-protein coupled receptor; olfactory receptor family 51 subfamily E member 2; KCNE1, potassium voltage-gated channel Isk-related family member 1.

basement membrane, assembly of the extracellular matrix, maintenance of hemidesmosome stability, maintenance of the cytoskeleton, epidermal proliferation, stem cell activation, and regulation of cell migration.[29–35] ITGA3 subunit binds with integrin beta1 to form integrin $\alpha_3\beta_1$ complex, which is predominantly expressed in keratinocyte stem cells[36] and

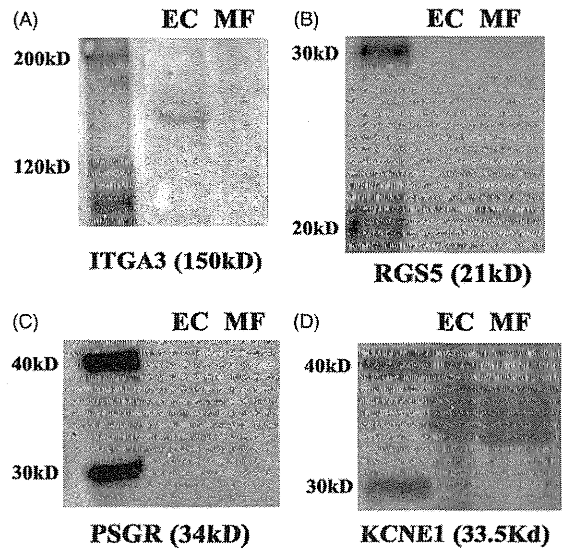

FIGURE 2 Expression of candidate markers for cultured human CECs by western blotting. Western blot analysis was performed using whole cell lysates from CECs and CSMFs. (A) A band for ITGA3 is detected in CECs, but not in CSMFs. (B) Bands for RGS5 are visible in the lanes for both CECs and CSMFs. (C) No band for PSGR is detected in both lanes. (D) Bands for KCNE1 are expressed in both lanes. The blots shown are representative of three independent experiments. CECs or EC, corneal endothelial cells; CSMFs or MF, corneal stromal myofibroblasts; ITGA3, Integrin alpha3; RGS5, regulator of G-protein signaling 5; PSGR, prostate specific G-protein coupled receptor; olfactory receptor family 51 subfamily E member 2; KCNE1, potassium voltage-gated channel Isk-related family member 1.

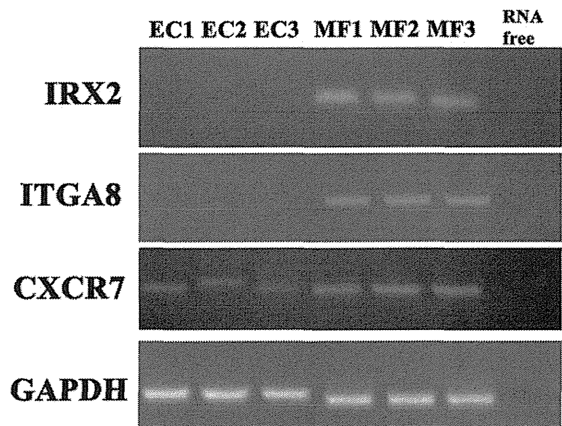

FIGURE 3 Selection of candidate markers for CSMFs contamination of cultured CECs by RT-PCR. CECs and CSMFs were prepared from three different cell sources. An RNA-free lane was the negative control. IRX2 gene is expressed all of the CSMFs, but not all the CECs. ITGA8 gene is expressed by all the CSMFs, but is weakly detected in only one of the samples of CECs. *CXCR7* gene expression is detected in all the samples from both CECs and CSMFs. Similar findings were obtained with repeated experiments. CECs or EC, corneal endothelial cells; CSMFs or MF, corneal stromal myofibroblasts; IRX2, iroquois homeobox 2; CXCR7, chemokine (C-X-C motif) receptor 7; ITGA8, Integrin alpha 8; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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functions as a receptor for laminin-332. Human CECs from donor corneas express integrin $\alpha_3\beta_1$[37] and our findings showed that cultured human CECs also express ITGA3, suggesting that ITGA3 plays a role in CEC functions and that such functions are preserved in cultured CECs.

Recently, new markers for CECs distinguishable from CSFMs such as glypican-4 and CD200 are reported.[29] They reported that monoclonal antibodies to glypican-4 and CD200 specifically stain CECs. These antibodies also can be used as separation markers for FACS sorting. In our microarray analysis, expression levels of Glypican-4 and CD200 were both similar between CECs and CSMFs (Yamaguchi, unpublished observation) implying that such difference of expression levels may occur with post-translational modification such as glycosylation. Together with our finding of ITGA3, these protein markers are useful for quality control of CEC purity.

The *Iroquois* genes (*Irx*) encode homeodomain-containing transcription factors that play a crucial role in the regionalization and patterning of tissues and organs during embryonic development. IRX2 is reported to be involved in neurogenesis in parts of the central nervous system, such as the retinal ganglion cell layer and the cerebellum.[38] Knockdown of IRX2 in zebrafish results in smaller eyes, lamination defect, and loss of retinogenesis waves.[39] Since CSMFs as well as CECs originate from the neural crest,[40] it is not surprising that IRX2 is expressed in CSMFs, but further studies are necessary for revealing why only CSMFs but not CECs express IRX2.

CONCLUSION

ITGA3 protein is a useful marker for distinguishing cultured human CECs from cultured CSMFs, while *IRX2* gene expression can detect contamination of cultured CECs by CSMFs. These molecules could be important markers for the production of highly purified cultured CECs for regenerative medicine.

DECLARATION OF INTEREST

The authors report that they have no conflicts of interest. Source of Funding: Knowledge cluster initiative grant from the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT). There is no commercial relationship in the form of financial support or personal financial interest.

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Supplementary material available online

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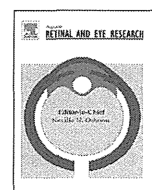

Ocular surface reconstruction using stem cell and tissue engineering

Takahiro Nakamura [a,*], Tsutomu Inatomi [b], Chie Sotozono [b], Noriko Koizumi [b], Shigeru Kinoshita [a]

[a] *Department of Frontier Medical Sciences and Technology for Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan*
[b] *Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan*

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ABSTRACT

Most human sensory information is gained through eyesight, and integrity of the ocular surface, including cornea and conjunctiva, is known to be indispensable for good vision. It is believed that severe damage to corneal epithelial stem cells results in devastating ocular surface disease, and many researchers and scientists have tried to reconstruct the ocular surface using medical and surgical approaches. Ocular surface reconstruction via regenerative therapy is a newly developed medical field that promises to be the next generation of therapeutic modalities, based on the use of tissue-specific stem cells to generate biological substitutes and improve tissue functions. The accomplishment of these objectives depends on three key factors: stem cells, which have highly proliferative capacities and longevities; the substrates determining the environmental niche; and growth factors that support them appropriately. This manuscript describes the diligent development of ocular surface reconstruction using tissue engineering techniques, both past and present, and discusses and validates their future use for regenerative therapy in this field.

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1. Introduction

The concept of an "ocular surface" is widely recognized in the field of ophthalmology, and our understanding of the role of ocular surface biology and immunology has been greatly improved by the numerous research studies carried out in this field (Thoft and Friend, 1979). Although the normal ocular surface comprises only 1/6 of the outer wall of the eye, it supports several of the eye's major functions, as it is covered with highly specialized corneal and conjunctival epithelia, formed by two phenotypically different types of epithelial cell. Over the past thirty years, several scientific discoveries such as the identification of corneal epithelial stem cells, the establishment of novel methods in epithelial culturing and the understanding of extracellular matrices and growth factors have enabled a novel surgical approach to treatment of ocular surface disorders, using regenerative medicine.

Based on tissue engineering, regenerative medicine is a newly developed area that uses somatic stem cells to generate biological substitutes and improve tissue functions (Langer and Vacanti, 1993). Success depends on three key factors: stem cells, extracellular matrices and growth factors. A variety of trials are currently in development, based on the utilization of stem cells and appropriate substrates to produce substitutes capable of reconstructing damaged and diseased tissues. In the field of ophthalmology, the production of tissue organs *in vitro* shows great promise, especially with regard to the anterior segment of the eye (Pellegrini et al., 1997).

Severe ocular surface disease (OSD) due to thermal and chemical burns, Stevens-Johnson syndrome (SJS), ocular cicatricial pemphigoid (OCP) or other conditions currently poses a serious clinical challenge for ophthalmologists worldwide. In these cases, the corneal epithelial stem cells located in the corneal limbus are destroyed, and coverage of the corneal surface by invading neighboring conjunctival epithelial cells results in neovascularization, chronic inflammation, ingrowth of fibrous tissue and stromal scarring. This severely compromises the ocular surface and seriously diminishes visual acuity (Chiou et al., 1998; Kinoshita et al., 2001; Tseng, 1989). Conventional treatment methods have generally proved unsatisfactory, and the long-term consequences of these ocular disorders are devastating. Clinically useful and effective surgical techniques for ocular surface reconstruction (OSR) are therefore needed for such patients.

* Corresponding author. Department of Frontier Medical Sciences and Technology for Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto 602-0841, Japan.
E-mail address: tnakamur@koto.kpu-m.ac.jp (T. Nakamura).

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In this present paper, we describe the history, recent advances, current developments and future challenges relating to OSR in both its basic science and clinical aspects, as well as providing novel clinical information for the treatment of severe OSD.

2. Ocular surface reconstruction

The concept of OSR is widely accepted in the field of ophthalmology, and our understanding of the role of the ocular surface has been greatly improved by numerous research studies. Various surgical procedures have been developed over the past 30 years to treat and reconstruct severely damaged or diseased ocular surface epithelia.

2.1. Conjunctival transplantation, keratoepithelioplasty and limbal transplantation

The concept of OSR was first reported in relation to an autologous conjunctival transplantation for unilateral chemical injury in Thoft's description of conjunctival tissue transplantation for unilaterally affected chemical injuries (Thoft, 1977). The surgery was performed by removing pathological scarred tissue from a patient's corneal surface and placing four pieces of conjunctival autograft taken from the contralateral eye at the limbus in order to reconstruct the cornea by regenerating conjunctival epithelial cells from these autografts. Subsequently, Thoft described the similar surgical technique of keratoepithelioplasty (Thoft, 1984), which employed a different tissue source (donor corneal lenticules) to regenerate corneal epithelial cells. Although the concept of corneal epithelial stem cells was not established at that time, the cell-level biological differences between regenerated corneal and conjunctival epithelia were known. Over time, keratoepithelioplasty has gradually gained acceptance despite initial disputes among researchers because it is a form of epithelial allograft (Kaufman, 1984). In fact, keratoepithelioplasty has proved to be dramatically effective in treating peripheral corneal ulcers, including Mooren's ulcer (Kinoshita et al., 1991), supplying both a regenerated corneal epithelium and an appropriate corneal substrate for inhibiting conjunctival invasion onto the cornea. Sun's group proposed the corneal limbal stem cell concept (Schermer et al., 1986), which had a tremendous impact on the development of keratoepithelioplasty, leading to autologous limbal transplantation (LT) (Kenyon and Tseng, 1989). Tsai and Tseng then introduced allogenic LT, aimed at achieving a permanent lifespan for regenerated corneal epithelium by means of stem cell transplantation, although intensive immunosuppressive therapy was also needed (Tsai and Tseng, 1994). These surgical procedures are classed as "cellular surgery"—a form of primitive regenerative medicine—as they are a form of *in vivo* expansion of corneal epithelial cells. Significantly, Kim and Tseng subsequently reported that amniotic membrane (AM) transplantation was capable of inhibiting pathological subepithelial scarring in OSR (Kim and Tseng, 1995). Since that time, AM transplantation combined with limbal allografts has been used to treat certain challenging occurrences of severe OSD (Tsubota et al., 1996).

2.2. Cultivated limbal epithelial transplantation (CLET)

While corneal epithelial transplantations (including keratoepithelioplasty and LT) have indisputably contributed to improved clinical outcomes for OSR in a range of clinical situations, an autologous LT needs quite a large section of limbal tissue from the healthy eye and cannot be applied if the disease is bilateral. In order to improve the surgical results of OSR for severe OSD, CLET needs to be developed *in vitro* from a small portion of limbal epithelium (Fig. 1).

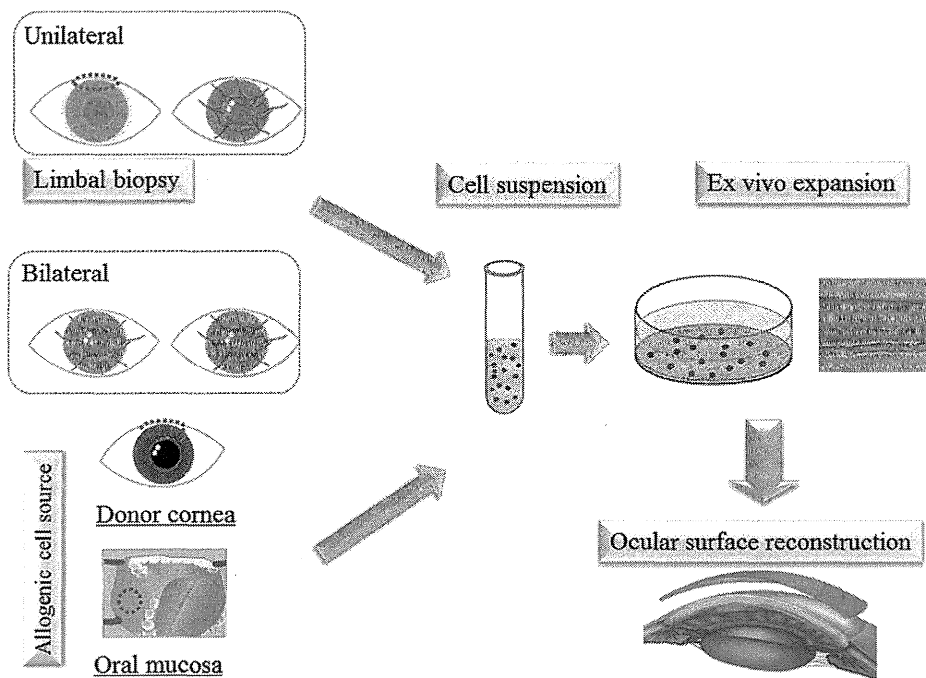

Fig. 1. Conceptual diagram of OSR via regenerative therapy. For patients with unilateral severe OSD, autologous corneal limbal epithelial cells from a biopsy of the uninjured eye (dashed circle) are used for cell culturing. For patients with bilateral severe OSD, allogenic cell sources (donor cornea or autologous oral mucosa) are used for cell culturing. The resultant cell suspensions were seeded onto the proper substrate under appropriate *in vitro* conditions. Finally, we successfully tissue-engineered the cultured sheet and transplanted it to the diseased corneal surface.

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2.2.1. The history of creating corneal epithelial sheets

In the initial stages of OSR during the 1980s, at least two investigative approaches were adopted in attempting to create an epithelial sheet. One was the direct sampling of corneal epithelial sheets, using dispase and gentle mechanical treatment. In rabbit experiments, the corneal epithelial sheet, taken directly from the *in vivo* cornea, attached itself to the corneal stroma but peeled away easily during lid movement, making it quite difficult to preserve the corneal surfaces (Gipson and Grill, 1982).

A second approach was to develop a cultivated epithelial cell sheet. In dermatological research, Green successfully established a procedure for cultivating epidermal cell sheets (Rheinwald and Green, 1975), focusing particular attention on the tissue-engineered cultivated corneal epithelial cell sheet. The cultivation of corneal epithelial cells on the scraped rabbit corneal stroma was examined by Friend et al. (1982), and several other substrates, such as collagen matrix and hydrogel coated with fibronectin, were also investigated in developing this procedure (Kobayashi and Ikada, 1991; Minami et al., 1993). However, until 1997, there were no clinical reports regarding the success of OSR using the cultivated corneal epithelial cell sheets, probably because of the poor understanding of surrounding corneal epithelial stem cell cultivation and/or its proper substrate. Some groups tried to reconstruct all three layers of corneal tissue—a "corneal equivalent"—using cell-lines arranged by natural and synthetic polymers (Griffith et al., 1999). This kind of corneal equivalent is now available for use in testing experimental drug toxicity and efficacy but not for clinical use (because of the use of immortalized cell lines).

2.2.2. Development of CLET

From the mid-1990s, attention has focused on the development of regenerative corneal epithelial cell therapy using tissue-engineered techniques as a new approach to OSR. The first successful OSR procedure using autologous CLET for patients with unilateral OSD was reported by Pellegrini et al. (Pellegrini et al., 1997). They developed a surgical method to reconstruct stratified corneal epithelial cell sheets on petrolatum gauze or a soft contact lens as carrier, treating two patients. It seems likely that their success can be accounted for by their adoption of a well-recognized epidermal keratinocyte-culturing method, including the use of mouse-derived 3T3 feeder layers to maintain epithelial stem cells. Since then, scientists worldwide have sought to devise novel and better methods for OSR. Again, in considering reconstruction of the ocular surface, the three key elements are the cell source and its proper substrate and growth factors. Following establishment of a suitable substrate, many researchers investigated the use of AM, fibrin and a temperature-responsive culture dish as carrier (Koizumi et al., 2000a; Nishida et al., 2004a; Rama et al., 2001; Tsai et al., 2000). From a clinical perspective, AM can serve not only as a proper epithelial carrier but also as a healthy substrate to cover a damaged ocular surface; the success of CLET using AM has been reported by a majority of groups worldwide (Baylis et al., 2011; Zhao and Ma, 2015). In addition, Sangwan has recently reported a novel surgical technique that combines AM transplantation and cultivation of small limbal explant with fibrin glue (Sangwan et al., 2012).

2.2.3. Clinical outcomes of autologous CLET

The ideal therapy for unilateral severe OSD—as caused, for instance, by thermal and chemical burns—is the use of autologous cultivated limbal epithelial sheets, with harvesting of much smaller pieces of limbal biopsy to prevent damage to the healthy or uninjured eye. With this in mind, several researchers have reported the successful use of autologous CLET for OSR (Grueterich et al., 2002; Nakamura et al., 2004b; Sangwan et al., 2003; Schwab et al., 2000; Tsai et al., 2000). These initial reports offer hope to patients with unilateral lesions or severe ocular surface damage. Long-term clinical assessment of autologous CLET was reported by Rama (Rama et al., 2010), confirming that autologous cultivated limbal stem cells represent an adequate long-term source for tissue-engineered transplants; clinical results reported success at up to 10 years in more than 75% of the patients treated. Importantly, this demonstrates the relationship between clinical results and the percentage of p63 (+) cells in cultivated graft. (Cultures in which p63-bright cells accounted for more than 3% of the total number of clonogenic cells were associated with successful transplantation in 78% of patients. In contrast, cultures in which such cells made up 3% or less of the total number of cells were associated with successful transplantation in only 11% of patients). In our long-term clinical cases (mean period 48 months; longest follow-up period 80 months), autologous CLET successfully reconstructed the corneal surface, but all cases showed varying degrees of mild superficial conjunctivalization extending from the limbal region (Fig. 2). These long-term clinical results strongly support the conclusion that tissue-engineered autologous CLET can be useful for reconstructing the ocular surface in cases of unilateral severe OSD. We further posit that the cultivation of limbal epithelial cells harvested from much smaller specimens is possible but difficult to achieve using conventional culturing techniques; these must be further developed to produce a sufficiently stratified epithelium that will hopefully include limbal stem cells.

2.2.4. Clinical outcomes of allogenic CLET

Although autologous CLET is the safest and most reliable procedure, bilaterally affected severe OSD cannot be treated by this means. In order to treat these bilateral cases, we have developed allogeneic CLET, using AM as a culture substrate (Kinoshita et al., 2004; Koizumi et al., 2001a). While acute-phase patients with persistent epithelial defects received allogeneic CLET for the purpose of covering the corneal surface and reducing ocular surface inflammation, chronic-phase patients received CLET to improve visual acuity. We have transplanted allogeneic cultivated corneal limbal epithelial cells in 39 eyes of 36 patients with severe OSD, including acute and chronic phases of SJS, OCP and thermal and chemical injuries (Fig. 3). During the postoperative 1–3 years, most of the transplanted sheets successfully survived on the ocular surfaces and maintained their transparency with the aid of immunosuppressive treatments.

It is worth noting that, in acute-phase patients, the severe preoperative ocular surface inflammation that had not been controlled by conventional treatments decreased rapidly post transplantation (Koizumi et al., 2001b); in chronic-phase patients, long-term visual outcome and epithelial stability varied. In a case of severe chemical injury, the transplanted corneal epithelial sheet was found to be transparent and stable as much as 10 years after transplantation, with only minimal conjunctival inflammation during the follow-up period. On the other hand, in SJS patients, mild to moderate ocular surface inflammation and subsequent rejection occurred after allogeneic CLET. While subconjunctival fibrosis had not progressed in SJS, conjunctival scarring (symblepharon and shortening of the conjunctival fornix) had progressed in OCP. Our clinical observations confirmed that the phenotypes of transplanted cells gradually changed from donor to host epithelial cells over a couple of years, but subepithelial scarring and neovascularization did not progress. This phenomenon can be explained as a mild rejection of the transplanted corneal epithelial cells. For that reason, we strongly believe that postoperative management, especially immunosuppressive therapy, is critical in ensuring survival of the transplanted graft. Although graft survival was brief in some chronic

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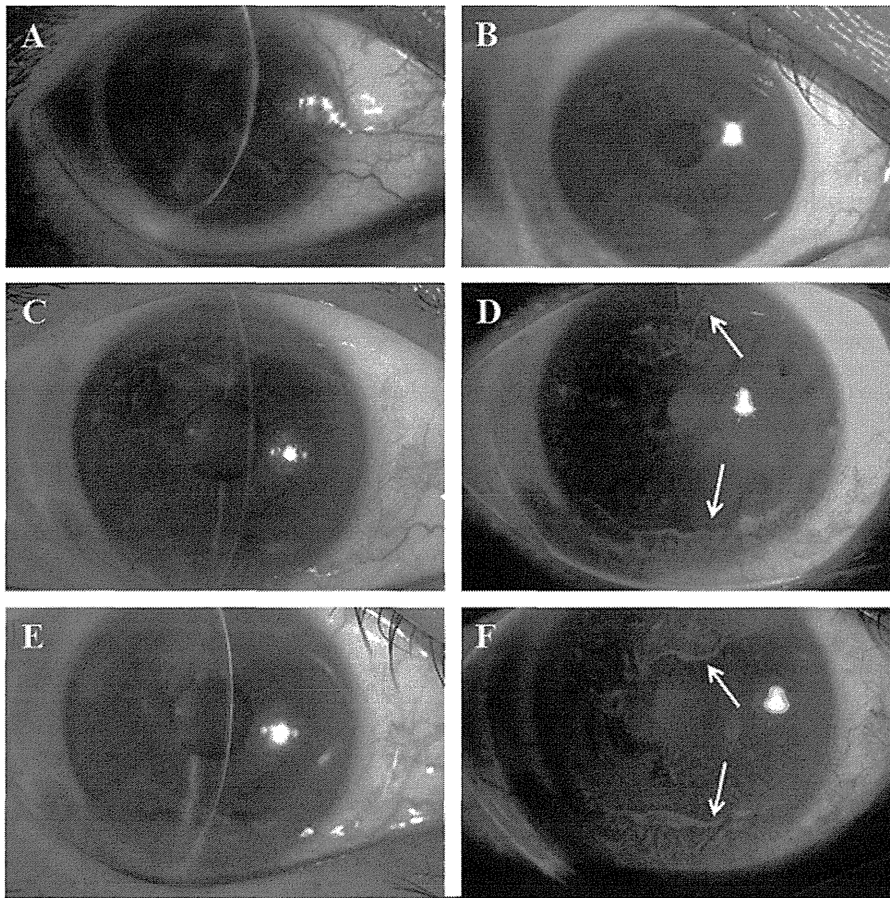

Fig. 2. Representative long-term clinical results of autologous CLET in patient with chemical injury. (A) Before transplantation, the eye manifested severe destruction of the ocular surface, with conjunctivalization and neovascularization. Postoperative appearance at 1 year (B), 5 years (C, D) and 7 years (E, F) shows a relatively smooth, epithelialized corneal surface with minimal corneal scarring and inflammation. During long-term follow-up, varying degrees of mild peripheral conjunctivalization were observed (D, F; white arrows).

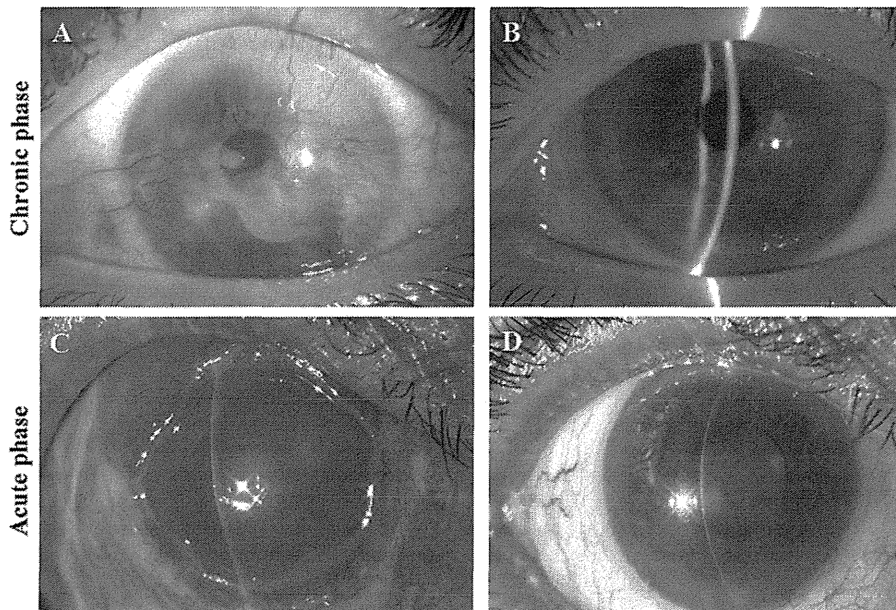

Fig. 3. Representative clinical results of allogenic CLET in patient with chronic (A, B) and acute (C, D) phase of chemical injury. Before transplantation, the eyes manifested conjunctivalization with corneal scarring (A) and persistent epithelial defects surrounded by inflammatory subconjunctival fibrosis (C). At 6 years (B) and 2 years (D) post-transplantation, the ocular surface was stable, without epithelial defects. Modified with permission from Kinoshita et al. (2004).

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cases, the ocular surface maintained its transparency and patients gained better visual function in comparison to their condition before surgery. Importantly, it was noted that the rejected cultivated transplants were easily removed from patients' ocular surfaces at the time of second surgery, and the exposed corneal stroma were found to be fairly transparent, with fewer scarring changes (Nakamura et al., 2003c).

2.2.5. *Phenotypic investigation of allogenic CLET*

Although our clinical assessments of allogenic CLET yielded favorable outcomes from the perspective of corneal stabilization, long-term phenotypic analyses of allogenic corneal epithelial transplants to the ocular surface would be required to confirm the direct evidence of graft survival. To this end, we compared our clinical observations with the results of long-term cell biological phenotype analysis of allogeneic CLET (Nakamura et al., 2010) (Fig. 4). In that report, we noted that, in clinical conjunctival phenotypic grafts, the transplanted cells were gradually replaced by surrounding conjunctival epithelial cells. In contrast, clinical corneal phenotypic grafts demonstrated that transplanted cells could indeed survive for a long period of time. Those findings have valuable clinical implications and provide novel information on allogeneic CLET, suggesting reasons for the phenotypic diversities of these grafts.

2.3. *Cultivated oral mucosal epithelial transplantation (COMET)*

As severe OSD is mostly bilateral, ophthalmologists worldwide have no choice but to select allogeneic LT or CLET, resulting in intensive and prolonged postoperative immunosuppressive therapy. These drawbacks have led us to examine whether the ocular surface could be reconstructed by using an autologous mucosal epithelium of non-ocular surface origin. In so doing, we (and other groups) have developed COMET as a substitute for corneal epithelial cells (Nakamura et al., 2003a, 2004a; Nakamura and Kinoshita, 2003; Nishida et al., 2004b).

2.3.1. *Development of COMET*

In the past, several groups have investigated the possibility of using oral mucosa for OSR. Ballen used oral mucosal grafts that included both epithelial and subepithelial tissues in both human and rabbit eyes, finding that they vascularized heavily with early fibrosis (Ballen, 1963). Gipson et al. transplanted oral mucosal epithelial cells freed by Dispase II treatment of underlying connective tissue onto the rabbit ocular surface (Gipson et al., 1986). They reported that it was feasible to transplant *in vivo* oral mucosal epithelium to corneal-limbal areas but that it was not maintained in central corneal regions. By general consensus, the character of epithelial cell is thought to depend on the underlying substrate, and AM is a good substrate for cultivating mucosal epithelium (Fig. 5). Based on these considerations, we first propose that oral mucosal epithelial cells cultivated on AM may be able to differentiate into cornea-like epithelial cells under cell culture conditions (Nakamura et al., 2003a; Nakamura and Kinoshita, 2003). After intensive investigations, we successfully generated a well-stratified and differentiated rabbit and human cultivated oral mucosal epithelial sheet that appeared very similar to normal *in vivo* corneal epithelium, successfully performing autologous transplantation of these cells onto rabbit corneas (Fig. 5). These results demonstrate that human cultivated oral mucosal epithelial sheets can function as an ocular surface epithelium and that COMET is a feasible method of OSR.

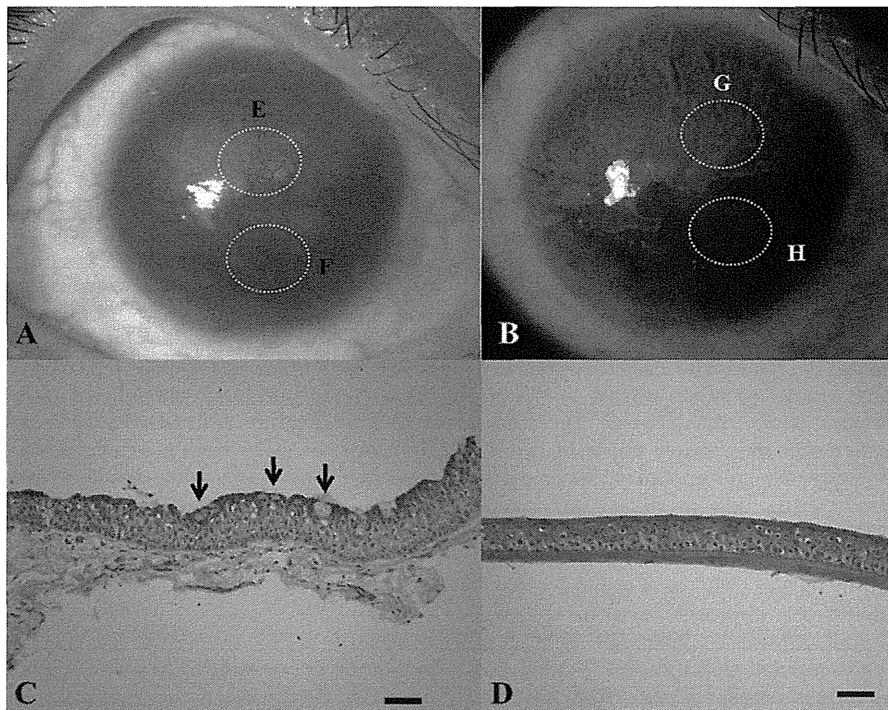

Fig. 4. Representative slit-lamp photographs after allogenic CLET in patient with SJS. Slit-lamp examination revealed that the upper part of the corneal surface was apparently covered with conjunctiva (A; white circle E). However, slit-lamp examination revealed that the lower-nasal part of the corneal surface was still transparent (A; white circle F). Fluorescein staining showed a comparatively clear demarcation between corneal and conjunctival phenotypes (B). The corneal phenotype area showed comparatively smooth epithelium with no fluorescein staining (B; white circle H), but the conjunctival phenotype area revealed light and stippled staining with fluorescein. (B; white circle G). The cross-section of white circle E disclosed 5 to 6 stratified layers of conjunctival-like epithelial cells and also included goblet-cell-like cells (black arrows) (C). Cross-section of white circle F showed 5 to 6 stratified cell layers and cornea-like epithelial cells (D). Scale bars: C, D = 50 μm. Modified with permission from Nakamura et al. (2010).

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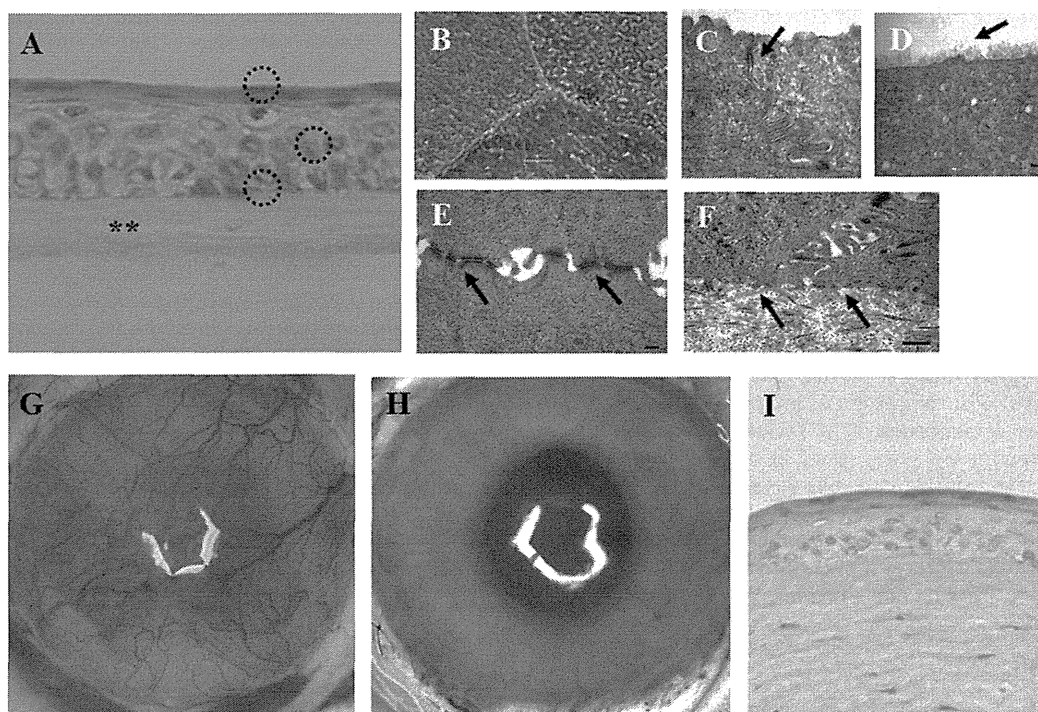

Fig. 5. Development of COMET. (A) The rabbit-cultivated oral mucosal epithelial sheet on AM had 4 to 5 layers of stratified, well-differentiated cells and appeared very similar to normal corneal epithelium. The apical surface of the cells was covered with numerous microvilli (B). In the superficial cell layer, what appeared to be tight junctions were evident between neighboring cells (C, arrow). The apical surface of the most superficial cells was covered with a glycocalyx-like material (D, arrow). The cultivated epithelial cells had numerous desmosomal junctions (E, arrows) and were attached to a basement membrane with hemi-desmosomes (F, Arrows). (G) Representative slit-lamp photographs of rabbit eye taken before transplantation and 10 days after transplantation (H). Before transplantation, the eye had total limbal stem cell destruction (G). Ten days after surgery, the corneal surface of the rabbit eye was covered with clear cultivated oral mucosal epithelium (H). The transplanted grafts adhered well to the host corneal stroma, with no evidence of subepithelial cell infiltration or stromal oedema (I). Modified with permission from Kinoshita et al. (2004), Nakamura et al. (2003).

2.3.2. Clinical outcomes of autologous COMET

In light of these experimental results, this method was initially applied to six eyes of four patients with severe OSD, and the ocular surfaces were successfully reconstructed (Nakamura et al., 2004a) (Fig. 6). During follow-up (13.8 ± 2.9 months), visual acuity showed improvement in all eyes, and the ocular surface remained stable. Although all eyes showed peripheral neovascularization with epithelial thickening, use of this novel tissue-engineering technique generated from autologous oral mucosa avoids the need to administer intensive and prolonged immunosuppressive therapy, so reducing the risk of postoperative complications.

We reported long-term clinical data on 19 eyes that received COMET (mean follow-up period 55 months; longest follow-up period 90 months) (Nakamura et al., 2011) (Fig. 6). The study included 19 eyes of 17 patients in the chronic phase of severe OSD; clinical results were evaluated and graded on a scale according to severity (Sotozono et al., 2007). Clinical safety was evaluated in cases of persistent epithelial defect (PED), ocular hypertension and infections. During follow-up, best-corrected visual acuity was improved in 18 eyes (95%), and visual acuity at the postoperative 36th month was improved in 10 eyes (53%). During the follow-up period, clinical conjunctivalization was significantly inhibited, and corneal opacification tended to improve. All eyes showed various degrees of superficial corneal vascularization, but this gradually reduced, and its activity was comparatively stable from 6 months after surgery. Symblepharon formation was also significantly inhibited; 7 of the 19 eyes showed PED at least once during the long-term follow-up. Ocular hypertension was observed in a total of 3 eyes. Corneal infection was observed mainly within 6 months after transplantation, and methicillin-resistant staphylococcus aureus was found to be the only cause of infection. Most recently, in order to clarify the effectiveness, disease-specific results and safety of COMET, all of the clinical data of all 72 patients treated with COMET since 2002 were analyzed (Sotozono et al., 2013). The findings of this retrospective study confirmed that long-term visual improvement can be obtained in end-stage severe OSDs, and that COMET offered substantial visual improvement even for patients with severe tear deficiency. The findings also showed that patients with corneal blindness resulting from severe OSDs such as SJS benefited from critical improvement of visual acuity. It was concluded that COMET is a safe and effective treatment for improvement of the visual prognosis of patients with severe OSD and that preexisting neovascularization and symblepharon were the prognostic factors. Because we have not precisely determined the clinical advantage and disadvantages of CLET and COMET, further clinical comparison of CLET and COMET is needed to develop a future clinical protocol for severe OSD.

In addition, Nishida et al. reported the success of OSR using carrier-free cultivated oral mucosal epithelial sheet in patients with severe OSD (Nishida et al., 2004b), but its long-term clinical outcomes are as yet unknown. Satake et al. reported that transplantation of cultivated oral mucosal epithelial sheets is a reliable procedure for reconstruction of a stable ocular surface (Satake et al., 2011). They also demonstrated that, during long-term follow-up, epithelialization of the corneal surface is very important both for obtaining a reliable long-term clinical results and in achieving a lower incidence of postoperative complications. In our own work, COMET was found to enable sustained reconstruction of the ocular surface epithelium in patients with severe OSD; management of

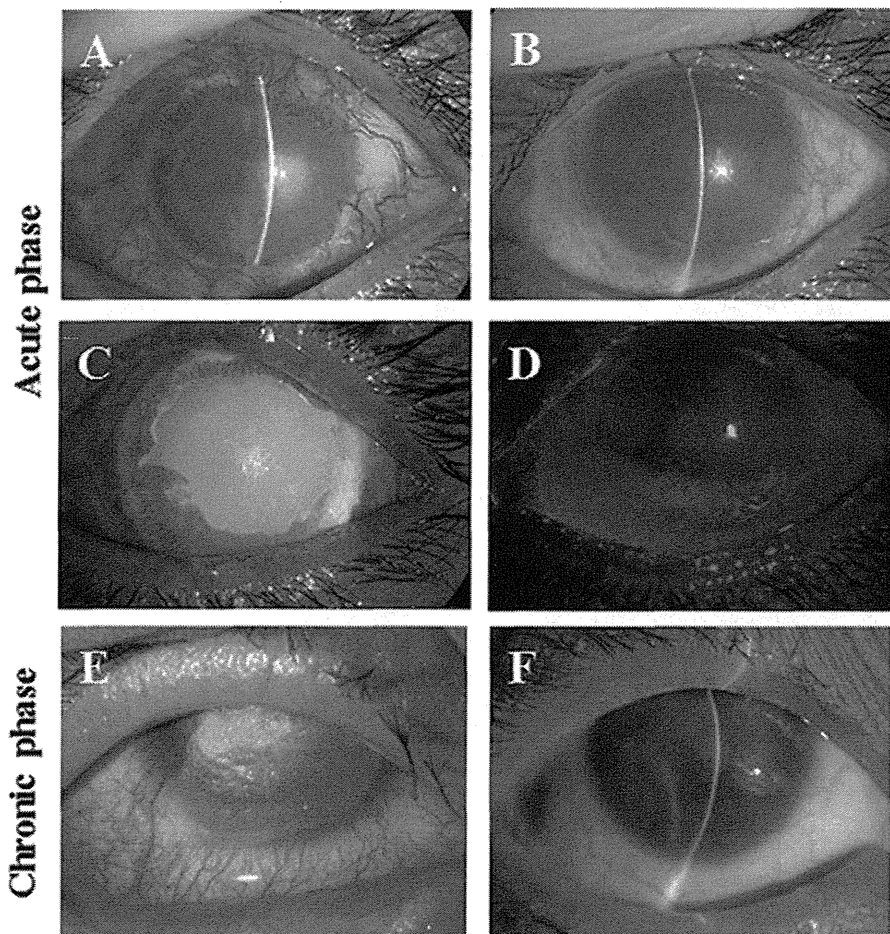

Fig. 6. Clinical outcomes of autologous COMET. Representative slit-lamp photographs taken before transplantation without (A) and with fluorescein (C) in patients with acute phase of chemical burn. Before transplantation, the eye manifested persistent epithelial defects surrounded by inflammatory subconjunctival fibrosis. The photographs were taken 17 months after transplantation without (B) and with fluorescein (D). The ocular surface was covered with transplanted cells and was stable without defects. (E, F) Long-term clinical progress of representative patient with severe OSD arising from SJS. Before transplantation, all eyes manifested severe destruction of the ocular surface with limbal stem cell deficiency (E). Postoperative appearance at 50 (F) months shows a relatively smooth, epithelialized corneal surface with minimal corneal neovascularization, scarring and inflammation. Modified with permission from Nakamura et al. (2004a), Nakamura et al. (2011).

postoperative PED and neovascularization may further increase the clinical efficacy of this type of surgery.

2.3.3. Phenotypic investigation of autologous COMET

Although our long-term clinical assessments of autologous COMET have yielded favorable results from the perspective of ocular surface stabilization, longevity and phenotypic analyses of cultivated oral mucosal epithelial sheets on the corneal surface are still required. As the effects of failed and successful grafts on the corneal surface remained unknown, we compared our clinical observations with the results of long-term cellular phenotype analysis of autologous COMET (Nakamura et al., 2007b) (Fig. 7). Our clinical, ultrastructural and cell biological examinations showed that the process of graft failure after COMET was responsible for the loss of transplanted cultivated oral mucosal epithelial sheets, and that this is followed by invasion of the corneal surface by the surrounding conjunctival epithelial cells. In the clinically successful transplanted eyes, transplanted cultivated oral epithelial cells survived and were found to have adapted well to the host corneal tissues (keratin 3 [+], Muc5ac [-]); there was no infiltration by inflammatory cells, nor was there any dissolution of the AM substrate. Based on our immunohistochemical results alone, we cannot determine whether cultivated oral epithelial cells can transdifferentiate into the corneal cell lineage. In addition, for successful COMET, it is essential to involve oral mucosal epithelial stem/progenitor cells in the cultivated sheet if long-term graft survival is to be expected. We previously reported that p75 was exclusively expressed in the basal cell layer of both the tips of the papillae and the deep rete ridges, and that these immunostaining patterns suggested a cluster organization (Nakamura et al., 2007a). Thus, we demonstrated that p75 may represent a novel marker for oral keratinocyte stem cell-containing populations. Unfortunately, we have yet to examine how many p75 (+) cells are involved in the transplanted graft, so further clinical study using the proper validation of the transplanted graft is needed to clarify this point. Studies are currently underway in our laboratory to shed further light on this observation. Our findings have valuable basic and clinical implications and provide useful insights into the mechanisms of both graft failure and graft survival after COMET.

2.3.4. Surgical variations

Severe OSD is sometimes accompanied by severe corneal stromal opacity and scarring. PKP without epithelial transplantation certainly results in PED because of the limited lifespan of the

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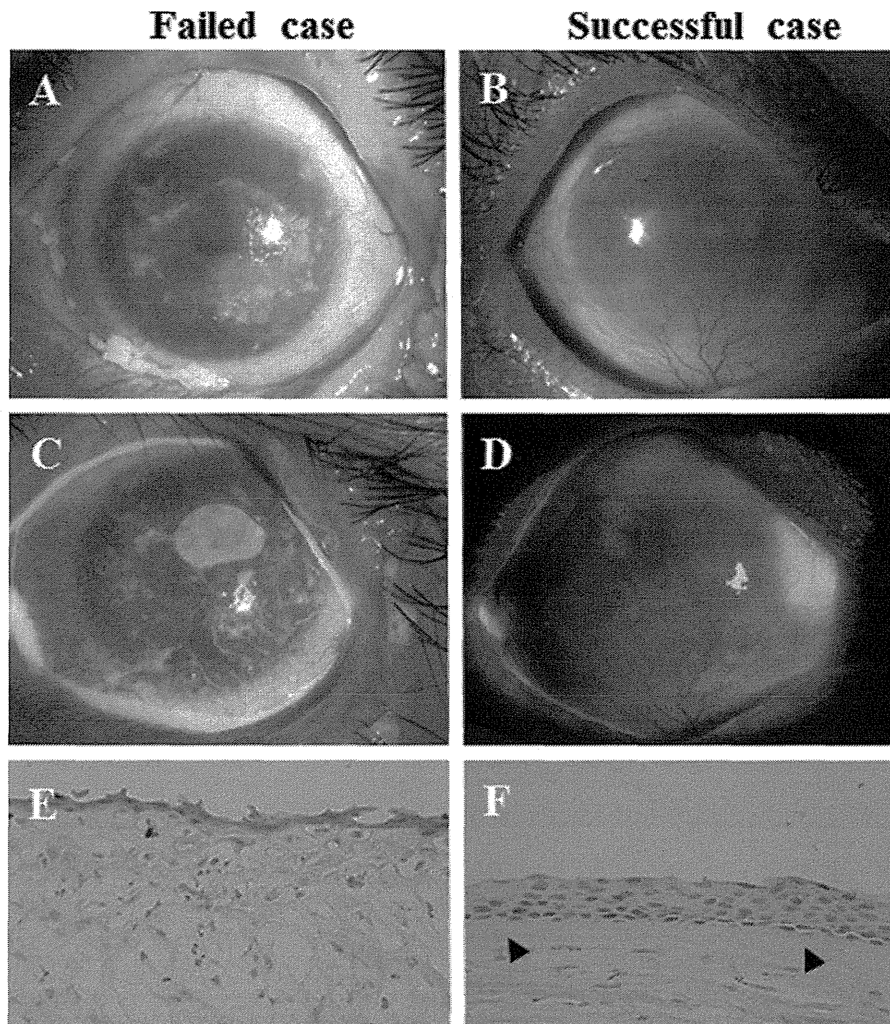

Fig. 7. Representative illustrations of samples from failed and successful COMET grafts. (A, C) 16 months after COMET, recurrent, small, persistent epithelial defects developed in a patient with SJS. (E) In cross-section, most areas showed 2 to 5 stratified layers and disorganized epithelium; microscopically, the AM substrate could not be observed. (B, D) Slit-lamp examination showed that the ocular surface was successfully reconstructed by COMET in a patient with chemical injury. Although in the successfully treated eye the ocular surface was stable and uniform and free of inflammation after initial transplantation, severe preoperative corneal stromal opacity that strongly affected these patients' visual acuity led us to perform PKP. (F) Microscopically, most areas contained 5 to 6 stratified layers of cells and cornea-like (cultivated oral mucosal epithelial sheet) epithelial cells on AM substrate (arrows) (original magnification: ×200). Modified with permission from Nakamura et al. (2007b).

corneal epithelium in patients with severe OSD. To improve the clinical outcome of these patients, their reconstructed cornea must be provided with a more stable epithelial supply such as cultivated epithelial stem/progenitor cell transplantation. Based on these findings, we proposed a two-step surgical strategy that apples a combination of COMET and PKP (Inatomi et al., 2006) (Fig. 8). In this procedure, PKP was performed around 6 months after the initial COMET; the ocular surface was found to be stable, and the donor cornea remained transparent after COMET. These clinical findings suggested that this surgical treatment may be useful for maintenance of the reconstructed ocular surface by providing cultivated oral mucosal epithelial stem/progenitor cells around the corneal graft.

Varying degrees of symblepharon formation (conjunctival shortening) and entropion frequently occur in patients with severe OSD. The resultant abnormal eyelid can often exacerbate severe OSD, as eyelid margin rotation or structural abnormalities disturb corneal wetting. Malfunction of the conjunctival fornix as a result of symblepharon formation can cause severe OSD such as dry eye, resulting from cicatricial entropion and restriction of ocular motility, making it necessary to reconstruct not only the ocular surface but also the eyelid formation in these patients. In previously reported clinical results of using the combination of COMET and eyelid surgery (Takeda et al., 2011), the ocular surfaces were successfully reconstructed with COMET and eyelid surgery in all 3 patients, with no serious complications during surgery (Fig. 8). The results of this clinical trial demonstrate that the combined procedure of COMET and eyelid surgery is a useful approach for treatment of severe OSD with associated eyelid abnormality.

In our clinical trials, COMET was used in two different transplantation scenarios: to reconstruct the corneal surface of a severe case of OSD, and to reconstruct the conjunctival fornix in patients with severe OSD-related symblepharon formation. In our clinical work, COMET was also used to reconstruct the conjunctival fornix and proved successful in treating severe OSD (Fig. 8). However, abnormal postoperative fibrovascular proliferation caused by primary severe OSD (SJS and OCP) must be considered in detail, as this remains critical to the long-term clinical prognosis.

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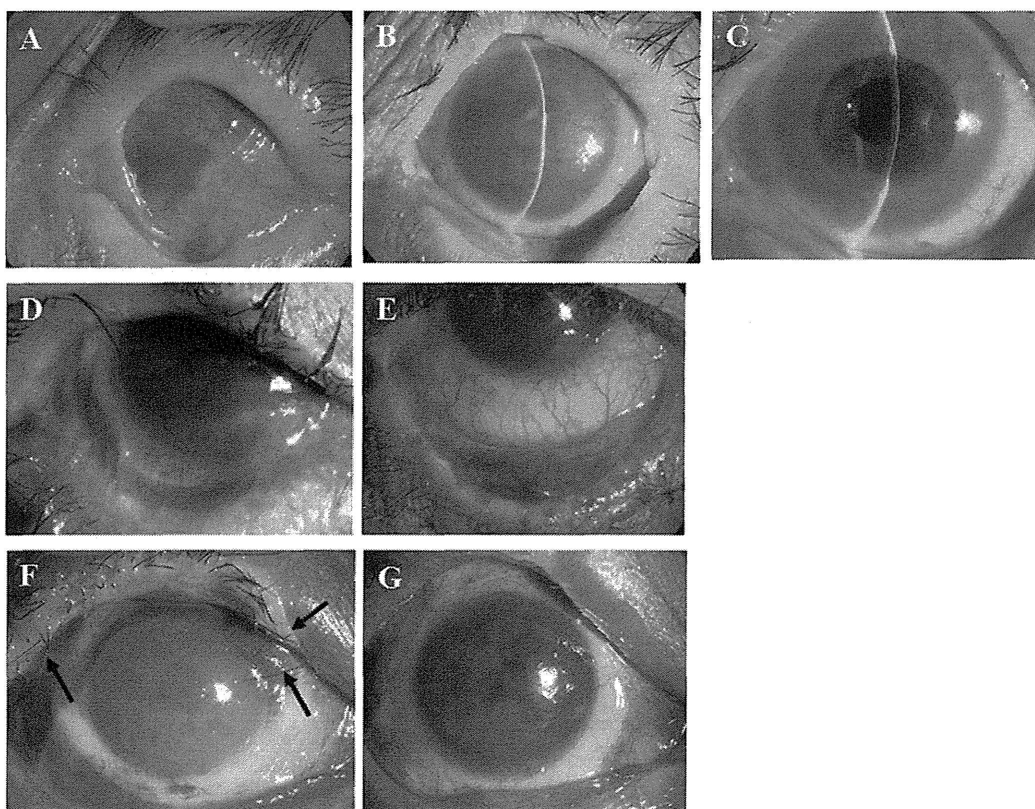

Fig. 8. (A–C) OSR with combination of COMET and PKP in patients with SJS. (A) Preoperative total conjunctivalization with severe symblepharon and partial parakeratinization. (B) Two months after initial COMET surgery. (C) Three months after PKP with cataract surgery. (D, E) Conjunctival fornix reconstruction with COMET in patients with idiopathic OSD. (D) Preoperative total conjunctivalization with severe symblepharon. (E) Six months after COMET, conjunctival fornix was successfully reconstructed. Modified with permission from Inatomi et al. (2006). (F, G) Representative illustrations of OSR using the combination of COMET and eyelid surgery in patients with thermal injury. (F) Preoperatively, there was persistent epithelial defect (PED), symblepharon and scarred entropion of the upper-eyelid. (G) Fifty months after surgery, the ocular surfaces were successfully reconstructed with COMET and eyelid surgery. Modified with permission from Takeda et al. (2011).

2.4. Recent pre-clinical trial

In view of these clinical trials, tissue-engineered CLET and COMET currently stand as established methods in the field of OSR. In an aim to further improve these surgical procedures, many groups worldwide have tried to develop treatments that use novel cell sources such as embryonic stem cells (Homma et al., 2004), mesenchymal stem cells (Ma et al., 2006), epidermal stem cells (Yang et al., 2008), immature dental-pulp stem cells (Monteiro et al., 2009), hair follicle bulge-derived stem cells (Meyer-Blazejewska et al., 2011) and umbilical cord stem cells (Reza et al., 2011). Most recently, we reported an attempt to overcome the problems of treating severe OSD with the most severe dry eye by transplanting a tissue-engineered cultivated nasal mucosal epithelial cell sheet to supply functional goblet cells and to stabilize and reconstruct the ocular surface (Kobayashi et al., 2015). This study represents a first step towards evaluating the use of tissue-engineered goblet cell transplantation of non-ocular surface origin for OSR. As a result of translational research and developments in the field of regenerative medicine for OSR, innovative advances have been made in the basic understanding and development of new therapeutic modalities such as the transplantation of tissue-engineered cultivated epithelial stem cell sheets. We strongly believe that an understanding of stem cell biology is crucial in developing the next generation of OSR, and we are hopeful that our efforts will enable treatment of all currently intractable forms of severe OSD.

3. Stem cells

It is generally believed that stem cells are critical for supplying and maintaining homeostasis within tissues or organs and for regenerating damaged tissues or organs. Based on previous studies, criteria for epithelial stem cells provide that they are relatively undifferentiated both biologically and morphologically, possess a high capacity for long-term self-renewal, are stimulated to proliferate in response to external stimuli and are usually located in well-protected, highly vascularized and innervated areas (Hall and Watt, 1989; Lajtha, 1979; Lavker and Sun, 1982; Leblond, 1981; Potten and Loeffler, 1990). In relation to OSR, many scientists worldwide have been examining the molecular mechanism of corneal stem cells in order to better understand the role of corneal homeostasis, as well as to elucidate the most useful approach to OSR. In clinical situations, the quality of a tissue-engineered transplant is known to be the key to success, and the selection of a large number of highly proliferating stem/progenitor cells enhances the reproducibility, quality and longevity of these transplants. It follows that basic understanding of corneal epithelial stem cells is of importance for the development and clinical evaluation of OSR.

3.1. Concept of corneal epithelial stem cell

3.1.1. XYZ hypothesis

As with other tissues and organs, the corneal epithelium must be maintained by corneal epithelial stem cells. The mechanism of corneal epithelial maintenance was first proposed as the XYZ

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hypothesis by Thoft et al. (Thoft and Friend, 1983). Describing as X the proliferation of corneal basal epithelial cells, the centripetal movement of peripheral cells as Y and the epithelial cell loss from the cell surface as Z, maintenance of the corneal epithelium can be defined by the equation: $X + Y = Z$. This clearly states that if the corneal epithelium is to be maintained, cell loss must be arranged by cell replacement. Using this hypothesis, it is possible to classify both diseases and treatments according to the specific component involved. Examining the X, Y and Z variables led to the limbal stem cell theory and to new insights into the pathogenesis and treatment of severe OSD.

3.1.2. Limbal stem cell theory

The presumed location of corneal epithelial stem cells in the limbus was first reported by Schermer et al., using a rabbit model (Schermer et al., 1986). Based on the cornea-specific keratin expression pattern, they proposed that corneal epithelial stem cells are located in the limbal regions, and that corneal basal cells correspond to "transient amplifying cells" in the diagram of "stem cells ~ transient amplifying cells ~ terminally differentiated cells". Subsequently, using 3H-thymidine labeling in mice, Cotsarelis et al. reported that label-retaining, slow-cycling cells are located only in the limbal region and that no such cells can be observed in the central corneal epithelium, suggesting that corneal epithelial stem cells are located in the limbus (Cotsarelis et al., 1989). The results of these cutting-edge investigations led to the advancement of limbal stem cell theory in understanding the pathogenesis of severe OSD and to clinical applications for OSR. However, we must exercise care in interpreting those results because the concept of "limbal stem cells" somehow differs from embryonic stem cells, induced pluripotent stem cells, hematopoietic stem cells and neural stem cells. In addition, although extensive experiments have been performed to test limbal stem cell theory, there is as yet no direct evidence that human corneal epithelial stem cells are located only in the limbal regions. While we completely agree that the limbus is an important area in the maintenance of corneal homeostasis, it is not impossible that a population of corneal stem cells may exist in the peripheral or central cornea, in much the same way as peripheral blood contains some stem cells. For that reason, continuous careful observation and direct examination will be needed to establish a proper understanding of limbal stem cell theory. The subject was well summarized and discussed in the excellent recent review (West et al., 2015).

3.1.3. Oligopotent stem cells

This well-established limbal stem cell theory presented challenges in discussing the location of the corneal epithelial stem cells. Using mouse models, Majo et al. demonstrated that central corneal epithelium could be serially transplanted for a long period of time, with capability for multipotential differentiation and unlimited cellular proliferation, implying that corneal epithelial stem cells were located not only in the limbal region but also in the central cornea (Majo et al., 2008). It is now known that the limbal region is not only the niche for corneal epithelial stem cells. Kawakita et al. reported that the central corneal epithelium can maintain itself when separated from the limbus in rabbits (Kawakita et al., 2011), and Chang et al. also showed that both human limbal and central corneal epithelial cells are capable of forming spheres in cultures that have stem cell properties (Chang et al., 2011). In examining this hypothesis, we found that the cornea still maintained its transparency six months after peripheral corneal and limbal epithelial ablation in rabbits, without inflammation and conjunctivalization (Fig. 9). Additionally, Bi et al. recently reported that human central corneal epithelial cells have *in vivo* self-healing ability under pathological conditions without support from limbal stem cells (Bi et al., 2013). While these findings require further detailed scrutiny, they may yet lead to reconsideration of the concept of corneal epithelial stem cells and of the cell source for OSR.

3.2. Candidate limbal stem cell markers

In terms both of basic knowledge and clinical practice, many scientists worldwide have been investigating the molecular marker for cornea epithelial stem cells in order to better understand the physiological maintenance of the cornea, and to identify the most useful tools for OSR. In the clinical setting, the quality and longevity of the cultivated epithelial transplant is known to be the key to success, and the manipulation of a large number of highly proliferating stem/progenitor cells using cell biological markers promotes the longevity of these transplants. Many putative corneal epithelial stem cell markers (e.g. p63, ABCG2, Integrin α9, Keratin 15, N-cadherin, NGF/TrkA, Integrin α6/CD71, Hes1, p75, Nectin 3, Importin 13, Nucleostemin, CD38/157, Lrig1, ABCB5, WNT7A) have been reported worldwide (Di Girolamo et al., 2008; Hayashi et al., 2008, 2007; Horenstein et al., 2009; Kawashima et al., 2009; Ksander et al., 2014; Kusanagi et al., 2009; Nakamura et al., 2014, 2008a; Ouyang et al., 2014; Pajoohesh-Ganji et al., 2006; Pellegrini et al., 2001; Qi et al., 2008; Wang et al., 2009; Watanabe et al., 2004; Yoshida et al., 2006) (Fig. 10). We posit that, in our work, most of these stem cell markers were expressed not only in the limbal region but also in the conjunctival basal cells. In that light, the appropriate corneal epithelial stem cell markers for use in OSR remain in need of further detailed investigation. We further consider that the current process for isolating cells must severely damage the cell's potential. In order to select the large number of stem cells needed for manipulation of the cultured sheet, innovative tissue-engineered technology is required. This important issue is seen as the first step towards the next generation of OSR using real corneal epithelial stem cells.

3.3. Single cell-based clonal analysis

It has been reported that one of the characteristics of epithelial stem cells is their greater proliferative capacity as compared to differentiated cells, using *in vitro* colony-forming efficiency assay from single cell. By means of this assay, Barrandon et al. previously reported the existence of three types of epidermal keratinocytes with different self-renewal capacities (Barrandon and Green, 1987). While holoclones (stem cells) have the highest reproductive capacity, in paraclones (transient amplifying cells), terminal differentiation is observed within a few generations (Fig. 11). Similar observations have subsequently been reported for the ocular surface epithelium (cornea and conjunctiva) (Pellegrini et al., 1999). They demonstrated that corneal epithelial stem cells are segregated in the limbal region while conjunctival epithelial stem cells are uniformly distributed in bulbar and fornical conjunctiva. We also reported that holoclone-type stem cells, previously identified in the skin and the ocular surface, were present in human oral mucosal epithelium, and that the low-affinity neurotrophin receptor p75 is a potential marker of oral keratinocyte stem/progenitor cells (Nakamura et al., 2007a).

3.3.1. Gene expression profiling of holoclone-type stem cells

In seeking an insight into the molecular mechanism of the human corneal epithelial stem cell, the gene expression profile of corneal epithelium was first reported by Nishida et al. (Kinoshita et al., 2001; Nishida et al., 1996). Subsequently, several groups conducted investigations using up-to-date experimental

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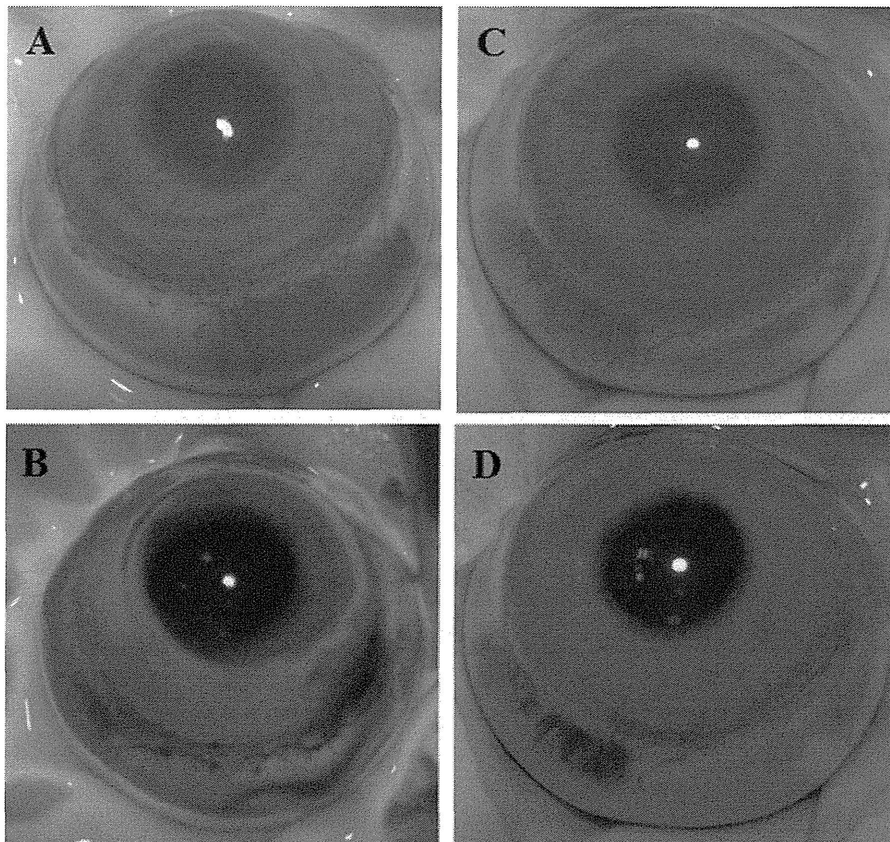

Fig. 9. Representative slit-lamp photographs of peripheral and limbal epithelial ablation in rabbits with or without fluorescein. (A, B) Immediately after ablation, fluorescein staining was clearly observed in all peripheral and limbal areas. (C, D) 6 months after ablation, cornea still maintained its transparency without inflammation or neovascularization.

procedures such as microarray and deep RNA sequencing (Bath et al., 2013; Figueira et al., 2007; Kulkarni et al., 2010; Nakatsu et al., 2013; Takacs et al., 2011; Utheim et al., 2009). However, the molecular mechanism and gene expression profile of holoclone-type corneal epithelial stem cells remain entirely unknown. In order to gain better understanding of the molecular mechanisms responsible for corneal homeostasis, we performed gene expression profiling of holoclone-type human corneal epithelial stem cells, using DNA chip analysis (Nakamura et al., 2014) (Table 1). Among the up-regulated genes in this assay, we focused on Lrig1 for further investigation because of its unique expression pattern in the ocular surface epithelium.

Lrig1 is a transmembrane glycoprotein, recently reported as a potential master regulator of epidermal and intestinal epithelial stem cells (Jensen and Watt, 2006; Powell et al., 2012; Suzuki et al., 2002; Wong et al., 2012). We demonstrated that Lrig1 was highly expressed in holoclone-type corneal epithelial stem cells and that it was essential for maintenance of the corneal epithelium during repair (Nakamura et al., 2014) (Fig. 12). Loss of Lrig1 impaired wound-induced corneal replacement and resulted in a cell-fate change from non-keratinized to keratinized epithelium. Interestingly, we discovered that Lrig1 controlled corneal homeostasis by negatively regulating the novel Stat3-dependent inflammatory pathway. Furthermore, corneal maintenance was arranged not only by corneal epithelial stem cells but also by bone marrow-derived inflammatory cells, whose functions and roles are well-regulated by the Lrig1-Stat3 inflammatory pathway (Fig. 13). These findings provide new insights into the underlying homeostatic regulation of Lrig1 (+) corneal epithelial stem cells and serve as a target for therapeutic exploitation, including OSR.

3.3.2. *miRNA profiling of holoclone-type stem cells*

Recently, particular attention has focused on microRNAs (miRNAs), a newly identified form of non-coding, regulatory RNA that modulate gene expression post-transcriptionally (He and Hannon, 2004). These play an important role in regulating a range of physiological and pathological processes, including cell proliferation, migration and differentiation and stem cell maintenance. Although some studies have addressed the function and role of miRNAs in the corneal epithelium (Lee et al., 2011; Lin et al., 2013; Shalom-Feuerstein et al., 2012; Yu et al., 2008), little is yet known about the molecular mechanism of miRNAs in corneal homeostasis. To better understand the subject, we performed miRNA profiling of holoclone-type corneal epithelial stem cells. Among the up-regulated miRNAs in this assay, we focused on several sets of miRNAs (1246, 3687, 4284 etc.) for further investigation. Extensive investigations are currently underway to clarify the molecular pathway of corneal epithelial stem cells by miRNAs. This basic understanding of corneal epithelial stem cells from different points of view holds promise for future regenerative therapy, including OSR.

4. Tissue engineering

When considering the development of OSR using tissue engineering, there are three important factors: 1) the cell source including stem cells, 2) a suitable biocompatible substrate and 3) suitable growth factors. In the past 20 years, this newly developed field of research has shown promising translational potential in the use of stem cells and biomaterials to generate biological substitutes and improve tissue functions. Successful generation of tissue-

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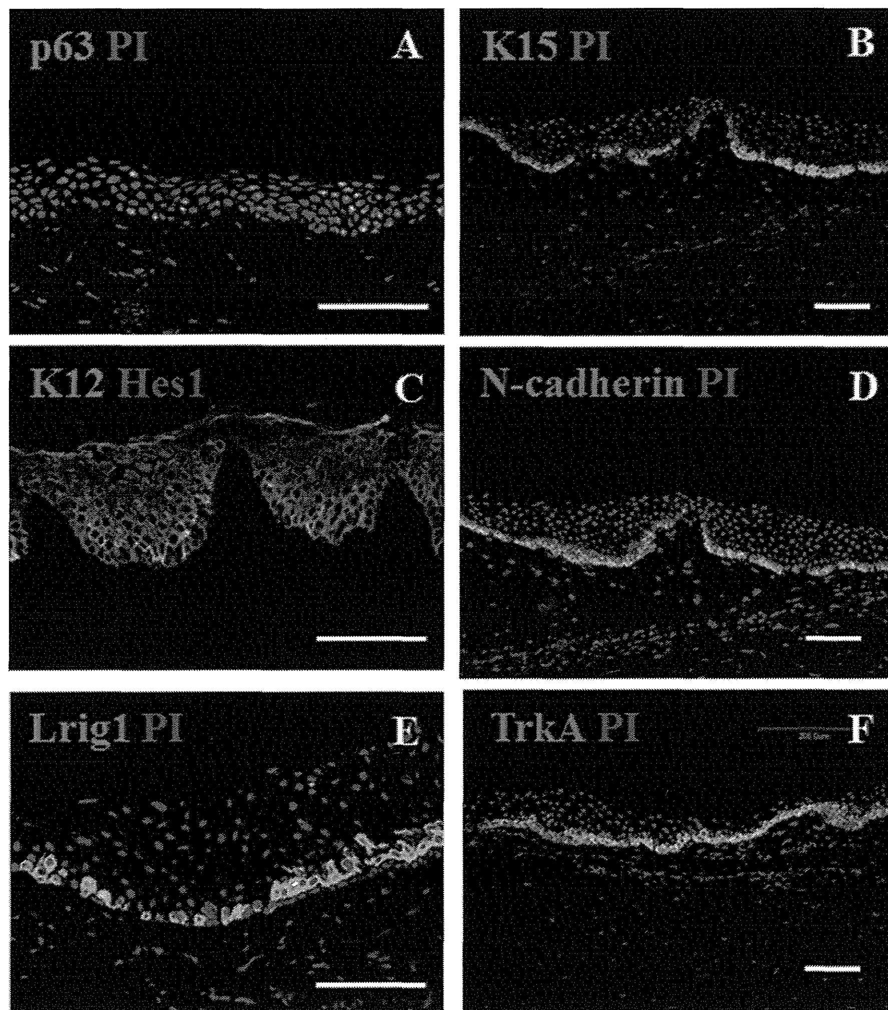

Fig. 10. Expression of proposed corneal epithelial stem cell markers. Immunohistochemistry for p63(A), Keratin 15(B), Hes1(C), N-cadherin(D), Lrig1(E) and TrkA(F) in human limbal region. Each marker (green) is clearly expressed in the basal cells of the limbus. Scale bars: 100 μm.

engineered sheet for OSR is highly dependent on the quality of underlying substrates. A useful substrate for OSR should have a highly biocompatible, non-immunogenic and non-inflammatory character, and should maintain corneal transparency and mechanical stability and promote cell adhesion and proliferation. Many cell substrates, such as biological scaffolds (e.g. AM), biosynthetic scaffolds (e.g. Fibrin, collagen) and synthetic scaffolds (e.g. temperature-responsive dish, contact lens), have been proposed as useful for OSR. Among these, AM is the most widely-used and represents the current gold standard substrate for OSR.

4.1. Amniotic membrane (AM)

4.1.1. Naive AM

Human AM is the innermost layer of the fetal sac and is composed of a monolayer of amniotic epithelial cells, a thick basement membrane and a subjacent avascular stroma. It has been used in surgical materials and biological dressing for the epidermal field (Trelford and Trelford-Sauder, 1979). In the context of OSR, previous studies have indicated AM's potential for conjunctival reconstruction in cases of severe OSD (De RÖTTH, 1940). A number of AM's characteristics make it a good fit for use in OSR; it contains various growth factors and cytokines inducing epithelialization and wound healing (Koizumi et al., 2000b), has anti-scarring and anti-inflammatory properties (Solomon et al., 2001; Tseng et al., 1999) and resembles the basement membrane of the corneal epithelium (Endo et al., 2004). From these basic and clinical observations, AM has come to be seen as an appropriate substrate for use in OSR. Clinical results of CLET and COMET using an AM substrate are encouraging, although there are still several variations and arguments in respect of its use. For example, there is no standard protocol to prepare the AM, and it also has donor-dependent variability and regional variations both physiologically and biologically. These concerns have prompted us to develop alternative substrates for OSR.

4.1.2. Dried AM

As mentioned above, AM has unique properties that can be useful in treating severe OSD. However, some biological and logistical issues remain. To date, a majority of ophthalmologists have used cryopreserved AM under conditions that are as sterile as possible; complete sterilization cannot be achieved using existing procedures. For clinical use, AM should ideally be sterile and free of contamination, and also be easily to obtain and preserve at room temperature; cryopreservation of AM needs an expensive and bulky deep freezer. To overcome these problems, we have

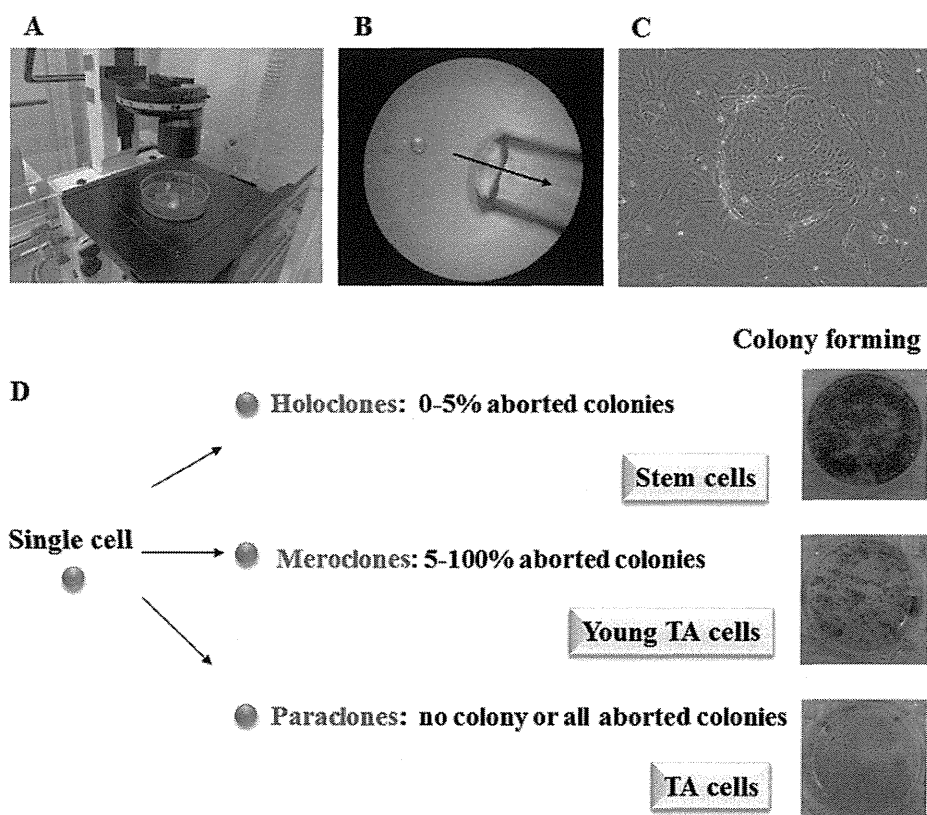

Fig. 11. Schema of single cell-based clonal analysis. (A, B) Using special equipment in the culture lab, a single cell can be manipulated under the microscope. (C) A colony can be formed from a single cell on the 3T3 feeder cells. (D) Holoclones (stem cells) have the highest reproductive capacity; in paraclones (transient amplifying (TA) cells), terminal differentiation is observed within a few generations.

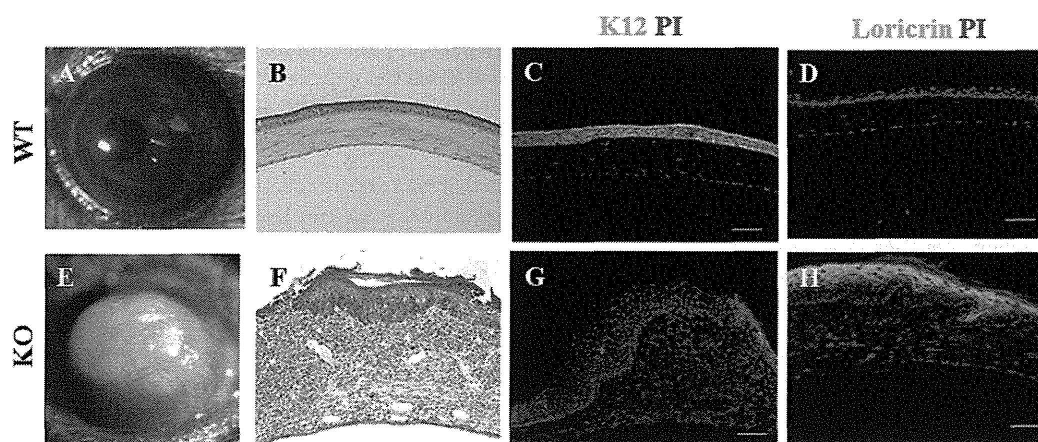

Fig. 12. Loss of Lrig1 results in a cell-fate change from corneal to keratinized epithelium. Slit-lamp photographs of Lrig1 wild-type (WT) (A) and knockout (KO) (E) mice corneas, and their histological appearances with hematoxylin and eosin staining (B, F). Lrig1 KO corneas formed corneal plaques with massive neovascularization and intense infiltration of inflammatory cells (E). Histological examination revealed extensive thickening and pathological keratinization of the corneal epithelium, with inflammation of the underlying corneal stroma (F). Immunostaining for keratin 12 and loricrin in Lrig1 WT (C, D) and KO (G, H) corneas. While the epithelium of the WT corneas specifically expressed keratin 12 (C), that of the Lrig1 KO corneas had gradually lost keratin 12 (D) and gained loricrin expression (H). Scale bars: 100 μm. Modified with permission from Nakamura et al. (2014).

successfully produced sterilized, freeze-dried AM using our own unique protocol (under vacuum conditions and vacuum-packed at room temperature with gamma-irradiation), and this biomaterial has been successfully used as a substrate in OSR (Nakamura et al., 2004c) (Fig. 14). On the basis of these results, we applied this biomaterial to OSR for patients with pterygium (Nakamura et al., 2006b). This prototype freeze-dried AM was improved by use of trehalose to protect its physical properties during the freezing process (Nakamura et al., 2008b). Most recently, Okabe et al. reported that hyper-dry AM, using far-infrared rays, depression of air and microwaves with γ-ray irradiation, is a useful biomaterial for tissue engineering (Okabe et al., 2014). In view of the particular