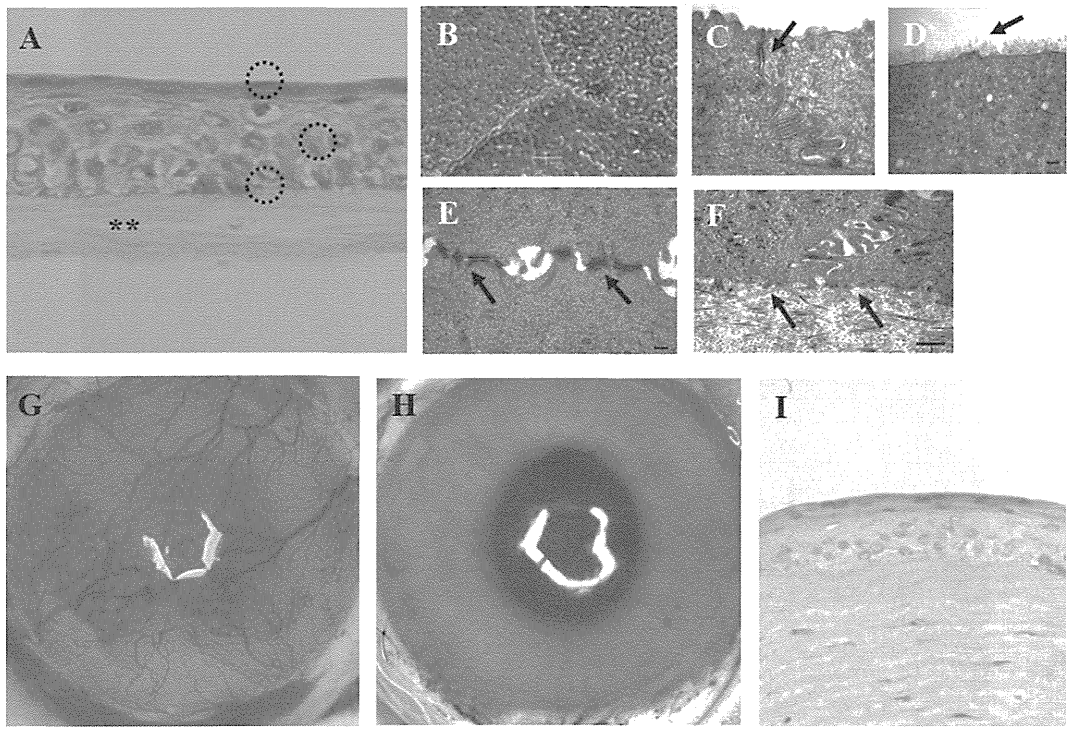

Fig. 5. Development of COMET. (A) The rabbit-cultivated oral mucosal epithelial sheet on AM had 4 to 5 layers of stratified, well-differentiated cells and appeared very similar to normal corneal epithelium. The apical surface of the cells was covered with numerous microvilli (B). In the superficial cell 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).

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 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

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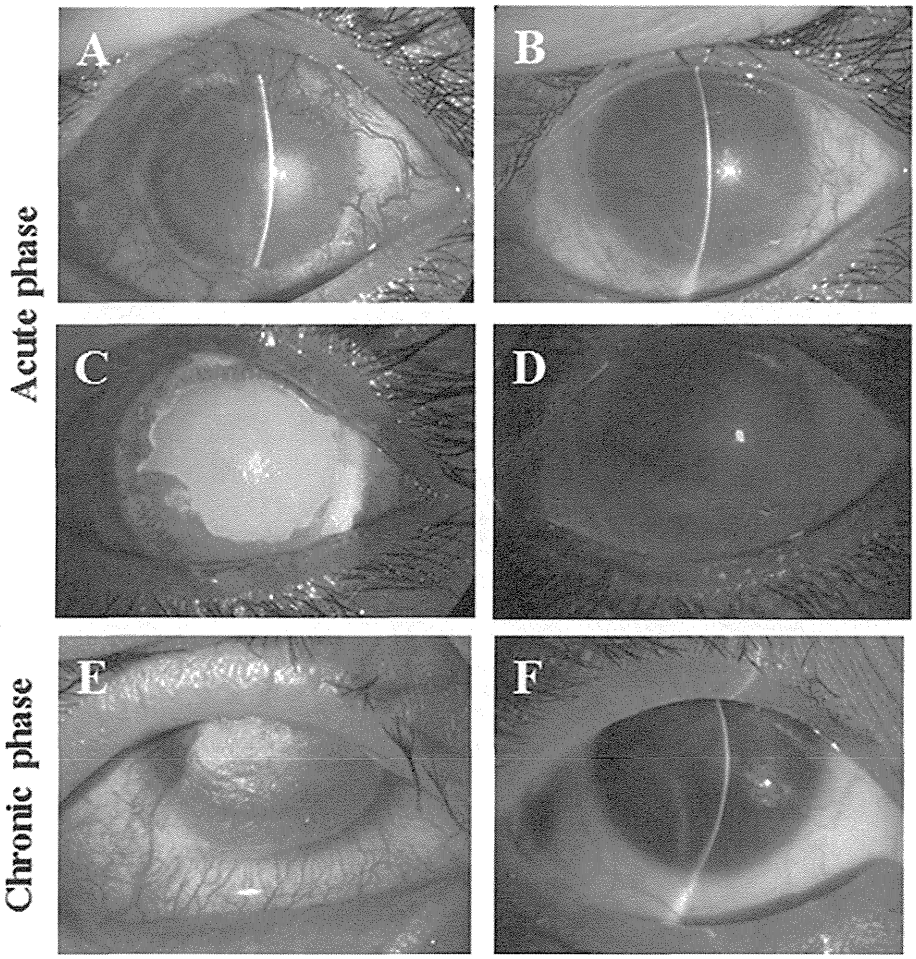

Fig. 6. Clinical outcomes of autologous COMET. Representative slit-lamp photographs taken before transplantation without (A) and with fluorescein (C) in patients with acute phase of chemical burn. Before transplantation, the eye manifested persistent epithelial defects surrounded by inflammatory subconjunctival 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).

stromal opacity and scarring. PKP without epithelial transplantation certainly results in PED because of the limited lifespan of the 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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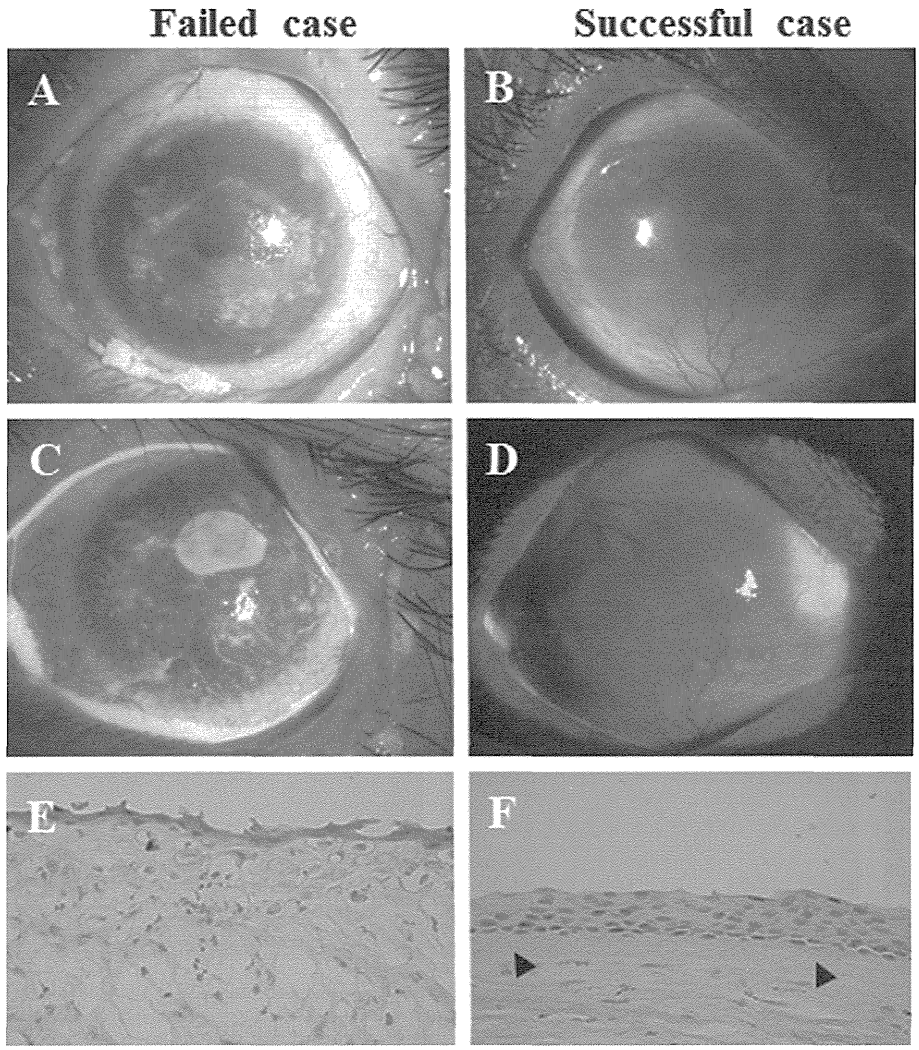

Fig. 7. Representative illustrations of samples from failed and successful COMET grafts. (A, C) 16 months after COMET, recurrent, small, persistent epithelial defects developed in a patient with SJS. (E) In cross-section, most areas showed 2 to 5 stratified layers and disorganized epithelium; microscopically, 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).

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

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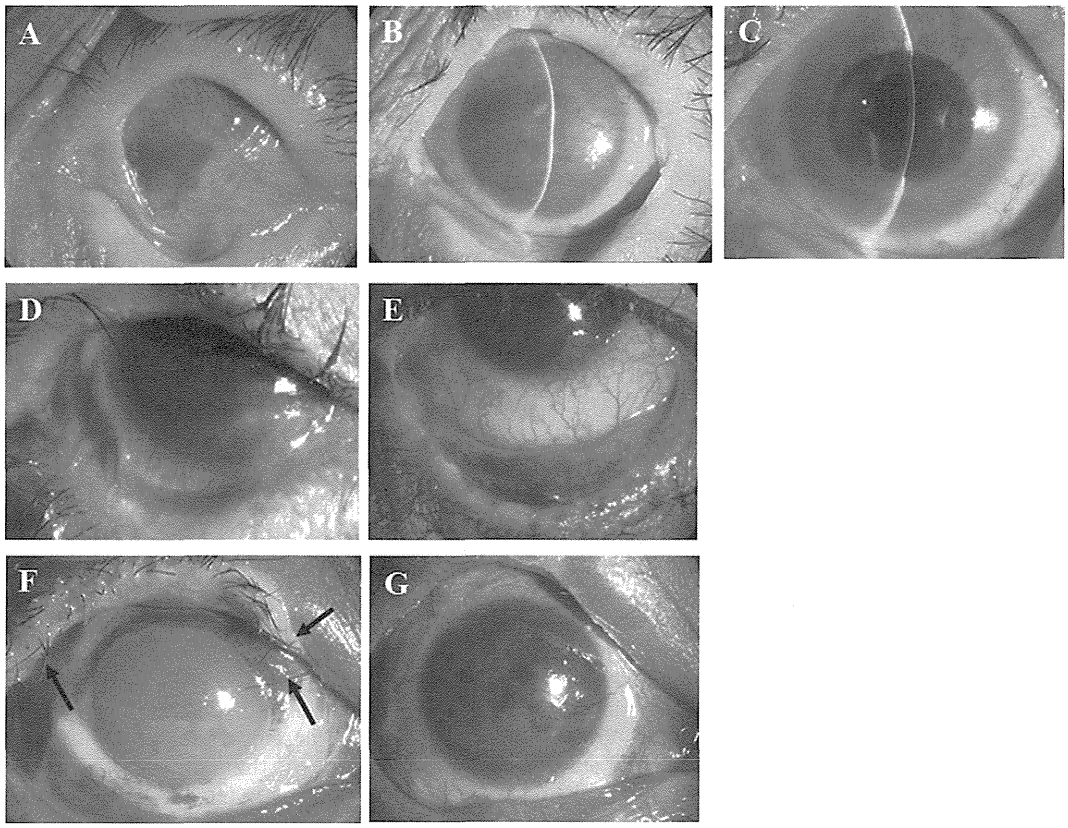

Fig. 8. (A–C) OSR with combination of COMET and PKP in patients with SJS. (A) Preoperative total conjunctivalization with severe symblepharon and partial parakeratinization. (B) Two months after initial COMET surgery. (C) Three months after PKP with cataract surgery. (D, E) Conjunctival fornix reconstruction 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).

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 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

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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

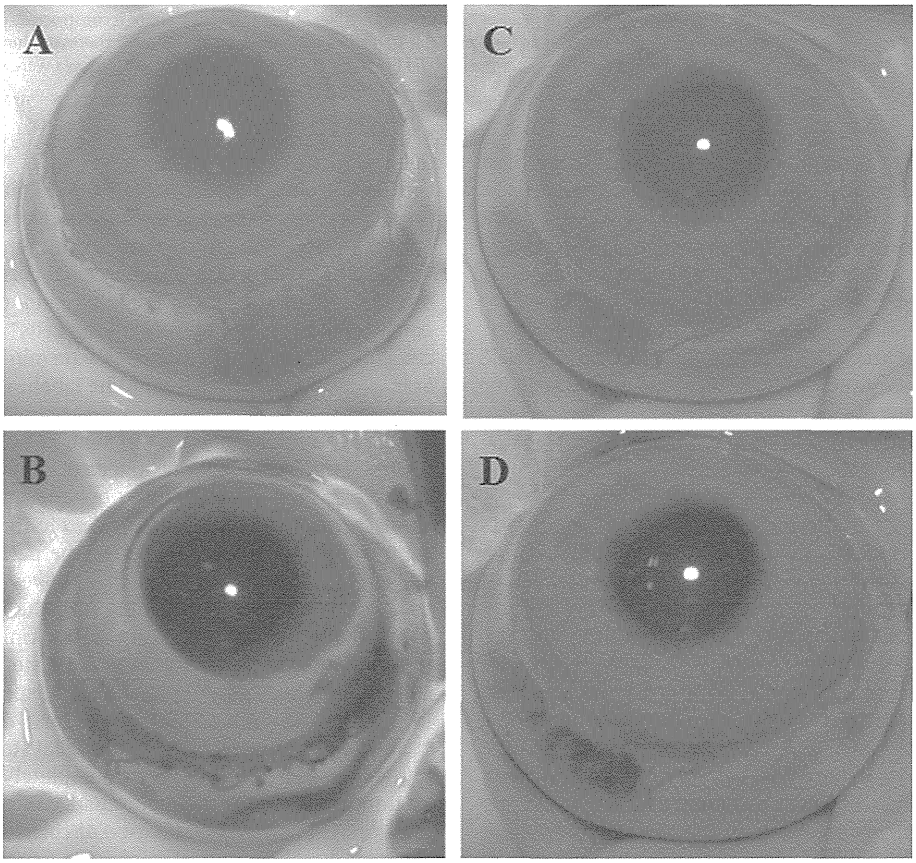

Fig. 9. Representative slit-lamp photographs of peripheral and limbal epithelial ablation in rabbits with or without fluorescein. (A, B) Immediately after ablation, fluorescein staining was clearly observed in all peripheral and limbal areas. (C, D) 6 months after ablation, cornea still maintained its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

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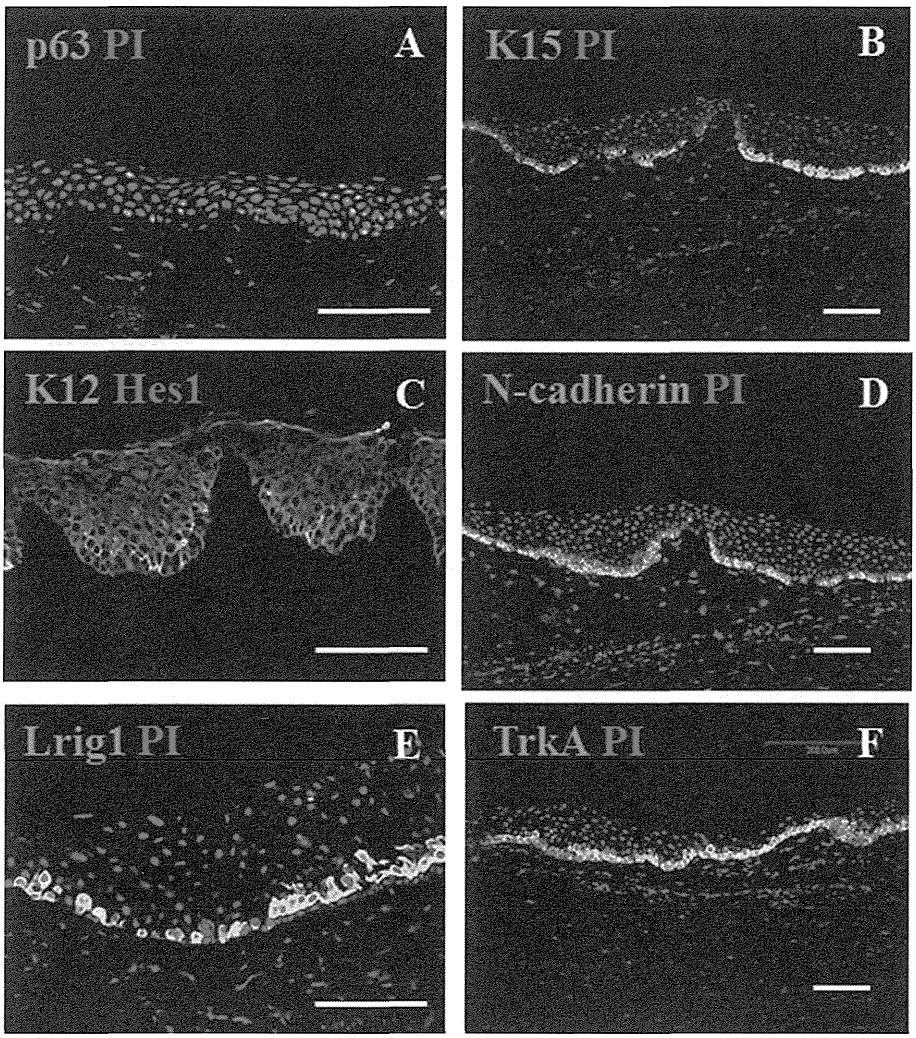

Fig. 10. Expression of proposed corneal epithelial stem cell markers. Immunohistochemistry for p63(A), Keratin 15(B), Hes1(C), N-cadherin(D), Lrig1(E) and TrkA(F) in human limbal region. Each marker (green) is clearly expressed in the basal cells of the limbus. Scale bars: 100 μm.

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 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.,

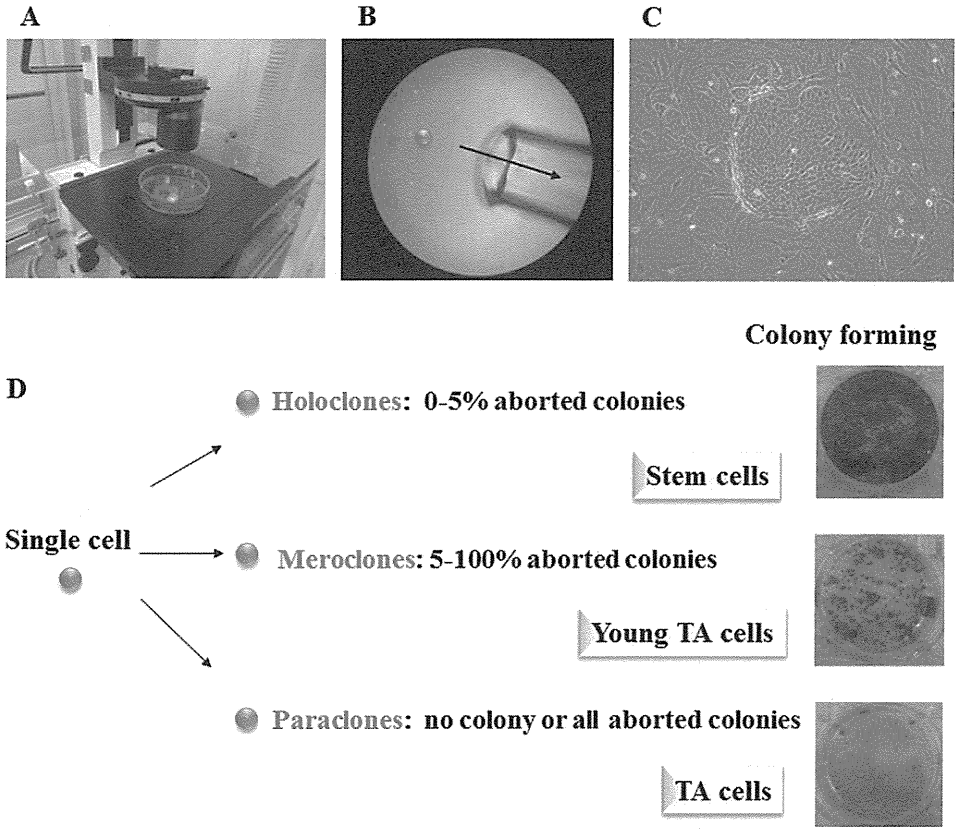

Fig. 11. Schema of single cell-based clonal analysis. (A, B) Using special equipment in the culture lab, a single cell can be manipulated under the microscope. (C) A colony can be formed from a single cell on the 3T3 feeder cells. (D) Holoclones (stem cells) have the highest reproductive capacity; in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

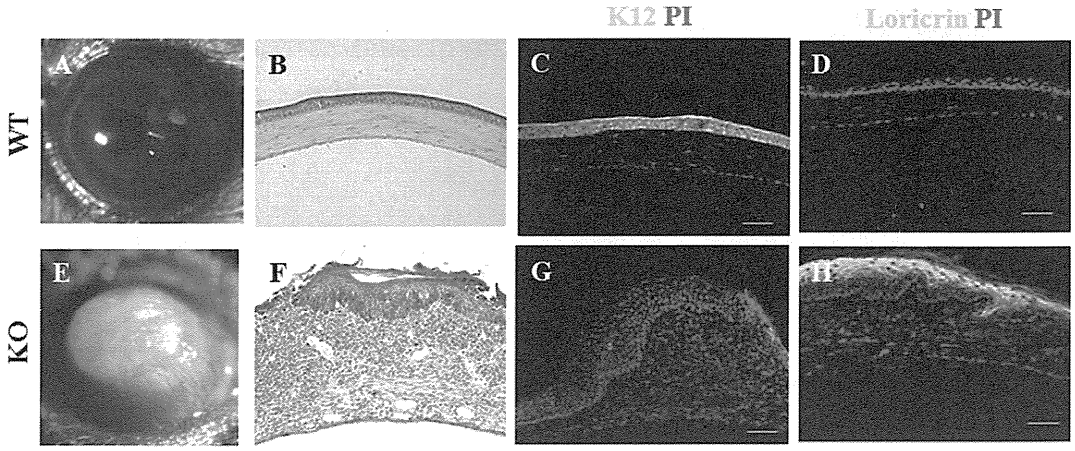

Fig. 12. Loss of Lrig1 results in a cell-fate change from corneal to keratinized epithelium. Slit-lamp photographs of Lrig1 wild-type (WT) (A) and knockout (KO) (E) mice corneas, and their histological appearances with hematoxylin and eosin staining (B, F). Lrig1 KO corneas formed corneal plaques with 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).

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).

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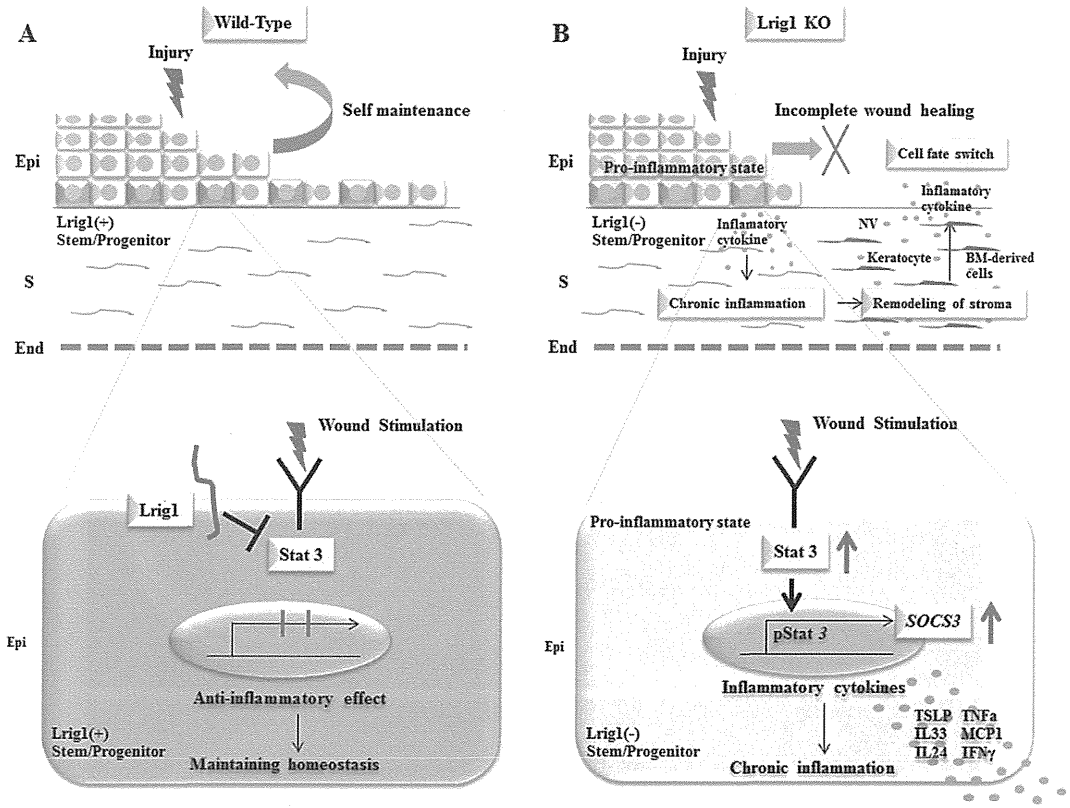

Fig. 13. Model of the function of Lrig1 in corneal homeostasis. (A) Normally, the cornea is self-maintained by Lrig1 (+) corneal stem/progenitor cells post-injury. Corneal transparency is maintained via negative regulation of the Stat3-dependent inflammatory pathway by Lrig1. (B) Loss of Lrig1 causes the pro-inflammatory state in Lrig1 (−) corneal stem/progenitor cells and impairs delayed/incomplete wound healing. Loss of Lrig1 activates the Stat3-dependent inflammatory pathway and induces chronic inflammation, resulting in remodeling of the corneal stroma. Inductive bone marrow-derived cells secrete inflammatory cytokines and cause cell-fate changes to keratinized epithelium. Epi: corneal epithelium; S: corneal stroma; End: corneal endothelium; NV: neovascularization. Modified with permission from Nakamura et al. (2014).

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-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-

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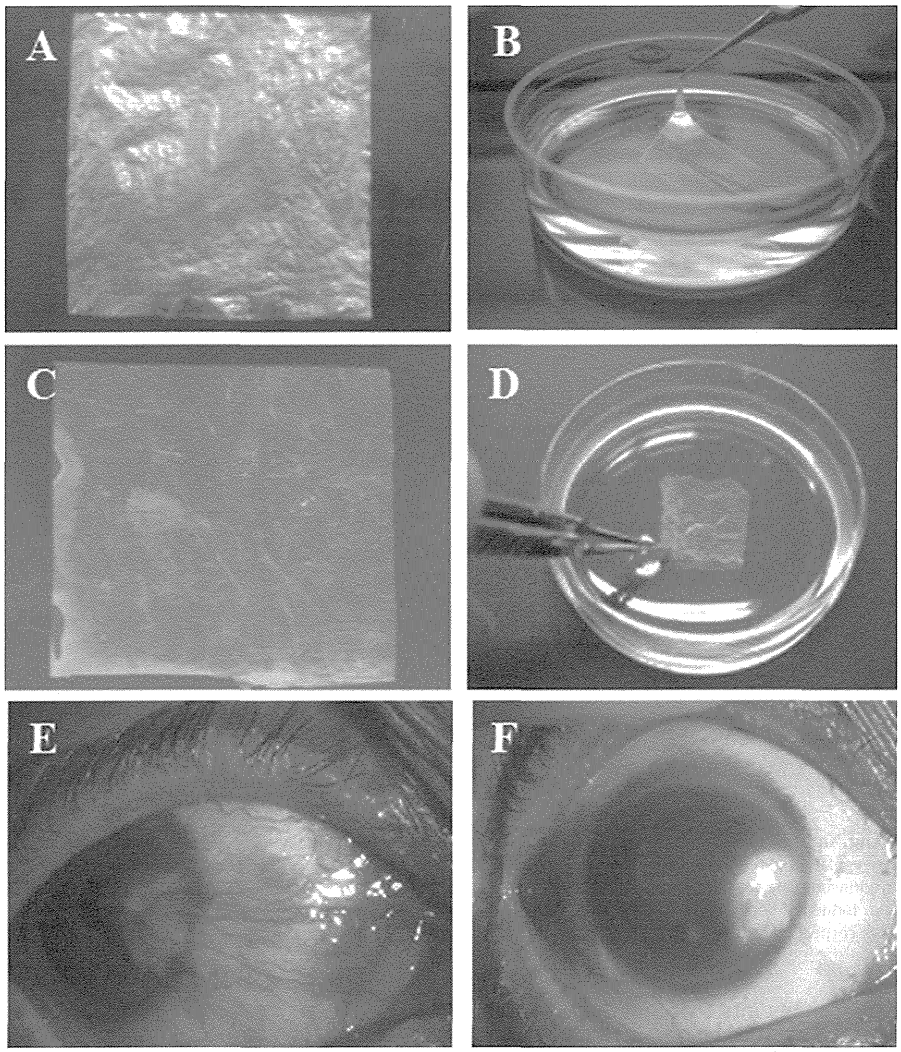

Fig. 14. Development of dried AM. (A) The sterilized, freeze-dried AM was wafer-like and very light and thin. (B) It became smooth and flexible on hydration. (C) Visually, trehalose-treated freeze-dried AM was similar to freeze-dried AM in the dry condition. (D) Trehalose-treated freeze-dried AM was smoother and more flexible than freeze-dried AM in the wet condition. Representative slit-lamp photographs taken before the sterilized, freeze-dried AM transplantation (E) and at 24 months after transplantation (F). Before transplantation, eyes manifested fibrovascular overgrowth of degenerative conjunctiva onto the cornea (E). At 24 months after transplantation, fibrosis was markedly suppressed, and the conjunctival surface was stable without inflammation (F). Modified with permission from Nakamura et al. (2004b), Nakamura et al. (2006b), Nakamura et al. (2008b).

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 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 focus on pathogenic organisms in recent years, suitable sterilization of both native and dried AM is vital, and these developments will contribute to the next generation of AM, using tissue engineering techniques. Following strict regulation in Japan, we are now trying

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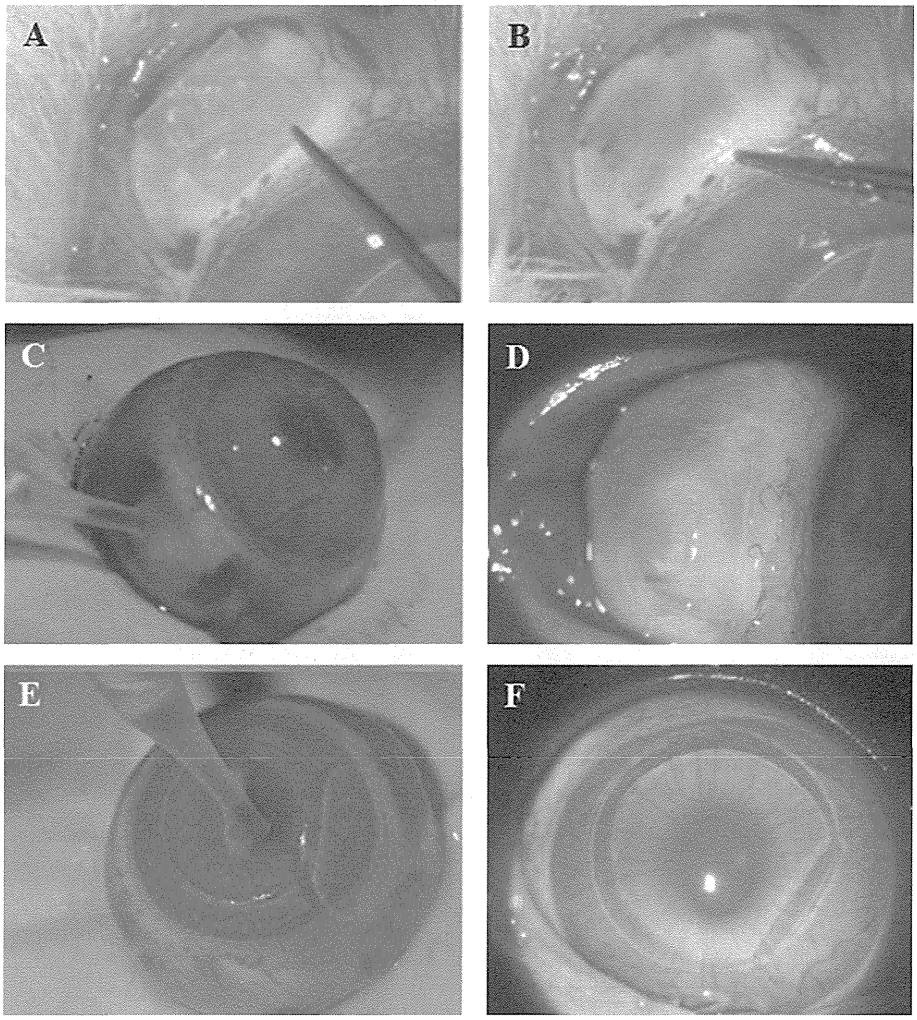

Fig. 15. Development of bio-adhesives. (A, B) Representative photos of fibrin glue-coated freeze-dried AM transplantation. FCFD-AM adhered immediately after transplantation onto the bare sclera, before fibrin glue-coated freeze-dried AM transplantation (A) and after fibrin glue-coated freeze-dried AM transplantation (B). Representative photographs of sutureless AM transplantation (C, D) and lamellar therapeutic keratoplasty (E, F) using chemically-defined bioadhesive. Modified with permission from Sekiyama et al. (2007), Takaoka et al. (2008), Takaoka et al. (2009).

to set up an AM banking center to organize the preparation and spread of AM.

4.2. Fibrin and the temperature-sensitive dish

Another promising substrate for clinical use in OSR is fibrin and the temperature-sensitive culture dish. The usefulness of a serum-derived fibrin substrate for cultured corneal epithelial cells has previously been reported (Hirayama et al., 2012; Rama et al., 2001, 2010). As fibrin substrate is absorbed after CLET and COMET, this method has some clinical benefits, in that the cultivated epithelial sheet can be transplanted directly onto the corneal surface. Although fibrin has achieved successful clinical outcomes, safety and logistical problems remain, such as the risk of disease transmission after operations (e.g. human parvovirus B19, prions) (Hino et al., 2000).

Another unique tissue-engineered technique has been developed using a temperature-sensitive culture dish for corneal epithelial cells (Nishida et al., 2004a, 2004b). This is an original system that allows the cultivated epithelium to be detached from the culture dish by changing the temperature. Both of these substrates (fibrin and the temperature-sensitive dish) may be more suitable for transplantation than AM because AM will remain permanently on the transplanted area if severe postoperative inflammation does not occur. However, since the ocular surface (including epithelium and subjacent stroma) is severely damaged in patients with severe OSD, we need to reconstruct the subjacent corneal stroma as well as the epithelial layer. In considering the biological clinical aspects, AM can serve as both an epithelial layer and as a healthy substrate covering a damaged corneal stroma. Further comparative clinical studies are needed to establish which substrate is most effective.

4.3. Development of novel cultured substrates

Although AM, fibrin and the temperature-sensitive culture dish have been widely used for OSR, a variety of alternative substrates suitable for generating tissue-engineered cultured sheet have been examined in preclinical or clinical applications. Among biosynthetic scaffolds, the cross-linked collagen scaffold (Dravida et al., 2008), fibroin membrane (Chirila et al., 2008), myogel extracted from skeletal muscle (Francis et al., 2009), collagen vitrigel (McIntosh Ambrose et al., 2009), compressed collagen (Levis et al., 2010; Mi et al., 2010), keratin films (Reichl et al., 2011) and chitosan hydrogels (Grolik et al., 2012) have all been studied. In the area of synthetic scaffolds, medical-use contact lenses (Di Girolamo et al.,

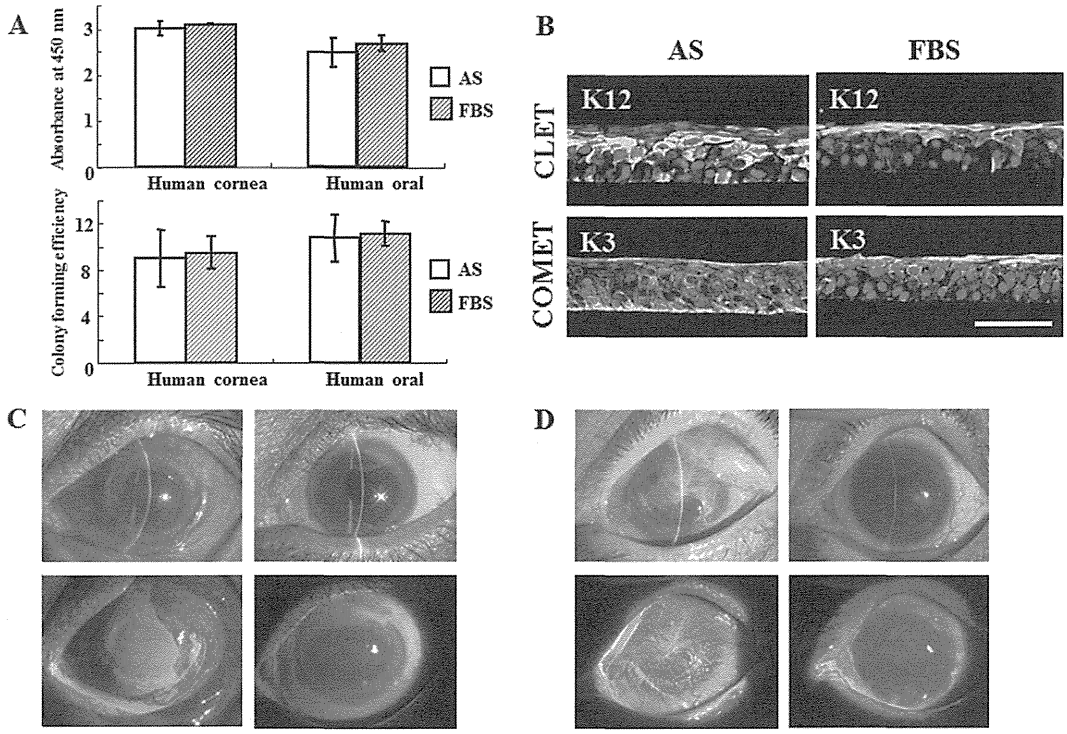

Fig. 16. Development of autologous serum-derived CLET and COMET. (A) BrdU proliferation assay and colony-forming efficiencies (CFE) showed that the proliferation indices and CFE of human corneal and oral mucosal epithelium cultivated using autologous serum (AS) and fetal bovine serum (FBS) were almost similar. (B) The expression patterns of K12 and K3 were similar in cultivated corneal and oral mucosal epithelial sheets derived from AS- and FBS-supplemented culture systems. (C) Representative clinical outcomes of autologous serum-derived CLET in patients with OCP. (D) Representative clinical outcomes of autologous serum-derived COMET in patients with OCP. Modified with permission from Nakamura et al. (2006a), Ang et al. (2006), Nakamura et al. (2006c).

Table 1
Upregulated genes in holoclone-type corneal keratinocytes (selected).

Gene symbol	Gene title	Proposed function
NLRP2	NLR family, pyrin domain containing 2	Activation of proinflammatory caspases
IL24	Interleukin 24	Anti-proliferative property
PTX3	Pentraxin-related gene, rapidly induced by IL-1 beta	Regulation of innate resistance to pathogens
CALD1	Caldesmon 1	Actin- and myosin-binding protein
KLK6	Kallikrein 6 (neurosin, zyme)	Serine protease
CRISP2	Cysteine-rich secretory protein 2	Regulation of ion channels' activity
JPH3	Junctophilin 3	Stabilization of the junctional membrane
MUM1	Melanoma associated antigen (mutated) 1	DNA damage response pathway
LRIG1	Leucine-rich repeats and Ig-like domains 1	Epidermal and intestinal stem cell marker
MTSS1	Metastasis suppressor 1	Cancer progression
KRT19	Keratin 19	Organization of myofibers
LRP11	Low density lipoprotein receptor-related protein 11	Receptor activity
DEFB4	Defensin, beta 4	Antibacterial activity
LGR5	Leucine-rich repeat-containing G protein-coupled receptor 5	Intestinal and hair follicule stem cell marker
KRT24	Keratin 24	Structural constituent of cytoskeleton

2009), nanofibers (Sharma et al., 2011; Zajicova et al., 2010) and electrospun 3D scaffolds (Ortega et al., 2013) have also been examined. In addition, biological scaffolds such as lens capsule (Galal et al., 2007) and decellular corneal stroma (Shafiq et al., 2012) have been investigated. All these proposed substrates have potential advantages and disadvantages, and careful investigation is again needed before treating patients with severe OSD using these substrates.

4.4. Bio-adhesive

Among established OSR protocols, the most standard method of AM transplantation, CLET and COMET using AM carrier involve suturing, which is time-consuming and is associated with disadvantages that include suture abscesses, granuloma formation and tissue necrosis. To overcome these problems, sutureless transplantation, using proper tissue-engineered bio-adhesive, is ideal for developing next-generation OSR. There have previously been some studies of sutureless techniques for OSR using fibrin glue (Szurman et al., 2006); we initially developed a novel sutureless technique for AM transplantation by generating a fibrin glue-coated dried AM (Sekiyama et al., 2007) (Fig. 15). Fibrin is derived from serum, so if a non-biologic and defined bio-adhesive can be successfully developed, it would prove ideal for safe and simple OSR. On that basis, we developed AM transplantation for OSR using a chemically defined bio-adhesive that is safe, biocompatible and biodegradable (Takaoka et al., 2008) (Fig. 15). Based on those results, we foresee the use of chemically defined bio-adhesives in a range of sutureless transplantations, including CLET, COMET and lamellar keratoplasty (Takaoka et al., 2009). We are currently applying this technique for lamellar keratoplasty, and the clinical results are quite promising so far.

5. Growth factors

5.1. Feeder layer factor

Based on the pioneering culture method by Rheinwald and

Green, long-term survival and serial expansion of epidermal stem cells is possible if the epithelial cells are co-cultured with mouse-derived 3T3 feeder layer (Rheinwald and Green, 1975). Since that time, 3T3 feeder cells have become the most widely-used for culturing corneal epithelial cells. The exclusion of animal material from the culture system offers significant clinical advantages for OSR, reducing the risk of transmission of animal-derived infections or unknown pathogens. Various candidate human-derived feeder cells such as MRC-5, amniotic epithelium, adipose tissue, mesenchymal stem cells and dermal fibroblast have been examined for their usefulness in generating cultivated epithelial sheet (Chen et al., 2007; Notara et al., 2007; Oie et al., 2010; Omoto et al., 2009; Sugiyama et al., 2008). However, widespread use of these culture systems has been hindered by the standardization of culture protocols because of the variable culture conditions of feeder cells.

5.2. Serum factor

The previously preferred method of cultivating epithelial sheets also requires the use of xenobiotic materials such as fetal bovine serum (FBS) in the culture system. However, the use of FBS in the culturing system is a major clinical concern, as bovine spongiform encephalopathy cannot be detected by any known assay. The use of autologous serum (AS) as an alternative to FBS is therefore significantly safer, excluding the need for bovine material in the culture process. Initially, we tried to determine whether AS from patients with severe OSD was as effective in supporting cell proliferation and differentiation in cultivated corneal and oral mucosal epithelial cells as culture methods using FBS (Nakamura et al., 2006a) (Fig. 16). We found that an AS-supplemented culture protocol was effective in supporting the proliferation of human corneal and oral mucosal epithelial cells, as well as the development of transplantable cultivated corneal and oral mucosal epithelial sheets. Based on these findings, we adapted this method for clinical application and reported the successful clinical use of cultivated corneal and oral mucosal epithelial sheets (Ang et al., 2006; Nakamura et al., 2006c). These clinical reports make important suggestions and represent progress in the pursuit of completely xenobiotic-free tissue-engineered transplants. In addition, we know from our clinical work that AS sometimes has donor-dependent variations (e.g. disease or age) that must be kept in view.

5.3. Development of feeder-free and serum-free systems

In light of the above mentioned factors, the development of feeder-free and serum-free culturing systems might be ideal for the next generation of OSR using tissue-engineering techniques. Yokoo et al. reported the generation of a cultivated corneal epithelial cell sheet for OSR in a completely serum-free and feeder-free culture system containing epidermal growth factor and B-27 (Yokoo et al. 2008). More recently, Miyashita et al. reported the long-term maintenance of corneal epithelial stem/progenitor cells using Rho Kinase inhibitor and keratinocyte growth factor (Miyashita et al., 2013). We believe that these studies represent an important step in the development of real feeder-free and serum-free transplantable cultivated epithelial sheets for safe and ideal OSR.

6. Future directions

Corneal regeneration has long been one of the great challenges for ophthalmologists and vision scientists worldwide. With advances in basic research in regenerative medicine and tissue engineering, great progress has been made in the fundamental understanding and development of novel therapeutic modalities such as CLET and COMET. However, although CLET and COMET currently represent the safest and most reliable form of newly developed transplantation, several issues remain to be overcome. First, autologous CLET is to date the most promising treatment for reconstructing the ocular surface in cases of unilateral severe OSD. However, in our long-term follow-up, we certainly observed some incidence of mild conjunctivalization in peripheral corneas. The current cultivated corneal epithelial sheet could not absolutely reproduce the corneal limbal niche, and recreation of the functional corneal limbal niche using innovative tissue-engineering technology may be needed to properly develop this surgical tool. In contrast, treatment of patients with bilateral severe OSD requires either allogeneic CLET or autologous COMET, depending on patient variables (e.g. type of disease, age). In the case of CLET, the risk of postoperative rejection must be addressed, requiring a basic knowledge of the immunological background of allogenic OSR and an appropriate protocol for postoperative management, especially with regard to immunosuppressive therapy. In the case of COMET, we must exercise caution because the cultivated oral mucosal epithelial sheet is not identical to *in vivo* corneal epithelium. This requires a basic understanding of epithelial cell biology in trying to characterize the cell source to be used. One candidate approach is to identify a novel, non-ocular surface cell source for use in OSR. The other candidate approach is to develop innovative genetically-modified biotechnology using induced-pluripotent stem cells or direct reprogramming. We strongly believe that greater knowledge of proposed and established surgical modalities, stem cell behavior, the surrounding extracellular matrix and beneficial growth factors will provide a foundation for the further development of treatments for severe OSD.

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