Dry eye disease (DED) is defined at TFOS/DEWS II as a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles. A major role of the lacrimal glands is the maintenance of a homeostatic environment on the ocular surface epithelium through tear secretion. The functional restoration of the lacrimal glands for severe DED began with the use of alternative tear substitutes, such as artificial tear drops, to sustain a stable tear film. An autologous serum eye drop has been recognized as a more physiologically relevant substitute for natural tears because it contains components, such as growth factors, fibronectin, and vitamins, that support proliferation, migration and differentiation of the ocular surface epithelium. On the other hand, the transplantation of autologous accessory salivary glands into the ocular conjunctiva has been reported in the clinic as an efficient way to reconstruct the ocular surface for severe DED. Tear/lacrimal gland alternative therapies are some of the therapeutic choices for severe DED and have already been used in the clinic. Recently, regenerative medicine, which has been improved by our knowledge of developmental biology, stem cell biology, and tissue engineering technology, has arisen as a new approach to restore the function of impaired organs. In the early decades of regenerative medicine, cell injection therapies using stem cells derived from tissues and stem cell activation therapies with cytokines have been investigated to restore the function of injured organs in cases of malignant disease, myocardial infarction, and hepatic dysfunction. Advances in tissue bioengineering technology allow us to generate two-dimensional tissues, including cultivated corneal epithelial cell sheets and oral mucosal epithelial cell sheets, to efficiently treat severe ocular surface diseases. In addition to therapies utilizing tear/lacrimal gland substitutes, recent exponential developments in biology have advanced regenerative medicine such that reconstruction of lacrimal gland function has recently emerged as a possible method to overcome severe DED. An organ replacement therapy based on the use of donor organs has promised a significant advantage for the restoration of organ functionality in patients with severe organ dysfunction. However, it has been difficult to solve medical issues related to donor organ transplantation, including the donor shortage and allogenic immunological rejection of the transplanted organ. Developments in engineering technology have contributed to the creation of novel medical tools, such as mechanical medical devices and artificial organs, which can be used to overcome some of the problems related to donor organ transplantation. One of the goals of current regenerative medicine is to replace an organ injured due to diseases, trauma, and aging with a fully functional 3D bioengineered organ created from stem cells in an ex vivo culture environment. Various types of technical innovation have been used to regenerate functional tissues/organs. One such innovation involves a 3D cell manipulation procedure that requires the precise arrangement of multiple kinds of cells, an optimized culture condition and a stem cell differentiation method to
induce the development of bioengineered organs from available cell sources.27

This review details the physiologic function and development of the lacrimal glands as well as recent advances in the restoration of lacrimal gland function obtained from both our works and published research. We present evidence that regenerative medicine may be utilized to make fully functional lacrimal glands in the future.

Functional Anatomy of the Lacrimal Glands

The lacrimal glands produce an aqueous layer, which makes up the major part of the pre-corneal tear film.4 Aqueous tears contain water, electrolytes, and various secreted substances, including lipocalin and lactoferrin.28,29 Tears play indispensable physiological roles on the ocular surface, such as lid lubrication, hydration, antimicrobial activity, and protection of the ocular surface epithelium.30

The lacrimal glands include the main lacrimal glands, which secrete the majority of the tears, and scattered small accessory lacrimal glands that exist in the ocular conjunctiva.2 The basic schematic of the lacrimal glands is that of a tubule-alveolar structure. The acini produce tear proteins and secrete fluids through aquaporin 5 (AQP5), a water channel, which is expressed on the luminal side of the cell. The fluids from the acini drain onto the ocular surface through the lacrimal gland excretory duct.2 Myoepithelial cells surrounding the acini make the acini shrink in response to nerve signals to help them to secrete tear products into the duct.31 An elaborate neural regulatory process governs lacrimal gland function in order to secrete tears.31,32 The tear-reflex loop pathway, which can secrete tears from the lacrimal glands and which depends on the stimulation of the ocular surface, is a representative example of the importance of neural regulation of tear secretion.33 The functionality of the lacrimal glands is essential for the maintenance of a physiologically normal ocular surface and visual function.

Lacrimal Gland Development During Embryogenesis

The elaborate 3D cell distribution of secretory gland structures, including lobules of acini, ducts and myoepithelial cells, is required for efficient organ function.2 This comprehensive structure, called a tubule-alveolar structure, is formed by a developmental process during ontogenesis.34 Representative ectodermal organs, including teeth, hair follicles, salivary glands and lacrimal glands, have a common embryonic developmental strategy resulting from reciprocal epithelial and mesenchymal interactions.35–37 An embryonic lacrimal gland bud, called the lacrimal gland germ, develops from the primitive ocular surface epithelium; this is followed by branching morphogenesis signaling between the embryonic epithelium and mesenchyme in the lacrimal gland bud (Figs. 1a, 1b).38 In mice, the development of the basic lacrimal gland structure is completed before birth, and the production of functional tears begins when the eye lids open.7

Various kinds of fundamental signaling underlying branching morphogenesis affect the developmental regulation of the lacrimal glands (Fig. 2).16,39–41 The expression of Pax6 in
embryonic ocular conjunctiva leads to the onset of the lacrimal gland bud. A condensed expression of fibroblast growth factor (Fgf) 10 in the peripheral mesenchyme induces bud formation from the epithelium. The formation and outgrowth of the bud is mediated by the local activation of a signaling cascade involving N-deacetylase/N-sulfotransferase, which modifies heparin sulfate, Fgf receptors and Src homology 2-containing tyrosine phosphatase 2. Fgf signaling regulates the Sox9-Sox10 pathway, the expression of which is required for lacrimal gland branching and differentiation. The Fgf signaling-regulated elongation of the lacrimal gland epithelial stalk requires Barx2 expression for the control of metalloprotei- nases. The signaling between the epithelium and mesen- chyme in the bud is required for the completion of branching morphogenesis. Signaling molecules in the bone morphogenetic protein (Bmp) family, including Bmp7, regulate the branching processes by managing mesenchymal proliferation and condensation. This series of complex biological developmental processes is critical for the creation of the histological architecture of the lacrimal glands.

Identification of Tissue Stem/Progenitor Cells in the Lacrimal Glands for Cell Injection Therapy

Tissue stem cells, which reside in adult tissues, are responsible for maintaining organ structure and function and are activated to a proliferative state for tissue regeneration when the tissue has been partially injured. Tissue stem cells have been recognized as a clinically available candidate cell source for cell injection therapy to restore injured organ function. It has been implied that the lacrimal glands have a regenerative capacity following partial tissue injury that is mediated by tissue stem cells. This stem cell population is posited to be similar to those in other secretory glands including salivary glands, the pancreas and mammary glands, although the detailed location of the tissue stem cells in the lacrimal glands is still unknown. In the salivary glands—the most similar ectodermal secretory glands to the lacrimal glands—stem/progenitor cells with regenerative capabilities reportedly exist in the intercalated ducts and the basal layer of the excretory ducts. Temporary injury of the murine lacrimal glands due to interventions such as cytokine injection or excretory lacrimal gland duct ligation demonstrated that, following a

Figure 2. Gene expression and molecular signaling during branching morphogenesis. Elaborate gene expression and molecular signaling networks between the epithelium and mesenchyme in the lacrimal gland germ are reportedly required for the differentiation and branching morphogenesis of the lacrimal glands. One of the lacrimal gland developmental hypotheses involves the existence of a signal center (SC). ECM, extracellular matrix; HS, heparan sulfates; HSSE, heparan sulfate-synthesizing enzymes; MMPs, matrix metalloproteinases.
Functional Restoration of the Lacrimal Glands

**FIGURE 3.** 3D regeneration of the bioengineered lacrimal gland using the organ germ method. (a) Conceptual illustration of 3D lacrimal gland regeneration using the organ germ method. (b) In vitro reproduction of development and branching morphogenesis in the bioengineered lacrimal gland. Scale bar: 100 µm. (c) Representative photograph of the induced duct structure in the epithelium of the bioengineered lacrimal gland (▼). Scale bar: 100 µm. (d) In vivo development of the bioengineered lacrimal gland after transplantation by the thread-guided procedure. The transplant after transplantation (▼) is shown in the left panel (Scale bar: 1 mm). The transplant at 30 days after surgery is shown in the right panel (Scale bar: 500 µm). (e) Immunohistology of the bioengineered lacrimal gland after transplantation. Aquaporin-5 (AQP5) were correctly expressed in luminal surface of the acini and duct in the left panel (AQP5, red, and E-cadherin, green). Calponin, a marker of myoepithelial cells (red) and E-cadherin (green) in the center panel, Calponin (red), neurofilament-H (NF-H; green) and DAPI (blue) in the right panel. Scale bars: 50 µm. Parts (b-e) reprinted from Hirayama M, Ogawa M, Oshima M, et al. Functional lacrimal gland regeneration by transplantation of a bioengineered organ germ. *Nat Commun*. 2013;4:2497.

suggested as contributing to repair processes. Recent studies have revealed characteristics of the tissue stem/progenitor cells in the lacrimal glands. Injection of a population of epithelial progenitor cells expressing c-kit+/dim/EpCAM+/Sca1+/CD34+/CD45− that were isolated from murine lacrimal glands were able to regenerate secretory acini in a lacrimal gland disease model. A small population of cells expressing cytokeratin15 (Krt15), which is commonly expressed in immature lacrimal gland epithelial cells in embryonic stages, has been observed in the basal cells of the intercalated duct in mature lacrimal glands, where the secretory gland tissue stem cells reportedly exist. In humans, lacrimal gland cells expressing stem cell markers such as ckit, ABCG2 and ALDH1 have been reported. Research into lacrimal gland regeneration using adult tissue stem/progenitor cells has continued to advance the clinical restoration of lacrimal gland functionality.

3D Functional Lacrimal Gland Organ Regeneration In Vivo

**Development of a Novel Cell Manipulation Method for 3D Organ Regeneration.** Establishment of procedures for the manipulation of several kinds of cells based on tissue bioengineering technology has been required for the generation of a 3D functional bioengineered organ. In addition, it is critical that the reconstructed 3D bioengineered organ be capable of coordinating with recipient tissues such as nerve and blood vessels. The organ germ method, which is a 3D tissue engineering technique to reconstitute a bioengineered organ germ, has emerged as a new strategy to initiate functional organ replacement regenerative therapy for severe organ dysfunction (Fig. 3a). Epithelial and mesenchymal cells, which are artificially assembled and compartmentalized at a high density using the organ germ method, were capable of reproducing the developmental process of organogenesis in vitro and building up the organ structure in a 3D collagen gel matrix. Bioengineered teeth and hair follicles, manipulated by the organ germ method, could successfully be engrafted and could develop into a fully functional organ with peripheral nerve coordination after transplantation. We investigated the possibility of restoring lacrimal gland function by transplanting a regenerated 3D lacrimal gland organ germ created by the organ germ method. The bioengineered lacrimal gland organ germ, which was regenerates using ED 16.5 mouse epithelial and mesenchymal cells isolated from the lacrimal gland germ, was able to reproduce the developmental branching morphogenesis process in organ culture (Fig. 3b). The organ germ method was successfully applied to generate a bioengineered hardier gland, which produces tear lipids in mice.

**Induction of a Duct Structure for Successful Engraftment of a Bioengineered Lacrimal Gland Germ.** Integration of the excretory duct and the lacrimal glands lobules is essential for delivery of tear fluids to the ocular surface. A bioengineered lacrimal gland lacking an excretory duct structure could not continue to develop the appropriate secretory gland architecture. Our thread-guided 3D culture and transplantation technique enables the in vitro bioengineered lacrimal glands to induce the excretory duct structure from the epithelial cell aggregate and concatenate the induced duct with a recipient lacrimal gland excretory duct in a mouse with the extra-orbital lacrimal gland removed (Figs. 3c, 3d). This procedure improved the success rate of bioengineered lacrimal gland transplantation. Further, the bioengineered lacrimal gland could achieve the appropriate secretory gland architecture including a tubule-alveolar structure (i.e., lobules consisting of acini, ducts, myoepithelial cells, and peripheral nerves).
Functional Restoration of the Lacrimal Glands

Restoration of Physiological Functions of the Lacrimal Glands. A primary merit of functional organ replacement therapy using 3D bioengineered organs has been the restoration of organ functions to a physiologically relevant state. Reflex tearing triggered via corneal thermoreceptors by a cooling stimulation on the ocular surface is a representative neural pathway for lacrimal gland function. The bioengineered lacrimal glands could demonstrate the reflex tearing by the cooling stimulation at the ocular surface through the host nervous system. This suggests that, during structural development, the bioengineered lacrimal gland germ successfully accepted host nerve innervation.

Normal tears contain not only water but also various kinds of tear proteins, such as lactoferrin. These contents support physiologic tear function, including tear stability, antibacterial protection and wound repair. Delivery of tear proteins and other contents of normal tears has been able to treat severe DED and diseased ocular surfaces, as demonstrated by clinical studies using tear substitutes such as autologous serum eye drops. We demonstrated that tears secreted from the bioengineered lacrimal glands contained tear proteins, including lactoferrin, which are produced in lacrimal gland acini. These results implied that the bioengineered lacrimal gland is much more physiologically relevant in vivo than previous attempts to restore lacrimal gland function.

One reason to work towards efficient lacrimal gland regeneration is to protect the ocular surface from DED. DED causes punctate keratitis (observed by fluorescein staining of the ocular surface), corneal epithelial thinning, and corneal stromal fibroblast activation. We demonstrated that the ocular surface of mice engrafted with the bioengineered lacrimal gland did not show the pathologic changes characteristic of DED. The functional replacement of the lacrimal glands using 3D bioengineered lacrimal glands has demonstrated the possibility of complete restoration of lacrimal gland functionality in order to maintain a healthy ocular surface in cases of DED.

Cutting-Edge Strategies to Regenerate the Lacrimal Glands in Humans

With the emergence of pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), technologies involving differentiation of stem cells to individual lineages have focused on the use of PSC-derived differentiated cells and tissues for regenerative medicine. A differentiation protocol using a chemically and biologically optimized culture environment has been reported for the differentiation of PSCs to target cells, such as neurons. In ophthalmology, iPSC-derived retinal pigment epithelial cells have been successfully transplanted into a patient to overcome a disease that can induce vision loss. A PSC differentiation technology using a self-organization process could generate biologically assembled 3D structures, such as retinal layers, and a series of ocular cell lineages, derived from SEAM. The primitive ocular epithelium induced by SEAM is expected to provide a source of cells for the generation of iPSC-derived functional bioengineered lacrimal glands using the organ germ method. Currently, a novel strategy has been proposed to induce target cells directly from PSCs based on our understanding of transcription factor regulation of cell identity during ontogenesis. One advantage of the direct conversion method is an efficient, immediate cell conversion from PSCs and somatic cells like fibroblasts to a targeted cell lineage. A previous report showed that the core transcription factors PAX6, OVOX2, KLF4, SOX9, TP63, and MYC could induce cells to a corneal epithelial cell lineage from human fibroblasts. Identification of a core transcription factor network that defines a particular cell lineage is the key to realizing the direct reprogramming of PSCs. Recently, a whole gene expression analysis based on microarray technology identified a subset of transcription factors (PAX6, FOXC1, SIX1, and SIX2) that are specifically and highly expressed in the ED16.5 mouse lacrimal gland epithelium compared with developmentally related organs such as the mature lacrimal glands, the mature/embryonic harderian glands and the embryonic ocular surface epithelium. These transcription factors have been implicated as part of a set of core transcription factors that define the lacrimal gland epithelial cell lineage. Specifically, the overexpression of PAX6, FOXC1, and SIX1 using transcription factor-coded synthetic mRNAs in human ESCs resulted in the differentiation of cells toward the lacrimal gland epithelial cell like phenotype accompanied by expression of a part of lacrimal gland development markers, including the immature lacrimal gland epithelial marker KRT15, the branching morphogenesis marker BAX2, and the tear secretion related markers AQP5 and lactoferrin. This result may suggest a possibility of regenerating lacrimal gland cells from human PSCs. A meta-analysis of gene expression in the human lacrimal glands has revealed the expression of specific genes and pathways in the lacrimal glands in comparison with the gene expression in other organs, such as the salivary glands, the corneal epithelium, and the conjunctiva. Integration of developmental biology and gene expression analyses, including next-generation sequencing technology, would provide us with new insights into lacrimal gland regeneration.

Future Directions

The demand for restoration of lacrimal gland function using regenerative medicine for patients with severe DED has further intensified with recent advances in biology and engineering. Research approaches for lacrimal gland regeneration have expanded from the use of tears and lacrimal gland alternatives to the induction of whole organs using stem cells (Fig. 4). Artificial lacrimal gland tear regulation via stimulation of neural pathways using medical devices has been proposed as a future therapy for DED. A step toward successful lacrimal gland regeneration is the establishment of technology that can construct functional secretory glands in 3D with a coherent distribution of multiple kinds of cells. The transplantation of bioengineered lacrimal gland germs has provided a proof of concept for the generation of functional bioengineered lacrimal gland structures in vivo. Previous reports demonstrated successful 3D reconstruction of the lacrimal glands using biohybrid materials, including a decellularized scaffold. The differentiation method involving self-organization of PSCs in 3D culture would be an attractive candidate for the generation of a 3D lacrimal gland organ or organoid. In addition, the latest bioengineering technologies, such as 3D cell printing, may contribute to the regeneration of the 3D lacrimal gland structure.

The use of available stem cell sources for the induction of lacrimal gland regeneration is one of the next challenges that must be overcome for the successful clinical application of lacrimal gland regenerative therapy. Cell injection therapy using tissue-derived stem cells in the lacrimal glands is nearing clinical application due to the discovery of specific cell markers that identify the epithelial progenitor cell populations in the lacrimal glands. Proof of the existence of such a progenitor cell in humans, an efficient culturing method for progenitor cell proliferation, the ability to efficiently obtain enough cell numbers for injection and full restoration of the damaged lacrimal glands, and investigations into optimization for human disease status would all strengthen this evidence for
future clinical application. It has been shown that the immature ocular epithelium, including embryonic lacrimal gland epithelial cells, has the ability to generate a 3D lacrimal gland structure through crosstalk with mesenchymal tissues. The primary ocular epithelium, which is induced from iPSCs by SEAM and its downstream differentiated cell lineages, may be an available cell candidate for lacrimal gland regeneration. The direct reprogramming of PSCs has been improved upon through the progression of analyses of gene regulatory networks. Identification of transcription factors promoting the differentiation of lacrimal gland epithelial phenotypes from human ESCs has opened the door to the generation of human lacrimal glands. Gene expression using synthetic mRNAs is closer to clinical use than approaches using vector-based procedures because the synthetic mRNAs have the advantage of not integrating into the genome of differentiated cells. Detailed analyses of regulatory gene networks involved in lacrimal gland development are expected to result in the discovery of appropriate cell sources for the regeneration of the lacrimal glands. A future determination of core transcription factors, which can induce the development of lacrimal gland tissue from PSCs or fibroblasts, would have a potential to advance the in vivo direct reprogramming of the lacrimal glands in chronically damaged tissues. A multidisciplinary approach involving various areas of science, including biology and engineering, is expected to make bioengineered organ regenerative therapy a future therapy to cure DED.

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