Functional lacrimal gland regeneration toward a next-generation organ replacement therapy

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Abstract

The lacrimal glands secret tear fluids, and maintain a homeostatic environment of healthy ocular surfaces. Dysfunction of the lacrimal glands causes the dry eye disease with tear shortage, which is one of prevalent eye diseases resulting in ocular discomfort, significant visual disturbances, and a reduced quality of life. Secretory gland structure of the lacrimal gland consists of acini, ducts and myoepithelial cells. It occurs from the lacrimal gland germ through reciprocal epithelial–mesenchymal interactions during ontogenesis. Strategies to restore the lacrimal gland function by regenerative medicine have expanded recently. Cell injection therapy using lacrimal tissue stem cells has been developed by establishing animal models that can induce partial damage in the lacrimal glands. Fully functional organ replacement using a bioengineered lacrimal gland has also been developed through a newly proposed three-dimensional stem cell manipulation, the organ germ Method. Currently, it has demonstrated an innovative concept to induce lacrimal gland cells from human pluripotent stem cells by a direct cell conversion procedure using artificial mRNAs based on our understanding of genetic-engineering biology. This study represented a significant advance in potential lacrimal gland organ regeneration from human pluripotent stem cells as a next regenerative therapy for dry eye disease. In this review, we will summarize recent progress in lacrimal gland regeneration research and the development of bioengineered lacrimal gland organ regenerative medicine.

Introduction

Current regenerative medicine, which has been advanced along with our understanding of developmental biology, stem cell biology, and tissue engineering, has estimated future prospects to realise next-generation medical therapies to restore injured organ function(1-3). Application of ideas of regenerative medicine, including stem cell transplantation of purified tissue-derived stem cells and stem cell activation using cytokine, has been achieved clinically for various organs(4, 5). For some kinds of severe diseases that can induce loss of organ function, such as malignant diseases, myocardial infarction, neurodegenerative diseases, and hepatic dysfunction, these stem cell therapies have been shown as attractive curable approaches(6-8). Recent advances in tissue engineering technique have generated a cell sheet transplant as a 2D tissue regeneration for efficient functional restoration of damaged organs(9). In the area of ophthalmology, the cell sheet transplant technique has clinically expanded as functional cell sheet transplantation, including cultivated corneal epithelial cell sheets and cultivated oral mucosal epithelial cell sheets, and has been used to treat severe ocular surface disorders(10-12). Therapies using regenerative medicine have already been essential in ophthalmology to overcome vision-threatening corneal and retinal eye diseases(13, 14).

Although a donor organ for transplantation surgery has provided promising benefits as a current clinical therapeutic approach for diseases with severe organ dysfunction, it would take much a significant amount of time to overcome profound medical issues from it, such as allogenic immunological rejection and shortage of donor(15). Technologies that can make new medical devices including mechanical device and bio-artificial organs would contribute to solving the issue of building a functional substitute organ(16-19). To replace a damaged organ by disease, injury, and aging from a functional bioengineered organ, which is reconstructed in vitro 3D environment using stem cells, has been one of the expected goals for regenerative medicine(20). In the next generation of regenerative medicine, it has been required to develop cell manipulation techniques *in vitro*, which uses precise arrangement of several different cell species and organ culture methods in a 3D environment, to realize functional bioengineered organ regeneration(21). A novel cell culture and differentiation method has been needed for an establishment of available cell sources for organ regeneration that can contribute to development of 3D organs (22, 23).

This review illustrates the physiology, function, diseases, and development of the lacrimal gland obtained from published research. We show the potential for novel, fully functional lacrimal gland regeneration as a next-generation regenerative medicine.

Physiology and function of the lacrimal glands

The principle role of the lacrimal glands is to maintain stable healthy physiological functions of the ocular surface microenvironment by secreting aqueous tears(24-26). Human have main lacrimal glands, which produce the bulk of aqueous tear volume, and small accessory lacrimal glands scattered throughout the ocular conjunctival epithelium(26). The lacrimal glands employ a tubular-alveolar system organized by the acini in which the ducts carrying fluid from the acini to a mucosal surface. The myoepithelial cells surround the acini and early duct elements to help the secretion of tears(26). The lacrimal glands

receive elaborate neural regulation to physiologically secret tears, i.e. tear-reflex loop system in response to a stimulation on ocular surface. Aqueous tears secreted from the lacrimal glands form layers of tear film on ocular surface with tear lipids and mucins for the microenvironment of corneal epithelium and for optical properties of visual functions(27-29). The lacrimal glands produce various kinds of tear proteins including lactoferrin working for biological functions such as moisturizing capacity and antimicrobial activity(30-32). Tear functions sustained by the lacrimal gland functionality are necessary to preserve the physiologically normal ocular surface epithelium and visual functions.

The dry eye disease

A tear deficiency caused by lacrimal gland dysfunction that results from systemic diseases and environmental exposures, such as Sjogren's syndrome and ocular cicatricial pemphigoid, or others, including aging, visual display work, contact lens use, dry environments and refractive surgery, lead to the onset of the dry eye disease (DED)(33-41). In DED, there are various ocular symptoms including ocular discomfort and significant decrease of visual function(42-45). In severe cases, it can be a cause of loss of vision due to a disruption of corneal epithelium homeostasis, such as keratinization, corneal ulcer, thinning, and perforation(46). Normal tears from the lacrimal glands contain water, salts, hydrocarbons, proteins and lipids(47-49). To substitute normal tear complexes, it has been expected to restore lacrimal gland function by using regenerative medicine(22, 50).

Organogenesis of the lacrimal glands during development

Various kinds of cells are distributed for constructing mature organs with efficient functionality in 3D histological architecture. The structure develops through a process of ontogenesis in the embryo. The lacrimal glands, as well as other ectodermal organs including teeth, hair follicles and salivary glands, demonstrate a common developmental strategy to form mature organs(26, 51). They occur from their organ germs via reciprocal epithelial and mesenchymal interactions(52). In embryonic ocular epithelium, the initial bud of the lacrimal gland germ is inducted by a process of branching morphogenesis (Figure 1a, 1b)(53) .

Figure 1

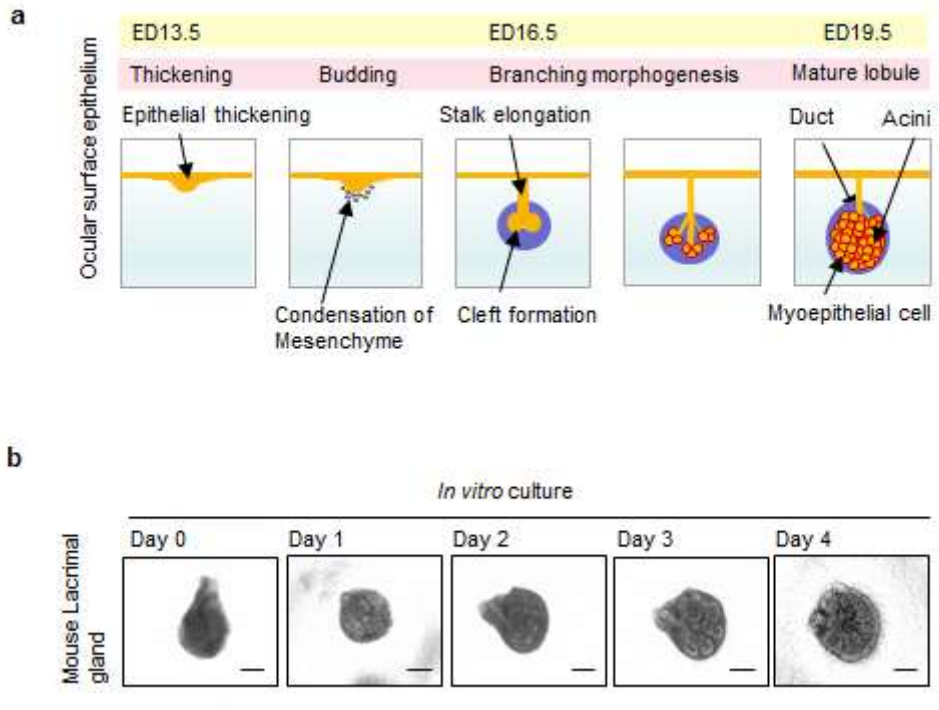


Figure 1. The organogenesis of the mouse lacrimal glands

a. The figure illustrates the lacrimal gland development during embryogenesis.

b. Representative images of the mouse lacrimal gland germ organ development *in vitro* culture environment. Scale bars, 100 μm. Modified and reprinted from Hirayama et al.[22]

Mouse lacrimal gland development reportedly starts at embryonic day (ED) 13.5 through a tubular invagination of the ocular conjunctival epithelium at the temporal region of the eye(54). After the invagination and elongation of the epithelium, the lacrimal gland germ expands the peripheral mesenchyme on ED 16.5, and rapidly proliferates for branching to make a lobule(55). The fundamental structure of the lacrimal glands are achieved by ED 19, and then, the production of tear contents such as proteins and lipids starts by postnatal day 7, at the time of eye opening. In mice, the harderian glands secret tear lipids for the protection of ocular surface(56). It develops on the nasal region of the conjunctival epithelium at ED 16 through a developmental branching process similar to that of the lacrimal glands. Mature harderian glands lie behind the eye(57, 58). In humans, the harderian glands reportedly degrade(58, 59). The delicate developmental biological series, such as branching morphogenesis, contribute to the modulation of the lacrimal gland development.

Cell injection therapy using tissue stem/progenitor cells in adult lacrimal glands

New therapeutic ideas for restoration of the lacrimal gland functions, including functional replacement therapy using accessory salivary glands(60) and regenerative medicine(61), have been developed. Currently, it has been reported that the lacrimal glands, as well as other secretory glands including salivary glands, the pancreas and mammary glands, have a capacity of self-renewal after tissue injury, which is mediated by tissue stem cells in adult tissue(62-64). The tissue stem cells derived from adult tissue has been one of the candidates as an available cell source for cell injection therapy to regenerate organ functions after partial injury(65, 66). The repair process that can proliferate and reconstruct a secretory gland structure after inflammation and cell death in the excretory duct-ligated salivary glands has been shown when the ligation is released(67-69). In the lacrimal glands, a possibility of an existence of tissue stem/progenitor cells in adult tissue has been reportedly implied. The murine lacrimal gland injury model induced by interleukin-1 has described the recovery process mediated by tissue stem or progenitor cells expressing nestin and Ki67 and mesenchymal cells after injury(70-72). A small population of Krt15 expressing epithelial cells existing in the basal cells in the inter-canulated duct in mature lacrimal glands, where reportedly secretory gland tissue stem cells locate, has newly founded(73). An epithelial cell progenitor population with c-kit+ dim/EpCAM+ /Sca1 - /CD34 - /CD45 -, which have been isolated from mouse lacrimal glands, express stem cell markers and the epithelial cell lineage marker, Runx1(74, 75). The epithelial progenitor cell populations can regenerate tubule-alveolar structure *in vivo*, and partially restore the lacrimal gland function by cell injection therapy. In humans, cells expressing stem cell markers including c-kit, ABCG2 and ALDH1 have been identified in the lacrimal glands(76, 77). Evidence of tissue regeneration using adult tissue stem/progenitor cells has been integrated to realise functional restoration of the lacrimal glands in clinical use(78, 79).

Fully functional lacrimal gland organ regeneration in vivo using the organ germ method

a. Emerging a novel 3D cell manipulation method; the organ germ method and its application to the lacrimal gland regeneration

For a regeneration of functional bioengineered organs, the development of tissue-engineering technology to manipulate multiple kinds of cells to reconstruct 3D organs has been

required(21). The organ germ method, a novel strategy to make bioengineered 3D organs through a reproduction of the developmental process during ontogenesis, has been proposed for the functional organ replacement and complete restoration of damaged organs(80). *In vitro* construct of cell compartmentalization of epithelial and mesenchymal cells at a high cell density, manipulated by the organ germ method, could develop in a type I collagen gel matrix as a bioengineered organ germ(20). These bioengineered organ germs, including teeth and hair follicle germs, successfully developed in vivo by transplantation surgery and achieved physiological functions through cooperation with peripheral tissues at the transplantation site(81-83). Developing the organ germ method expands a possibility to regenerate other ectodermal secretory organs; i.e. the salivary glands and lacrimal glands (Figure 2a, 2b)(23, 50, 84-88). We investigated the utility of the organ germ method to regenerate a bioengineered lacrimal gland and restore its physiological function. The bioengineered lacrimal gland germ, reconstituted of the epithelial and mesenchymal cells from the lacrimal gland germ of an ED 16.5 mouse, could reproduce the process of branching morphogenesis in organ culture *in vitro* (Figure 2c). We could apply the organ germ method to make bioengineered harderian gland germs.

Figure 2

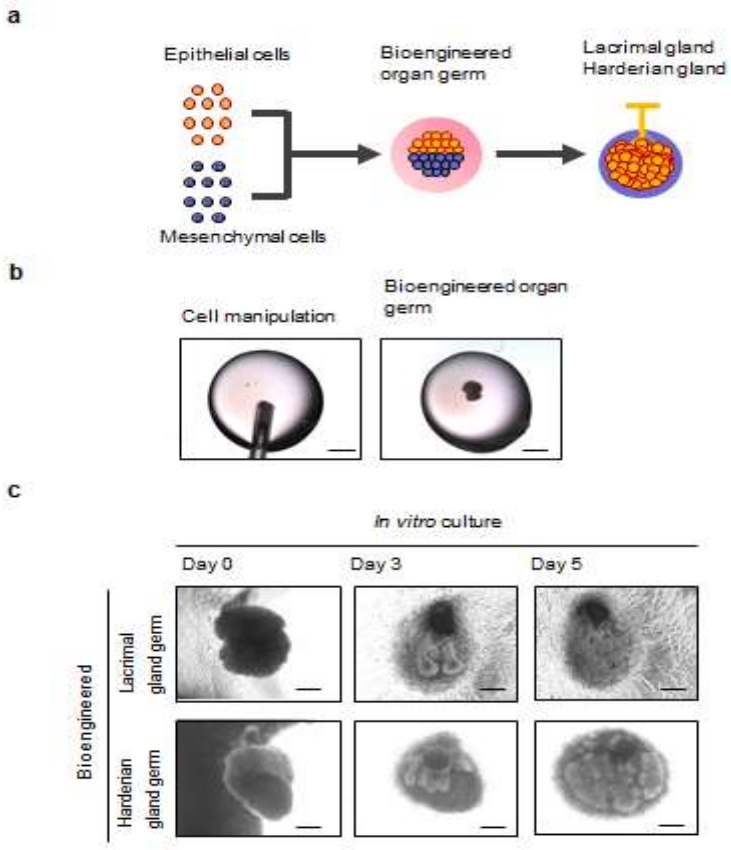


Figure 2. The organ germ method for bioengineered organ regeneration

a. Schematic illustration of the strategy for functional bioengineered lacrimal gland regeneration. The bioengineered lacrimal gland germs are reconstituted from epithelial and mesenchymal cells by the organ germ method.

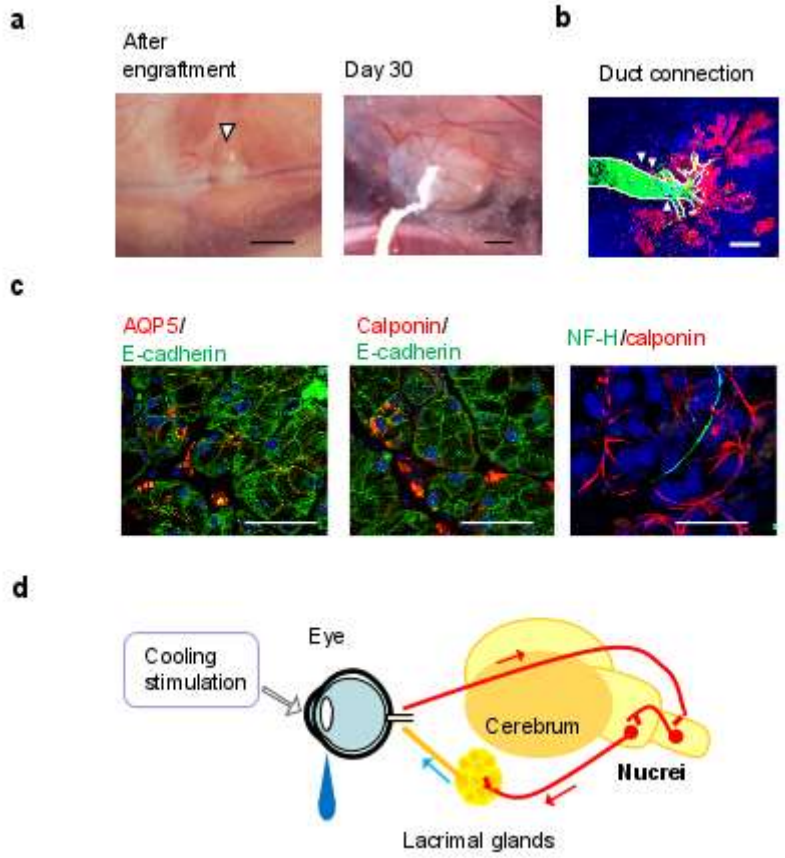
b. Representative images of making the bioengineered organ germs. The embryonic mesenchymal cells is injected into the center of a collagen drop (left panel). The isolated epithelial cells are subsequently injected into the drop adjacent to the mesenchymal cell aggregate (right panel).

c. Images of the development of the bioengineered lacrimal glands. The bioengineered lacrimal glands and harderian glands develops *in vitro* culture. Scale bar, 100 μm. Modified and reprinted from Hirayama et al.[22]

b. Transplantation of a bioengineered lacrimal gland organ germ

A duct connection between the bioengineered lacrimal gland and the host lacrimal excretory duct of the host mouse is essential to drain tears to the ocular surface. The bioengineered lacrimal gland developed the excretory duct structure from the epithelial part, and successfully connected to the host lacrimal excretory duct *in vivo* by our thread-guided transplantation technique in an extra-orbital lacrimal gland removed mouse model (Figure 3a, 3b)(81). The bioengineered lacrimal gland and the bioengineered harderian gland reproduced the developmental process after the engraftment and achieved the proper secretory gland structure, such as acini, duct, myoepithelial cells and peripheral nerve fibres (Figure 3c). The bioengineered lacrimal gland can develop their secretory gland structure and integrate to peripheral tissues after transplantation.

Figure 3



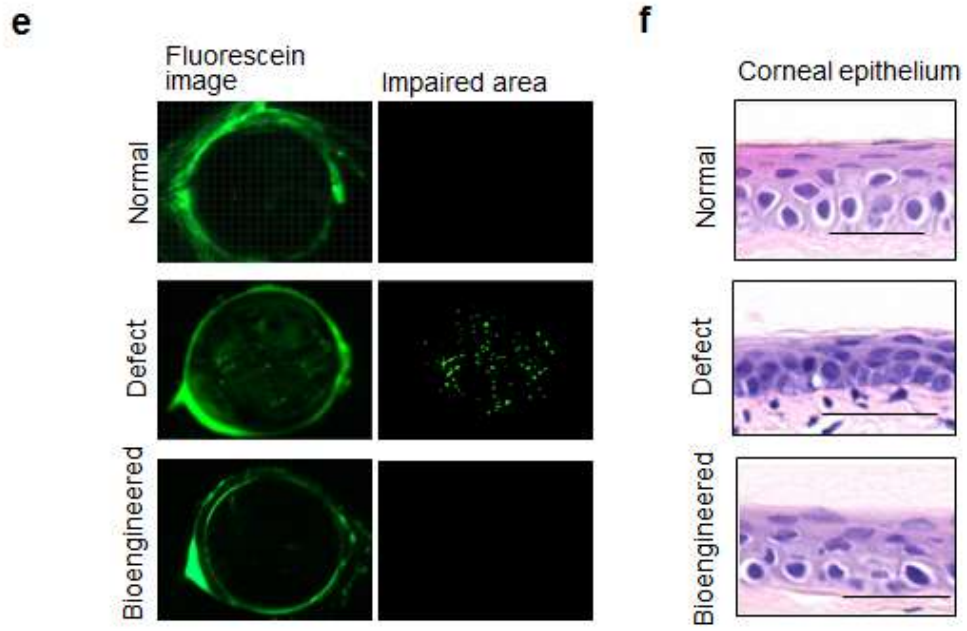


Figure 3. Transplantation and function of the bioengineered lacrimal glands.

a. The bioengineered lacrimal gland germ after transplanting into a mouse with the extra-orbital lacrimal gland removed (arrowhead) (left panel; Scale bar, 1 mm) and at 30 days after transplantation (right; Scale bar, 500 μm). Modified and reprinted from Hirayama et al.[22]

b. The duct connection between the bioengineered lacrimal gland (red) and recipient lacrimal excretory duct (arrowheads). FITC-gelatin (green), which was injected from the host lacrimal excretory duct, could reach to the bioengineered lacrimal gland. Scale bars, 100 μm. Modified and reprinted from Hirayama et al.[22]

c. Immunohistochemical analysis of the bioengineered lacrimal gland after transplantation. The bioengineered lacrimal gland achieved correct secretory gland structure with aquaporin-5 (red, left), calponin (red, center) and neurofilament-H (NF-H, green, right). Scale bars, 50 μm. Modified and reprinted from Hirayama et al.[22]

d. The figure illustrates the neural tear reflex loop. Cooling stimulation on the ocular surface leads tear secretion from the lacrimal gland through the central nervous system.

e. The images of the corneal surface of a normal lacrimal gland (upper), a lacrimal gland-defect mouse (center) and a bioengineered lacrimal gland-engrafted mouse (lower). The punctate staining area by fluorescein showed impaired area on corneal surface. Scale bar, 1 mm. Modified and reprinted from Hirayama et al.[22]

f. Representative images of the corneal epithelium. A normal mouse (upper), lacrimal gland defective mouse (center) and bioengineered lacrimal gland transplanted mouse

(lower) are shown. Scale bars, 25 μm. Modified and reprinted from Hirayama et al.[22]

c. Tear secretion and ocular surface protection effect of the bioengineered lacrimal gland

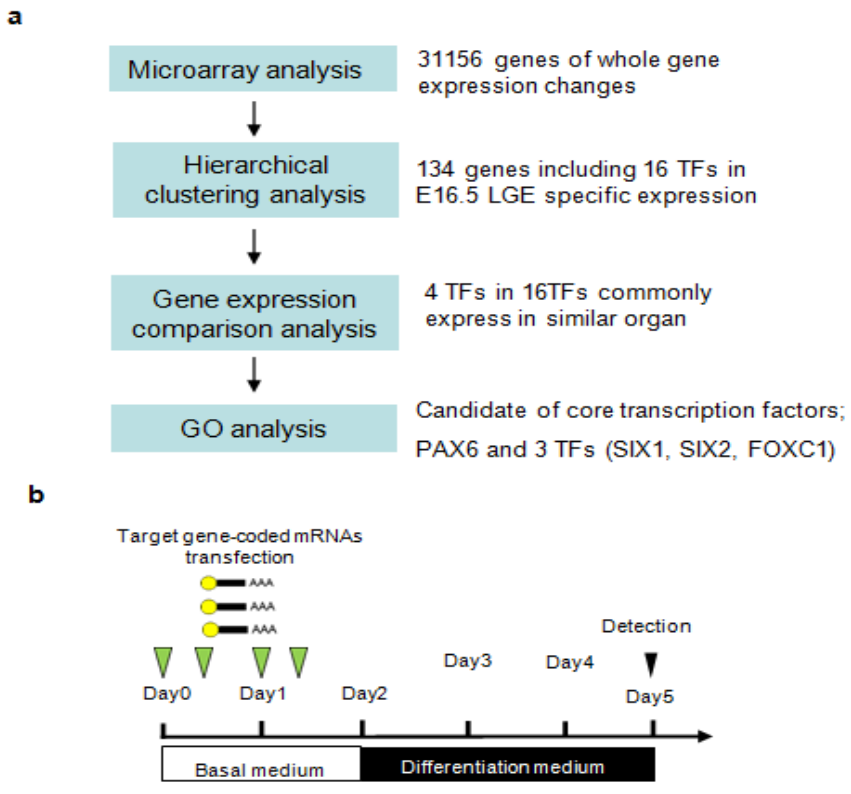
The Advantage of functional replacement using bioengineered lacrimal glands has been reconstructing more physiologically functions of the lacrimal glands(53). First, a coordination with neural functions, such as a lacrimation reflex, is critical to protect the ocular surface (Figure 3d)(89-91). We demonstrated that the bioengineered lacrimal glands could produce tears in response to a cooling stimulation on ocular surface via host neural systems(92, 93). The bioengineered lacrimal glands can work in corporation with host neural functions. Second, tears contain tear proteins, such as lactoferrin and lipids, which are important for physiological tear function including tear stability, wound repair and anti-bacterial effects(29). In clinical therapy, autologous serum eye drop and albumin eye drop have been applied to substitute tear protein function for severe lacrimal gland dysfunction (94-96). We clarified that tears from the bioengineered lacrimal glands contained major tear proteins such as lactoferrin. These results indicated that the bioengineered lacrimal gland could reconstruct more physiologically lacrimal gland function. The goal of the bioengineered lacrimal gland replacement therapy is to protect the ocular surface from DED. Punctate keratitis diagnosed by fluorescein staining of the ocular surface(97, 98), corneal epithelial thinning, and corneal stromal fibroblast activation(99, 100) were observed in our DED model mouse with an extra-orbital lacrimal gland defect. We demonstrated that these pathological changes were prevented by the transplantation of the bioengineered lacrimal gland (Figure 3e, 3f). This study proved that the bioengineered lacrimal gland could develop in vivo and achieve enough function to sustain a healthy ocular surface in DED.

Induction of lacrimal gland cells from human pluripotent stem cells

Technologies for organ regeneration using available cell sources including pluripotent stem cells have been expected to realize the future clinical application of the bioengineered organ replacement therapy. In ophthalmology, it has been developed us a differentiation method of retina cells and corneal epithelium cells using specifically modified culture environment with chemical / biological reagents(101, 102). The iPSC-derived retinal pigment epithelial cell sheet has been engrafted in

clinical therapy to restore retina function in a blind patient(14). A novel strategy based on direct cell conversion procedure has been proposed recently to induct lacrimal gland epithelial cell lineage from human pluripotent stem cells(103). Embryonic lacrimal gland epithelial cells, which have organ-inductive potential for bioengineered organs, are attractive candidate to be inducted from stem cells for LG organ regeneration(73). Whole gene expression analysis of mouse embryonic lacrimal gland tissues and adult lacrimal glands using systems medicine approach has identified a set of genes, Pax6, Foxc1, Six1 and Six2, as core transcription factors specifically enriched in ED16.5 mouse lacrimal gland epithelium (Figure 4a). Overexpression of the set of genes of PAX6, FOXC1, and SIX1, using synthetically modified mRNAs has demonstrated rapid and efficient induction of lacrimal gland epithelial like cells with characteristic morphological changes and expression of lacrimal gland epithelial cell markers and branching morphogenesis markers, including KRT15, BARX2, AQP5, and LTF, from human embryonic stem cells (Figure 4b and 4c, Table 1). This study is the first report suggesting a possibility to differentiate lacrimal gland cells from human pluripotent stem cells.

Figure 4



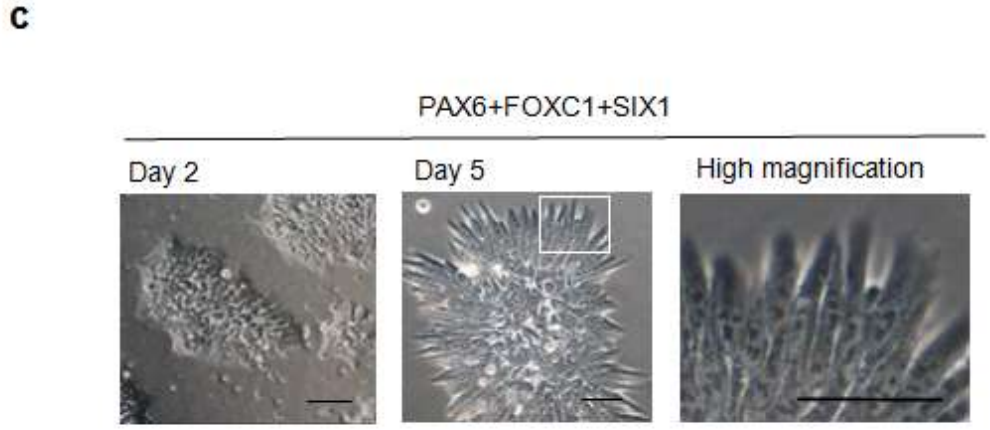


Figure 4. Direct conversion method of the lacrimal gland epithelium from human ES cells using synthetic mRNA transfection.

a. The figure illustrates a systems biology approach to find the lacrimal gland enriched transcription factors.
b. A brief protocol of the synthetic mRNA transfection for the differentiation of the lacrimal gland epithelium like cells from human ES cells.
c. Photographs of the differentiated cells after the induction using 3 transcription factors encoded mRNAs. Scale bar, 100um. Modified and reprinted from Hirayama et al.[103]

Conclusions and future directions

The interest to regenerate lacrimal gland function for DED using regenerative medicine has been expanded recently. Various strategies of the lacrimal gland regeneration has developed from cell injection therapy using tissue stem cells to organ induction using pluripotent stem cells. Bioengineered lacrimal gland germs exhibit 3D histological structure and proper physiological functions, including tear production in response to neural stimuli and ocular surface protection. Attempts to differentiation of lacrimal gland cells and / or lacrimal gland organs from stem cells would be one of the next goals of lacrimal gland regenerative medicine toward clinical application. Studies to develop tissue-engineering technology for 3D organ induction from stem cells will be requested to realize the future practical clinical applications of organ replacement regenerative therapy. Differentiation of lacrimal gland cells in 3D culture environment is one of the attractive concepts to make 3D lacrimal gland organs from stem cells. Our direct conversion technique of the lacrimal gland epithelial like cells by overexpression of core transcriptional factors using synthetic modified mRNAs

is a proof-of-concept for lacrimal gland cell induction from human pluripotent stem cells, which can express markers for organ inductive potential including branching morphogenesis markers. In addition, the establishment of cell fate conversion procedure using synthetic mRNAs provides substantial advances to overcome problems of genomic integrity in differentiated cells with vector-based genetic modification(104). Whole gene expression analysis in human lacrimal glands has been reported to find enriched, specific genes in the lacrimal glands(105). It has been expected that the bioengineered organ regenerative therapy become an available therapeutic strategy to cure DED as the next-generation regenerative therapy.

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References

1. Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H, et al. Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nature medicine. 2006;12(4):459-65. Epub 2006/04/04.
2. Okano H, Yamanaka S. iPS cell technologies: significance and applications to CNS regeneration and disease. Molecular brain. 2014;7:22. Epub 2014/04/02.
3. Sekine H, Shimizu T, Yang J, Kobayashi E, Okano T. Pulsatile myocardial tubes fabricated with cell sheet engineering. Circulation. 2006;114(1 Suppl):I87-93. Epub 2006/07/06.
4. Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky N, et al. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. Nature. 2002;418(6893):50-6. Epub 2002/06/22.
5. Appelbaum FR. The current status of hematopoietic cell transplantation. Annual review of medicine. 2003;54:491-512. Epub 2002/11/05.
6. Lindvall O, Kokaia Z. Stem cells for the treatment of neurological disorders. Nature. 2006;441(7097):1094-6. Epub 2006/07/01.
7. Copelan EA. Hematopoietic stem-cell transplantation. The New England journal of medicine. 2006;354(17):1813-26. Epub 2006/04/28.
8. Segers VF, Lee RT. Stem-cell therapy for cardiac disease. Nature. 2008;451(7181):937-42. Epub 2008/02/22.
9. Haraguchi Y, Shimizu T, Matsuura K, Sekine H, Tanaka N, Tadakuma K, et al. Cell sheet technology for cardiac tissue engineering. Methods Mol Biol. 2014;1181:139-55. Epub 2014/07/30.
10. Nishida K, Yamato M, Hayashida Y, Watanabe K, Yamamoto K, Adachi E, et al. Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. The New England journal of medicine. 2004;351(12):1187-96. Epub 2004/09/17.
11. Tsubota K, Satake Y, Kaido M, Shinozaki N, Shimmura S, Bissen-Miyajima H, et al. Treatment of severe ocular-surface disorders with corneal epithelial stem-cell transplantation. The New England journal of medicine. 1999;340(22):1697-703. Epub 1999/06/03.
12. Hirayama M, Satake Y, Higa K, Yamaguchi T, Shimazaki J. Transplantation of cultivated oral mucosal epithelium prepared in fibrin-coated culture dishes. Investigative ophthalmology & visual science.

2012;53(3):1602-9. Epub 2012/02/11.

13. Osakada F, Hirami Y, Takahashi M. Stem cell biology and cell transplantation therapy in the retina. Biotechnology & genetic engineering reviews. 2010;26:297-334. Epub 2010/01/01.

14. Mandai M, Watanabe A, Kurimoto Y, Hirami Y, Morinaga C, Daimon T, et al. Autologous Induced Stem-Cell-Derived Retinal Cells for Macular Degeneration. The New England journal of medicine. 2017;376(11):1038-46. Epub 2017/03/16.

15. Abouna GM. Organ shortage crisis: problems and possible solutions. Transplantation proceedings. 2008;40(1):34-8. Epub 2008/02/12.

16. Oshima M, Inoue K, Nakajima K, Tachikawa T, Yamazaki H, Isobe T, et al. Functional tooth restoration by next-generation bio-hybrid implant as a bio-hybrid artificial organ replacement therapy. Scientific reports. 2014;4:6044. Epub 2014/08/15.

17. Fort A, Fort N, Ricordi C, Stabler CL. Biohybrid devices and encapsulation technologies for engineering a bioartificial pancreas. Cell transplantation. 2008;17(9):997-1003. Epub 2009/01/31.

18. Wolf AV. The artificial kidney. Science. 1952;115(2982):193-9. Epub 1952/02/22.

19. Copeland JG, Smith RG, Arabia FA, Nolan PE, Sethi GK, Tsau PH, et al. Cardiac replacement with a total artificial heart as a bridge to transplantation. The New England journal of medicine. 2004;351(9):859-67. Epub 2004/08/27.

20. Ikeda E, Tsuji T. Growing bioengineered teeth from single cells: potential for dental regenerative medicine. Expert opinion on biological therapy. 2008;8(6):735-44. Epub 2008/05/15.

21. Nakao K, Morita R, Saji Y, Ishida K, Tomita Y, Ogawa M, et al. The development of a bioengineered organ germ method. Nature methods. 2007;4(3):227-30. Epub 2007/02/27.

22. Hirayama M, Ogawa M, Oshima M, Sekine Y, Ishida K, Yamashita K, et al. Functional lacrimal gland regeneration by transplantation of a bioengineered organ germ. Nature communications. 2013;4:2497. Epub 2013/10/03.

23. Ogawa M, Oshima M, Imamura A, Sekine Y, Ishida K, Yamashita K, et al. Functional salivary gland regeneration by transplantation of a bioengineered organ germ. Nature communications. 2013;4:2498. Epub 2013/10/03.

24. Dilly PN. Contribution of the epithelium to the stability of the tear film. Transactions of the ophthalmological societies of the United Kingdom. 1985;104 (Pt 4):381-9. Epub 1985/01/01.

25. Dilly PN. Structure and function of the tear film. Advances in experimental medicine and biology. 1994;350:239-47. Epub 1994/01/01.

26. Schechter JE, Warren DW,

Mircheff AK. A lacrimal gland is a lacrimal gland, but rodent's and rabbit's are not human. The ocular surface. 2010;8(3):111-34. Epub 2010/08/18.

27. Schoenwald RD, Vidvauns S, Wurster DE, Barfknecht CF. The role of tear proteins in tear film stability in the dry eye patient and in the rabbit. Advances in experimental medicine and biology. 1998;438:391-400. Epub 1998/06/23.

28. Holly FJ. Formation and stability of the tear film. International ophthalmology clinics. 1973;13(1):73-96. Epub 1973/01/01.

29. Mishima S. Some Physiological Aspects of the Precorneal Tear Film. Archives of ophthalmology. 1965;73:233-41. Epub 1965/02/01.

30. Broekhuyse RM. Tear lactoferrin: a bacteriostatic and complexing protein. Investigative ophthalmology. 1974;13(7):550-4. Epub 1974/07/01.

31. Seal DV, McGill JI, Mackie IA, Liakos GM, Jacobs P, Goulding NJ. Bacteriology and tear protein profiles of the dry eye. The British journal of ophthalmology. 1986;70(2):122-5. Epub 1986/02/01.

32. Ohashi Y, Dogru M, Tsubota K. Laboratory findings in tear fluid analysis. Clinica chimica acta; international journal of clinical chemistry. 2006;369(1):17-28. Epub 2006/03/07.

33. Shoja MR, Besharati MR. Dry eye after LASIK for myopia: Incidence and risk factors. European journal of ophthalmology. 2007;17(1):1-6. Epub 2007/02/13.

34. De Paiva CS, Chen Z, Koch DD, Hamill MB, Manuel FK, Hassan SS, et al. The incidence and risk factors for developing dry eye after myopic LASIK. American journal of ophthalmology. 2006;141(3):438-45. Epub 2006/02/24.

35. Paulsen AJ, Cruickshanks KJ, Fischer ME, Huang GH, Klein BE, Klein R, et al. Dry eye in the beaver dam offspring study: prevalence, risk factors, and health-related quality of life. American journal of ophthalmology. 2014;157(4):799-806. Epub 2014/01/07.

36. Galor A, Kumar N, Feuer W, Lee DJ. Environmental factors affect the risk of dry eye syndrome in a United States veteran population. Ophthalmology. 2014;121(4):972-3. Epub 2014/02/25.

37. Galor A, Feuer W, Lee DJ, Florez H, Carter D, Pouyeh B, et al. Prevalence and risk factors of dry eye syndrome in a United States veterans affairs population. American journal of ophthalmology. 2011;152(3):377-84 e2. Epub 2011/06/21.

38. Ahn JM, Lee SH, Rim TH, Park RJ, Yang HS, Kim TI, et al. Prevalence of and risk factors associated with dry eye: the Korea National Health and Nutrition Examination Survey 2010-2011. American journal of ophthalmology. 2014;158(6):1205-14 e7. Epub

2014/08/26.
39. Uchino M, Nishiwaki Y, Michikawa T, Shirakawa K, Kuwahara E, Yamada M, et al. Prevalence and risk factors of dry eye disease in Japan: Koumi study. Ophthalmology. 2011;118(12):2361-7. Epub 2011/09/06.
40. Lee AJ, Lee J, Saw SM, Gazzard G, Koh D, Widjaja D, et al. Prevalence and risk factors associated with dry eye symptoms: a population based study in Indonesia. The British journal of ophthalmology. 2002;86(12):1347-51. Epub 2002/11/26.
41. Uchino M, Yokoi N, Uchino Y, Dogru M, Kawashima M, Komuro A, et al. Prevalence of dry eye disease and its risk factors in visual display terminal users: the Osaka study. American journal of ophthalmology. 2013;156(4):759-66. Epub 2013/07/31.
42. Kaido M, Ishida R, Dogru M, Tsubota K. Visual function changes after punctal occlusion with the treatment of short BUT type of dry eye. Cornea. 2012;31(9):1009-13. Epub 2012/02/24.
43. Alves M, Fonseca EC, Alves MF, Malki LT, Arruda GV, Reinach PS, et al. Dry eye disease treatment: a systematic review of published trials and a critical appraisal of therapeutic strategies. The ocular surface. 2013;11(3):181-92. Epub 2013/07/11.
44. Mantelli F, Massaro-Giordano M, Macchi I, Lambiase A, Bonini S. The cellular mechanisms of dry eye: from pathogenesis to treatment. Journal of cellular physiology. 2013;228(12):2253-6. Epub 2013/05/23.
45. Kaido M, Dogru M, Yamada M, Sotozono C, Kinoshita S, Shimazaki J, et al. Functional visual acuity in Stevens-Johnson syndrome. American journal of ophthalmology. 2006;142(6):917-22. Epub 2006/12/13.
46. Tsubota K, Satake Y, Shimazaki J. Treatment of severe dry eye. Lancet. 1996;348(9020):123. Epub 1996/07/13.
47. Yokoi N, Bron AJ, Georgiev GA. The precorneal tear film as a fluid shell: the effect of blinking and saccades on tear film distribution and dynamics. The ocular surface. 2014;12(4):252-66. Epub 2014/10/07.
48. Bron AJ, Tiffany JM, Gouveia SM, Yokoi N, Voon LW. Functional aspects of the tear film lipid layer. Experimental eye research. 2004;78(3):347-60. Epub 2004/04/27.
49. Hirayama M, Murat D, Liu Y, Kojima T, Kawakita T, Tsubota K. Efficacy of a novel moist cool air device in office workers with dry eye disease. Acta ophthalmologica. 2013;91(8):756-62. Epub 2013/01/03.
50. Hirayama M, Oshima M, Tsuji T. Development and prospects of organ replacement regenerative therapy. Cornea. 2013;32 Suppl 1:S13-21. Epub 2013/10/23.
51. Sakai T. Epithelial branching morphogenesis of salivary gland:

exploration of new functional regulators. The journal of medical investigation : JMI. 2009;56 Suppl:234-8. Epub 2009/01/01.

52. Pispa J, Thesleff I. Mechanisms of ectodermal organogenesis. Developmental biology. 2003;262(2):195-205. Epub 2003/10/11.

53. Hirayama M, Kawakita T, Tsubota K, Shimmura S. Challenges and Strategies for Regenerating the Lacrimal Gland. The ocular surface. 2016;14(2):135-43. Epub 2016/01/08.

54. Makarenkova HP, Ito M, Govindarajan V, Faber SC, Sun L, McMahon G, et al. FGF10 is an inducer and Pax6 a competence factor for lacrimal gland development. Development. 2000;127(12):2563-72. Epub 2000/05/24.

55. Dean C, Ito M, Makarenkova HP, Faber SC, Lang RA. Bmp7 regulates branching morphogenesis of the lacrimal gland by promoting mesenchymal proliferation and condensation. Development. 2004;131(17):4155-65. Epub 2004/07/29.

56. Satoh Y, Gesase AP, Habara Y, Ono K, Kanno T. Lipid secretory mechanisms in the mammalian harderian gland. Microscopy research and technique. 1996;34(2):104-10. Epub 1996/06/01.

57. Tsau C, Ito M, Gromova A, Hoffman MP, Meech R, Makarenkova HP. Barx2 and Fgf10 regulate ocular glands branching morphogenesis by controlling extracellular matrix remodeling. Development. 2011;138(15):3307-17. Epub 2011/07/14.

58. Payne AP. The harderian gland: a tercentennial review. Journal of anatomy. 1994;185 (Pt 1):1-49. Epub 1994/08/01.

59. Park SH, Satoh Y, Kumagai S, Seyama Y. Localization of porphyrin in mouse Harderian glands. Archives of histology and cytology. 1996;59(2):189-95. Epub 1996/05/01.

60. Sant' Anna AE, Hazarbassanov RM, de Freitas D, Gomes JA. Minor salivary glands and labial mucous membrane graft in the treatment of severe symblepharon and dry eye in patients with Stevens-Johnson syndrome. The British journal of ophthalmology. 2012;96(2):234-9. Epub 2011/04/30.

61. Zoukhri D. Mechanisms involved in injury and repair of the murine lacrimal gland: role of programmed cell death and mesenchymal stem cells. The ocular surface. 2010;8(2):60-9. Epub 2010/04/30.

62. Walker NI. Ultrastructure of the rat pancreas after experimental duct ligation. I. The role of apoptosis and intraepithelial macrophages in acinar cell deletion. The American journal of pathology. 1987;126(3):439-51. Epub 1987/03/01.

63. Scoggins CR, Meszoely IM, Wada M, Means AL, Yang L, Leach SD. p53-dependent acinar cell apoptosis

triggers epithelial proliferation in duct-ligated murine pancreas. American journal of physiology Gastrointestinal and liver physiology. 2000;279(4):G827-36. Epub 2000/09/27.

64. Walker NI, Bennett RE, Kerr JF. Cell death by apoptosis during involution of the lactating breast in mice and rats. The American journal of anatomy. 1989;185(1):19-32. Epub 1989/05/01.

65. Sumita Y, Liu Y, Khalili S, Maria OM, Xia D, Key S, et al. Bone marrow-derived cells rescue salivary gland function in mice with head and neck irradiation. The international journal of biochemistry & cell biology. 2011;43(1):80-7. Epub 2010/10/12.

66. Lombaert IM, Brunsting JF, Wierenga PK, Faber H, Stokman MA, Kok T, et al. Rescue of salivary gland function after stem cell transplantation in irradiated glands. PloS one. 2008;3(4):e2063. Epub 2008/05/01.

67. Takahashi S, Schoch E, Walker NI. Origin of acinar cell regeneration after atrophy of the rat parotid induced by duct obstruction. International journal of experimental pathology. 1998;79(5):293-301. Epub 1999/04/08.

68. Cummins M, Dardick I, Brown D, Burford-Mason A. Obstructive sialadenitis: a rat model. The Journal of otolaryngology. 1994;23(1):50-6. Epub 1994/02/01.

69. Feng J, van der Zwaag M, Stokman MA, van Os R, Coppes RP. Isolation and characterization of human salivary gland cells for stem cell transplantation to reduce radiation-induced hyposalivation. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology. 2009;92(3):466-71. Epub 2009/07/25.

70. You S, Kublin CL, Avidan O, Miyasaki D, Zoukhri D. Isolation and propagation of mesenchymal stem cells from the lacrimal gland. Investigative ophthalmology & visual science. 2011;52(5):2087-94. Epub 2010/12/24.

71. Zoukhri D, Fix A, Alroy J, Kublin CL. Mechanisms of murine lacrimal gland repair after experimentally induced inflammation. Investigative ophthalmology & visual science. 2008;49(10):4399-406. Epub 2008/07/01.

72. You S, Avidan O, Tariq A, Ahluwalia I, Stark PC, Kublin CL, et al. Role of epithelial-mesenchymal transition in repair of the lacrimal gland after experimentally induced injury. Investigative ophthalmology & visual science. 2012;53(1):126-35. Epub 2011/10/26.

73. Hirayama M, Liu Y, Kawakita T, Shimmura S, Tsubota K. Cytokeratin expression in mouse lacrimal gland germ epithelium. Experimental eye research. 2016;146:54-9. Epub 2015/12/15.

74. Gromova A, Voronov DA, Yoshida M, Thotakura S, Meech R, Dartt DA, et al. Lacrimal Gland Repair Using

Progenitor Cells. Stem cells translational medicine. 2017;6(1):88-98. Epub 2017/02/09.

75. Voronov D, Gromova A, Liu D, Zoukhri D, Medvinsky A, Meech R, et al. Transcription factors Runx1 to 3 are expressed in the lacrimal gland epithelium and are involved in regulation of gland morphogenesis and regeneration. Investigative ophthalmology & visual science. 2013;54(5):3115-25. Epub 2013/03/28.

76. Tiwari S, Ali MJ, Vemuganti GK. Human lacrimal gland regeneration: Perspectives and review of literature. Saudi journal of ophthalmology : official journal of the Saudi Ophthalmological Society. 2014;28(1):12-8. Epub 2014/02/15.

77. Tiwari S, Ali MJ, Balla MM, Naik MN, Honavar SG, Reddy VA, et al. Establishing human lacrimal gland cultures with secretory function. PloS one. 2012;7(1):e29458. Epub 2012/01/19.

78. Kagami H, Wang S, Hai B. Restoring the function of salivary glands. Oral diseases. 2008;14(1):15-24. Epub 2008/01/05.

79. Purnell B. New release: the complete guide to organ repair. Introduction. Science. 2008;322(5907):1489. Epub 2008/12/06.

80. Ikeda E, Morita R, Nakao K, Ishida K, Nakamura T, Takano-Yamamoto T, et al. Fully functional bioengineered tooth replacement as an organ replacement therapy. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(32):13475-80. Epub 2009/08/12.

81. Toyoshima KE, Asakawa K, Ishibashi N, Toki H, Ogawa M, Hasegawa T, et al. Fully functional hair follicle regeneration through the rearrangement of stem cells and their niches. Nature communications. 2012;3:784. Epub 2012/04/19.

82. Oshima M, Mizuno M, Imamura A, Ogawa M, Yasukawa M, Yamazaki H, et al. Functional tooth regeneration using a bioengineered tooth unit as a mature organ replacement regenerative therapy. PloS one. 2011;6(7):e21531. Epub 2011/07/19.

83. Asakawa K, Toyoshima KE, Ishibashi N, Tobe H, Iwadate A, Kanayama T, et al. Hair organ regeneration via the bioengineered hair follicular unit transplantation. Scientific reports. 2012;2:424. Epub 2012/05/31.

84. Ogawa M, Tsuji T. Functional salivary gland regeneration as the next generation of organ replacement regenerative therapy. Odontology / the Society of the Nippon Dental University. 2015;103(3):248-57. Epub 2015/07/16.

85. Ogawa M, Tsuji T. Functional Salivary Gland Regeneration. Methods Mol Biol. 2017;1597:135-51. Epub 2017/04/01.

86. Hirayama M. [Proof of a

Concept for Bioengineered Organ Replacement to Restore Lacrimal Gland Function]. Nippon Ganka Gakkai zasshi. 2015;119(11):799-806. Epub 2015/12/22.

87. Hirayama M, Tsubota K, Tsuji T. Bioengineered Lacrimal Gland Organ Regeneration in Vivo. Journal of functional biomaterials. 2015;6(3):634-49. Epub 2015/08/13.

88. Hirayama M, Tsubota K, Tsuji T. Generation of a Bioengineered Lacrimal Gland by Using the Organ Germ Method. Methods Mol Biol. 2017;1597:153-65. Epub 2017/04/01.

89. Rock JR, Hogan BL. Developmental biology. Branching takes nerve. Science. 2010;329(5999):1610-1. Epub 2010/10/12.

90. Kumar A, Brockes JP. Nerve dependence in tissue, organ, and appendage regeneration. Trends in neurosciences. 2012;35(11):691-9. Epub 2012/09/20.

91. Dartt DA, Hodges RR, Zoukhri D. Signal transduction pathways activated by cholinergic and alpha 1-adrenergic agonists in the lacrimal gland. Advances in experimental medicine and biology. 1998;438:113-21. Epub 1998/06/23.

92. Robbins A, Kurose M, Winterson BJ, Meng ID. Menthol activation of corneal cool cells induces TRPM8-mediated lacrimation but not nociceptive responses in rodents. Investigative ophthalmology & visual science. 2012;53(11):7034-42. Epub 2012/09/07.

93. Parra A, Madrid R, Echevarria D, del Olmo S, Morenilla-Palao C, Acosta MC, et al. Ocular surface wetness is regulated by TRPM8-dependent cold thermoreceptors of the cornea. Nature medicine. 2010;16(12):1396-9. Epub 2010/11/16.

94. Kojima T, Higuchi A, Goto E, Matsumoto Y, Dogru M, Tsubota K. Autologous serum eye drops for the treatment of dry eye diseases. Cornea. 2008;27 Suppl 1:S25-30. Epub 2008/10/01.

95. Tsubota K, Goto E, Fujita H, Ono M, Inoue H, Saito I, et al. Treatment of dry eye by autologous serum application in Sjogren's syndrome. The British journal of ophthalmology. 1999;83(4):390-5. Epub 1999/08/06.

96. Shimmura S, Ueno R, Matsumoto Y, Goto E, Higuchi A, Shimazaki J, et al. Albumin as a tear supplement in the treatment of severe dry eye. The British journal of ophthalmology. 2003;87(10):1279-83. Epub 2003/09/26.

97. Ward KW. Superficial punctate fluorescein staining of the ocular surface. Optometry and vision science : official publication of the American Academy of Optometry. 2008;85(1):8-16. Epub 2008/01/05.

98. Korb DR, Herman JP, Finnemore VM, Exford JM, Blackie CA. An evaluation of the efficacy of

fluorescein, rose bengal, lissamine green, and a new dye mixture for ocular surface staining. Eye & contact lens. 2008;34(1):61-4. Epub 2008/01/09.

99. Xiong C, Chen D, Liu J, Liu B, Li N, Zhou Y, et al. A rabbit dry eye model induced by topical medication of a preservative benzalkonium chloride. Investigative ophthalmology & visual science. 2008;49(5):1850-6. Epub 2008/04/26.

100. Nyunt AK, Ishida Y, Yu Y, Shimada S. Topical apolipoprotein A-1 may have a beneficial effect on the corneal epithelium in a mouse model of dry eye: a pilot study. Eye & contact lens. 2008;34(5):287-92. Epub 2008/09/10.

101. Eiraku M, Takata N, Ishibashi H, Kawada M, Sakakura E, Okuda S, et al. Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature. 2011;472(7341):51-6. Epub 2011/04/09.

102. Hayashi R, Ishikawa Y, Sasamoto Y, Katori R, Nomura N, Ichikawa T, et al. Co-ordinated ocular development from human iPS cells and recovery of corneal function. Nature. 2016;531(7594):376-80. Epub 2016/03/10.

103. Hirayama M, Ko SBH, Kawakita T, Akiyama T, Goparaju SK, Soma A, et al. Identification of transcription factors that promote the differentiation of human pluripotent stem cells into lacrimal gland epithelium-like cells. npj Aging and Mechanisms of Disease. 2017;3(1).

104. Li M, Sancho-Martinez I, Izpisua Belmonte JC. Cell fate conversion by mRNA. Stem cell research & therapy. 2011;2(1):5. Epub 2011/02/25.

105. Aakalu VK, Parameswaran S, Maienschein-Cline M, Bahroos N, Shah D, Ali M, et al. Human Lacrimal Gland Gene Expression. PloS one. 2017;12(1):e0169346. Epub 2017/01/13.

Table 1. Contribution of transcription factors for the differentiation of lacrimal gland epithelium like cells. Modified and reprinted from Hirayama et al.[103]

Combination of TF	Morphological change (Day 5)	Expression of PAX6, BARX2, KRT15, AQP5, and LTF (Day 5)
None	Spindle	No
PAX6	Spindle	No
FOXC1	Smooth	No
SIX1	Smooth	No
SIX2	Round	BARX2*
PAX6+FOXC1	Elongate	BARX2, KRT15, AQP5
PAX6+SIX1	Smooth / spindle	No
PAX6+SIX2	Round / spindle	No
FOXC1+SIX1	Spindle	No
FOXC1+SIX2	Elongate	BARX2, AQP5*
PAX6+FOXC1+SIX1	Elongate	PAX6, BARX2, KRT15, AQP5, LTF
PAX6+FOXC1+SIX2	Spindle / elongate	BARX2*

The table was reprinted and modified from Hirayama et al (103).

