

Regeneration of Lacrimal Gland Function to Maintain the Health of the Ocular Surface

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Dry eye is a multifactorial disease that is one of the most common diseases worldwide. A major cause of dry eye is the deficiency of aqueous tears, which are mainly secreted from the lacrimal gland. The lacrimal gland plays an important role in maintaining the health of the ocular surface and protecting it from environmental exposure. Dry eye can lead to ocular irritation and discomfort, as well as severe ocular surface diseases (e.g., ocular infections, corneal ulcerations, and ocular surface keratinization). These severe diseases can be induced by an atrophied or injured lacrimal gland; current therapies cannot completely restore the function of lacrimal gland. To develop more definitive therapies, it is important to understand lacrimal gland biology at the molecular level, as well as inflammatory processes affecting the function of the gland. During severe inflammation, the tissue structure of the lacrimal gland is destroyed; it is replaced by scar formation during wound healing, which leads to lacrimal gland dysfunction. Using an animal model of lacrimal gland dysfunction, many investigators have studied molecular mechanisms of inflammation in the lacrimal gland. To restore lacrimal gland function, the lacrimal acini must be restored in their niche. Notably, organ transplantation therapies have been reported to restore lacrimal gland function, directly or indirectly, in animal models. In this review, we describe the current understanding of the lacrimal gland as the therapeutic target for dry eye diseases, as well as recent advances in the field of lacrimal gland cell-based therapy to treat severe dry eye diseases.

Keywords: lacrimal gland, lacrimal functional unit, progenitor cell, regeneration

A healthy ocular surface is covered by a stable tear film, which is a barrier that protects the ocular surface from the external environment. The tear film maintains a wet ocular surface, protects the ocular surface epithelium from exposure and physical damage, and maintains epithelial homeostasis. The main lacrimal glands (LGs) secrete a large fraction of the aqueous component of tears. LGs are also responsible for basal and reflex tearing, which is stimulated by ocular surface sensation. However, the extent of the contribution of accessory LGs to tear secretion on the ocular surface remains unclear.

Aqueous tears are necessary to maintain ocular surface stem cells within their niche; thus, healthy LGs prevent corneal and conjunctival epithelial stem cell deficiency. The loss of aqueous tears can lead to corneal epithelial injury, which is associated with severe inflammation. This can stimulate corneal stem cells to proliferate as transit-amplifying cells (TACs). TACs differentiate into terminally differentiated cells, which can restore the damaged corneal epithelium.¹ Continuous corneal epithelial damage promotes an abnormal cellular cycle, which may result in a corneal epithelial stem cell deficiency. Moreover, the complete loss of aqueous tears may induce corneal epithelial stem cell deficiency, as well as ocular surface epithelial keratinization and/or keratinocyte invasion from the skin.² Therefore, a stable tear film guarantees a stable ocular surface microenvironment.

The tear film consists of three layers: the inner mucin layer, the middle aqueous layer, and the outer lipid layer. An insufficient aqueous layer is one of the main causes of dry eye disease, as the proper balance of these layers is important

to maintain a healthy ocular surface. Although all three components are important, the worst cases of dry eye result from the complete loss of the aqueous layer, which is induced by LG dysfunction. Such conditions destroy ocular surface epithelial homeostasis and lead to the shortening or loss of the conjunctival sac, as well as the keratinization of the ocular surface.² When LG function is partially damaged, punctal occlusion or punctal plug therapy can be performed because the ocular surface requires sufficient aqueous tears to recover epithelial homeostasis. However, complete LG dysfunction cannot be treated by punctal occlusion; in this case, the regeneration of the LG is necessary. Although LGs serve important physiological functions, research regarding LG function and pathology remains inadequate; thus, there is a limited understanding of the mechanism of LG inflammation and the repair process that occurs after LG damage.³ Surgical transplantation of autologous minor salivary glands to a subconjunctival location has been reported in cases of severe ocular surface damage associated with LG dysfunction; it successfully maintained a wet ocular surface.⁴

This review discusses the importance of LGs for maintenance of the ocular surface. Recent advances in the understanding of LG pathophysiology during the damage and recovery phases (obtained in mouse model experiments from our laboratory, as well as from published research) are also presented here. We review the evidence regarding possible mechanisms of LG inflammation and the potential for regenerative medicine to restore LG function in the future.



MECHANISM OF LACRIMAL GLAND DYSFUNCTION IN ANIMAL MODELS

Many animal models have been reported to study dry eye associated with aqueous tear deficiency, but their causes and mechanisms are straightforward. To understand the mechanism of LG dysfunction, we must consider a variety of causes, including autoimmune reactions, obstruction of the lacrimal punctum, and corneal sensory nerve degeneration. For these specific causes, several simple animal models of LG dysfunction have been established, involving bone marrow transplantation, mechanical obstruction of the LG duct, and inflammatory cytokine injection into the LG; these have revealed mechanisms for the induction of LG inflammation, as well as the regeneration abilities of the LG.^{5,6} In this review, we focus on defined animal models of LG dysfunction: graft-versus-host disease (GVHD) after bone marrow transplantation, LG duct obstruction, and interleukin-1 α (IL-1 α) injection mouse models.

LG INFLAMMATION AND SCAR FORMATION IN GVHD MOUSE MODEL

Dry eye is the most frequent complication of chronic GVHD, occurring in 40% to 76% of patients; its severity is related to the severity of GVHD.⁷ The main cause of dry eye in GVHD is lymphocytic infiltration of the accessory and major LGs, which sometimes leads to fibrosis of the acini and ducts. Aqueous tear production may be obstructed by the accumulation of normal-appearing granules, together with amorphous material and cellular debris, in the acini and ducts; these granules are associated with the expression of several aging markers.⁸

Epithelial-mesenchymal transition (EMT) has been reported to contribute to the mechanism of fibrosis in multiple organs.^{9,10,11} EMT is involved in embryonic development, organ fibrosis, and cancer metastasis.¹² However, organ fibrosis is different from the EMT that occurs during embryonic development because the fibrosis is irreversible. There are two types of epithelial-mesenchymal differentiation: EMT and epithelial mesenchymal transdifferentiation (irreversible EMT [iEMT]). Organ fibrosis consists of scar formation involving activated myofibroblasts that cannot redifferentiate into normal epithelial cells or fibroblasts. iEMT is defined as the complete loss of epithelial cell markers and acquisition of mesenchymal markers with fibrotic morphology, mostly with α -smooth muscle actin (α -SMA) expression. iEMT is triggered by various stimuli, including transforming growth factor- β and the disruption of the basal membrane.^{13,14} These triggers of iEMT are also involved in the pathogenesis of GVHD. iEMT is involved in exocrine gland fibrosis associated with GVHD, which might induce dry eye associated with aqueous tear deficiency.

In human LGs, myoepithelial cells are found between the basal lamina and acinar and ductal cells, and contract the ductal cells to secrete aqueous tears by stimulation.¹⁵ Fibroblast-specific protein-1 (FSP1) expression was observed on the basal side of LG epithelia in GVHD, but not in Sjögren's syndrome. FSP1⁺ spindle-shaped cells were observed around FSP1⁺ myoepithelia, which might indicate that these cells originated from the myoepithelium. To prove this hypothesis, a cell-tracing study was performed. In GVHD LGs, myoepithelia exhibited altered morphology with disrupted basal lamina and activation of matrix metalloproteinase-9. Collectively, the data indicated that myoepithelia in GVHD might acquire the mesenchymal phenotype.¹⁶ Notably, it was unclear whether this differentiation was irreversible. However, no in vivo or in vitro studies have found that myofibroblasts from tissue fibrosis

could differentiate into normal fibroblasts or normal epithelium. Thus, the cell-tracing study more likely detected a case of iEMT, which is similar to the process by which fibrosis occurs in other organs.^{9,10,11}

EMT has been also reported in a mouse model of LG dysfunction after IL-1 α injection.¹⁷ After a single injection of IL-1 α into normal murine LGs, immune cells infiltrated the LGs and acinar epithelial cells lost their secretory function. Subsequently, aqueous tear deficiency was induced.¹⁷ This IL-1 α injection method reduced tear secretion for 7 days; then, tear secretion spontaneously recovered. You et al.¹⁸ reported that the EMT might be related to the mechanism of this LG dysfunction. During the healing of injured LGs after IL-1 α injection, Snail, an important EMT marker and transcription factor, is activated in LG epithelial cells, and is associated with E-cadherin inhibition and vimentin promotion. Snail expression changes occurred earlier than changes in vimentin; notably, Snail is the initiating factor for LG restoration involving the EMT.¹⁸

In this model of the self-repairing of LGs, this EMT is reversible because LG function was recovered after inflammation. This was associated with recovery and reduced scar formation. Therefore, this EMT might involve a different underlying mechanism than that of iEMT in cases of GVHD. After IL-1 α -induced inflammation, vimentin expression increased in LGs, then gradually decreased to the baseline level in healthy LGs.¹⁸ In injured LGs, expression of nestin, which is recognized as a stem cell marker, was also increased, in a manner similar to vimentin. The hypothesis is that epithelial progenitor cells transition into mesenchymal cells via the EMT, then migrate to the damaged areas for tissue repair by subsequently transitioning into acinar or ductal epithelial cells. This hypothesis includes both EMT and mesenchymal-epithelial transition during LG wound healing, which may occur during the tissue development stage. To prove this hypothesis, further research is necessary, including studies regarding whether those mesenchymal cells exhibit potential to differentiate into acinar or ductal epithelial cells. In addition, another study has reported that myoepithelial cells include a nestin⁺ population during the LG repair phase; these myoepithelial cells may be the source of progenitor/stem cells in LGs.¹⁹ Because cells undergoing EMT change their morphologies and phenotypes during wound healing, they are difficult to analyze. However, further analysis might be a key aspect of future research (see Ref. 3 for review).

Ductal ligation in organs has been a conventional and promising method to induce organ dysfunction, including inflammation and fibrosis, by obstructing the fluid pathway.²⁰⁻²³ Ductal ligation and release in animal models have been useful in the analysis of the tissue wound-healing process and recovery of functions. In the salivary gland, the ductal ligation model induced the proliferation of various cell types, including acinar, ductal, and myoepithelial cells, during tissue repair after ligation release.²³ Recently, two studies^{24,25} have been reported regarding LG dysfunction in animal models (murine and rabbit) by ductal ligation. Both studies show that the ligation-injured LGs decrease in size and weight dramatically and exhibit impaired tear secretion with inflammatory cell infiltration. In the rabbit model, following reopening of the ligated LG duct, immunohistochemical analysis demonstrates that a significantly greater number of K14⁺ cells are observed as cell clusters and duct-like structures associated with significantly greater numbers of Δ Np63⁺ cells (Δ Np63 is a stem cell marker in epithelial cells) around duct-like structures, compared with control. Nestin⁺ cells are also increased in a manner similar to that of the K14⁺ cells.²⁵ Furthermore, EMT-associated markers in LG tissues were unregulated after ductal ligation-induced injury and ligation release, in a manner

TABLE. Lacrimal Gland Progenitor Markers

Mouse Model	Cell Origin	Markers for Progenitors	Ref. No.
Normal	Unknown	c-kit ⁺ dim, EpCAM ⁺ , Sca1 ⁻ , CD34 ⁻ , CD45 ⁻	27
IL-1a injection	Myoepithelia	Ki67 ⁺ , α -SMA ⁺ , nestin ⁺	14, 16
Duct ligation	Duct/myoepithelia	Nestin ⁺ , K14 ⁺ , ABCG2 ⁺ , Δ Np63 ⁺ , K15 ⁺	21, 22

Lacrimal gland progenitor markers reported according to animal model; normal adult mouse, lacrimal gland IL-1a injection mouse model, and lacrimal main duct ligation mouse/rabbit model studies are shown.

similar to that of an IL-1a injection model. Cells obtained from injured LGs could proliferate from a single cell and were passaged >12 times. These cells also tested positively for ocular surface stem/progenitor cell-related markers, including K14, ABCG2, Δ Np63, and K15.

Similar to the rabbit model, our study investigated main duct ligation in the mouse model for the first time.²⁴ Because of the small LG size and difficulty in performing the technique required to ligate the main duct of the mouse LG, no one has previously reported this model. Here, both the duct and the artery running alongside it were ligated. Therefore, the mechanisms of tissue damage in this model arose from both tear flow obstruction and ischemic reaction,²⁶ in a manner different from the rabbit model. The ligated artery supplies approximately 60% of blood flow to the LG in the normal state; the remaining blood supply was maintained.²⁴ This model exhibited a proliferative state after inflammation, which is consistent with the previous partial injury model of LGs. Similar to the rabbit model, immunohistochemical analysis showed that nestin⁺ cells were significantly increased in the interstitial tissues with an elongated morphology after surgery. The proliferative cells were analyzed by a proliferative cell marker, PCNA. PCNA⁺ cells existed in the basal layer of the duct, whereas nestin⁺ cells were found in the interstitial tissues. After LG ligation, the number of acini significantly decreased, while the number of ducts significantly increased, consistent with reports of other animal LG injury models. This procedure is easily reproducible; notably, it generated stable injury in LGs over time with inflammation after surgery. After inflammation at the early stage, proliferative changes were observed involving PCNA⁺ cells and nestin⁺ cells.²⁴ Although the specific cell population in LGs that contributes to the regeneration is unclear, this model will be useful for investigations of such cells in LGs.

FUNCTIONAL LG RESTORATION AND THE CONTRIBUTION OF STEM CELLS AFTER LG INJURY

Stem/progenitor cells have been isolated and expanded for cell injection therapy. Such cell injection therapy has been reported to be effective in restoring gland function in mice²⁷ and in humans.²⁸ A recent study has shown the isolation and characterization of epithelial cell progenitors (EPCPs) from LGs of uninjured adult mice.²⁹ The investigators sorted EPCPs by c-kit⁺dim/EpCAM⁺/Sca1⁻/CD34⁻/CD45⁻, which are tentative markers of EPCPs. In healthy organs, stem/progenitor cells are quiescent to maintain their homeostasis; thus, these cells are difficult to identify and trace with specific markers. After epithelial cells were cultivated in monolayers, they began to lose their specific characteristics.³⁰ To maintain their progenitor properties, they required complex cell-cell and cell-matrix interactions, in the presence of growth factors.²⁹ When EPCPs were cultivated with 3D colony formation, rather than monolayers, isolated EPCPs successfully formed acini and ducts (i.e., when grown in reaggregated 3D cultures).

Furthermore, when EPCPs isolated from adult wild-type mouse LGs were transplanted into LGs of TSP-1^{-/-} aqueous tear-deficient mice, LG structure and function was restored. That study provided the first evidence that cellular transplantation of normal adult EPCPs could repair LG dysfunction in animal models.²⁹ In the LG, specific stem cell markers are not yet known, but several possible combinations of markers have been reported (Table).

Stem cell microenvironment or “niche” has been recognized as very important in stem cell maintenance. Umazume et al.³¹ have shown that the expression of epithelial stem/progenitor cell markers is altered in the LGs of those with Sjögren’s syndrome (nonobese diabetic and *MRL-lpr/lpr* mice) during LG inflammation. Mishima et al.³² have sorted cells by the expression of the adenosine triphosphate-binding cassette superfamily G member 2 (ABCG2), a critical factor for the side population (SP) phenotype. They reported that SP cells do not directly regenerate injured LGs themselves, but that the SP cell-specific secretory protein, clusterin, is an essential factor for the recovery of hypofunctioning glands.³² Clusterin inhibits reactive oxygen species-induced cell damage by acting as a radical scavenger, possibly affecting SP cell function. This study also suggested that clusterin is important in the stem/progenitor cell niche involved in the regeneration of LGs. There are several issues that must be clarified to understand stem/progenitor cells in LGs. An important topic in cell therapy is the induction process involved in differentiation to functional LGs, which could lead to a potential breakthrough for LG research.

POSSIBILITY OF LACRIMAL FUNCTIONAL RESTORATION BY REGENERATIVE MEDICINE

LGs exhibit a complex and beautiful architecture of epithelial acini, connecting ductal branching structures, and vascular and neuronal networks from the ocular surface, which function together to produce and secrete aqueous tears. Tears are secreted by the lacrimal functional unit, which is composed of the ocular surface, the main and accessory LGs, lacrimal puncta, and the neural network connecting them; notably, this functional unit is extremely complicated.³³⁻³⁵ The LG complex system maintains the health of the ocular surface tissues, and reflexively regulates aqueous tear homeostasis (Fig. 1).

Generally, simple and small components are better for generating transplantable tissue that involves cells. Thus, a single cell suspension was initially involved in the mouse exocrine dysfunction model.³⁶ However, there are multiple disadvantages of this cell transplantation method: it is easily affected by the microenvironment at the transplanted location; it is difficult to affect the cellular homeostasis of LGs; and it is difficult to understand the timing of cell transplantation.

Recent advances in bioengineered organ technology enable successful generation of bioengineered LGs in vitro, via epithelial-mesenchymal interaction during the developmental stage in a mouse model.^{37,38} Moreover, these bioengineered

Integrated Lacrimal Functional Unit

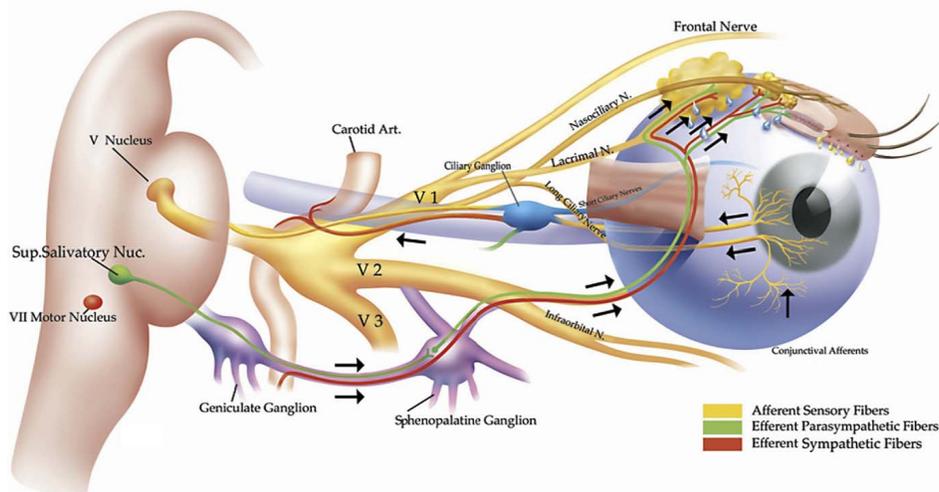


FIGURE 1. The lacrimal functional unit. The lacrimal functional unit has a complex neural network connecting the sensory tissues and secretory glands that maintain homeostasis on the ocular surface. Illustration from Beuerman et al. *The Lacrimal Functional Unit in Dry Eye and Ocular Surface Disorders*. Pflugfelder SC, Beuerman RW, and Stern ME, eds. New York: MarchelDekker, Inc.; 2004:11-39.

LGs, accompanied by ducts, could be transplanted into LG-excision mouse models with functional innervation and vascularization, leading to lacrimal functional unit recovery in a mouse transplant model. Physiologically, the bioengineered LG in vitro might be a better replacement for the main LG in this experiment. However, it is expected that generating a main LG associated with duct formation similar in size to that of humans will be quite difficult to achieve clinically.

When autologous minor salivary glands, obtained from the lip, were transplanted into a subconjunctival area associated with ocular surface reconstruction surgery, the wettability of the ocular surface was improved.^{4,39} Although the underlying mechanisms behind innervation, vascularization, and duct formation are not clear, these clinical cases suggest that small gland transplantation to a subconjunctival area may be

effective in providing aqueous fluid to the ocular surface in cases of severe dry eye associated with aqueous tear deficiency.

Induced pluripotent stem cells (iPS cells) are expected to be used as a cellular source for tissue-specific stem cells.⁴⁰ Overexpression of tissue-specific transcription factors in human pluripotent stem cells can successfully induce the LG epithelial cell phenotype.⁴¹ Using such induced epithelial and mesenchymal cells, small functional LGs may be generated via epithelial-mesenchymal interactions ex vivo. These generated LGs might be similar to an accessory LG rather than the main LG, but when transplanted, may work as functional LGs. Although a great deal of work remains, bioengineered small LGs generated from iPS cells may be an option to treat severe keratinization of the ocular surface in the future (Fig. 2).

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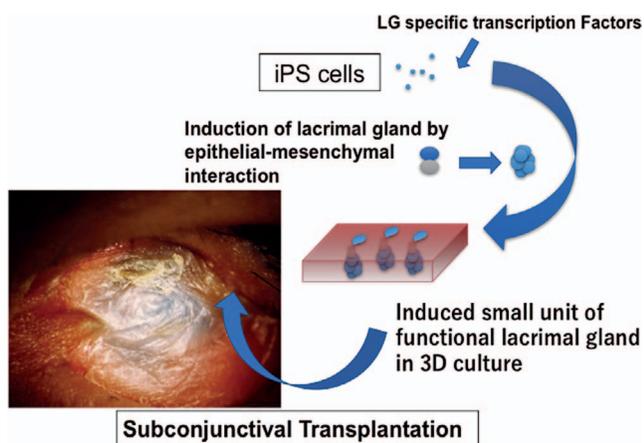


FIGURE 2. Hypothetical schema of the transplantation of accessory LG generated from induced pluripotent stem cells. Theoretically, LG-specific epithelial and mesenchymal cells can be induced by induction of their specific transcription factors. Using those cells, a small functional unit of LGs might be generated ex vivo by an epithelial-mesenchymal interaction from iPS cells with several critical factors in 3D culture. The cultivated functional LGs might be transplantable to the subconjunctival area, where the accessory lacrimal gland is located.

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