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Video Article

Manipulating the Murine Lacrimal Gland

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Abstract

The lacrimal gland (LG) secretes aqueous tears necessary for maintaining the structure and function of the cornea, a transparent tissue essential for vision. In the human a single LG resides in the orbit above the lateral end of each eye delivering tears to the ocular surface through 3 - 5 ducts. The mouse has three pairs of major ocular glands, the most studied of which is the exorbital lacrimal gland (LG) located anterior and ventral to the ear. Similar to other glandular organs, the LG develops through the process of epithelial branching morphogenesis in which a single epithelial bud within a condensed mesenchyme undergoes multiple rounds of bud and duct formation to form an intricate interconnected network of secretory acini and ducts. This elaborate process has been well documented in many other epithelial organs such as the pancreas and salivary gland. However, the LG has been much less explored and the mechanisms controlling morphogenesis are poorly understood. We suspect that this under-representation as a model system is a consequence of the difficulties associated with finding, dissecting and culturing the LG. Thus, here we describe dissection techniques for harvesting embryonic and post-natal LG and methods for *ex vivo* culture of the tissue.

Video Link

The video component of this article can be found at http://www.jove.com/video/51970/

Introduction

The lacrimal gland (LG) is responsible for aqueous tear secretion critical for visual acuity and the health, maintenance, and protection of the cells of the ocular surface. LG dysfunction results in one of the most common and debilitating ocular disorders: aqueous deficient Dry Eye Disease, which is characterized by ocular irritation, light sensitivity and decreased vision[1]. In the human the LG resides in the orbit above the lateral end of the eye where 3 - 5 excretory ducts deposit tears onto the ocular surface. The mouse has three pairs of major ocular glands, the most studied of which is the lacrimal gland (LG) located anterior and ventral to the ear (exorbital) with tears traveling to the eye via a single excretory duct. Similar to other glandular organs, the LG develops through the process of epithelial branching morphogenesis in which a single epithelial bud within a condensed mesenchyme undergoes multiple rounds of bud and duct formation to form an intricate interconnected network of secretory acini and ducts (**Figure 1**)[2]. During development the epithelium becomes vascularized as well as heavily innervated by the parasympathetic nerves of the pterygopalatine ganglion and to a lesser extent by sympathetic nerves from the superior cervical ganglion[3]. Interactions between each of these cells types *i.e.* neuronal, epithelial, endothelial and mesenchymal cells, are essential to the function and maintenance of the adult tissue. However, the underlying molecular mechanisms coordinating LG development and regeneration as well as how inter-cell type communication guides these processes remains unclear.

The advent of embryonic *ex vivo* culture techniques has allowed the identification of developmental and regenerative pathways in multiple branching organs[4]. Culturing *ex vivo* gives the researcher the ability to manipulate the organ (mechanical, genetic or chemical) under defined conditions as well as to characterize organ development and cell-cell interactions in real time. The exorbital LG of the mouse is highly amenable to this technique and recent studies have defined signaling systems that regulate its development[2,5]. However, despite the need to understand molecular cues underpinning LG development and regeneration, it currently remains understudied, likely due to the technical difficulties in isolating the organ. In this paper, we describe how to isolate and perform *ex vivo* culture of the embryonic murine LG to define developmental programs.

Protocol

All animal work was performed under strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, San Francisco.

1. Mouse Embryonic Lacrimal Glands (LG): Harvesting and Microdissection

1. Following procedures approved by the Institutional Animals for Scientific Research Ethics committee, euthanize timed pregnant CD-1 (or transgenic) females with rising CO_2 inhalation, followed by confirmation by cervical dislocation to harvest embryos on the appropriate embryonic day. Designate the day of vaginal plug discovery e0.
   Note: Lacrimal glands (LGs) can be harvested from the e14 single bud stage onwards. However, e16 (5 - 10 buds) embryos give the most consistent results for *ex vivo* culture.
2. Sterilize the ventral side of the mouse using 70% ethanol. Pinch the skin and make an incision at the midline with sterile surgical scissors; cut through the skin and peritoneum to expose the abdominal cavity. Remove the 2 uterine horns by cutting along the mesometrium at the top of each uterine horn and place in cold PBS (Phosphate Buffered Saline) or DMEM F12 media supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin.
3. Using forceps (#5), remove embryos from the amniotic sacs and place into a second sterile Petri dish containing cold PBS. Be careful to not touch the eyes or head area.
4. Place embryo onto a dissecting microscope with a transillumination base. If the embryo is <e17, perform **steps 1.5 - 1.7**. If embryos are >e17, these steps may be helpful but are not essential, therefore proceed to **step 1.8**.
5. Sever the head from the torso just below the lower mandible (step 1, **Figure 2**) using a sterile scalpel (#10 blade). Rotate head so that the mandible is now on the dissecting plate. Use forceps to stabilize the head and a scalpel to remove about ¼ of the dorsal side of brain (step 2, 1st cut, **Figure 2**) – this is especially important once cartilage stiffens around e15.
6. Orient the head so that the scalpel can pierce the nose between the eyes. Make a cut through the center of the head, so that the eyes are now on separate halves (step 2, 2nd cut, **Figure 2**).
7. Using two #5 forceps, take one half of the head and remove excess tissue (see step 3, **Figure 2**). Be sure to orient the head so the LG (located in the lower posterior corner of the eye) is not lost in this removal process. Remove excess cerebral, cartilage, and nasal tissue surrounding and below the eye. Since the gland is barely visible at this point, ensure the eye is properly oriented after excess tissue removal to avoid losing the location of the LG.
8. Carefully grab the skin from the posterior, bottom corner of the eye with both forceps. Remove the skin by carefully pulling open with the forceps to expose the LG. Use additional illumination above and below the tissue to help visualize the LG.
   Note: The LG, distinguishable by its appearance as a bud on a duct within a darker, condensed mesenchyme, should be visible at this point.
9. Carefully begin to dissect the LG and associated mesenchyme (refer to diagram and images) away from the surrounding tissue using forceps, being careful not to grab the LG or its associated duct. Once the gland is free from the surrounding tissue, gently remove the entire gland by gripping the epithelia surrounding the eye at the base of the LG duct. Take care to preserve the mesenchyme surrounding the epithelium, which can be observed as a condensed mesenchyme into which the epithelium invaginates.
   NOTE: Some additional removal of tissue (any small bone fragments, muscle, and connective tissue) may be necessary to avoid signaling/ growth factors from neighboring tissue.
10. For culture, harvest 4 - 5 glands and associated mesenchyme per plate (LGs are plated immediately upon dissection); collect a minimum of 14 glands per 100 µl of RNA lysis buffer for RT-PCR[6].

2. Prepare Plates for *Ex vivo* Culture

This procedure is based on that employed for the developing salivary gland[7,8].

1. Add 200 µl of DMEM F12 (supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin) culture media with 50 µg/ml ascorbic acid and 50 µg/ml holo-transferrin to a glass-bottomed 50 mm diameter microwell dish[7].
   Note: DMEM F12 was chosen as we previously found it to maximize morphogenesis in the embryonic salivary gland.
2. Float a 13 mm polycarbonate membrane filter with 0.1 µm pores on top of media.
3. Optional Step: Dilute 3-D laminin 1:1 with DMEM/F12 to make a final concentration of 3 mg/ml (use 200 µl pipette to gently mix; centrifuge for 1 min if bubbles appear). Add 15 µl of this diluted 3-D laminin to filter.
   Note: This dilution was found to be optimal for maintaining the LG in its 3D state without compromising epithelial branching. However, it is not essential for culture of LG.
4. Place 4 - 5 LG onto filter or into laminin. Culture LGs *ex vivo* for 24 - 48 hr (**Figure 3**) or fix with 4% PFA for 20 min for further analysis by qPCR or immunostaining (**Figure 4**). For qPCR analysis of embryonic glandular tissue, please refer to Rebustini *et al.,* 2011. For immunofluorescent analysis of embryonic glandular tissues please refer to the following citations: Hoffman *et al.,* (2002) and Steinberg *et al*., (2005).

3. Mouse Postnatal and Adult Lacrimal Glands (LG): Dissection

1. Euthanize the postnatal mouse according to appropriate animal ethics committee's standards. For postnatal pups day 8 or younger, euthanize by severing the head with sterile scissors. For postnatal day 8 or older, spray fur with 70% ethanol.
2. To locate the LG, lay mouse lateral side up under a dissecting microscope with illumination from above. Note: The post-natal and adult LG are bordered by the carotid artery, the masseter muscle and the dermis (see **Figure 5**).
3. Using small dissection scissors, make small incision in the epidermis laterally from where the LG should be located.
4. Using forceps, pull open the epidermis toward the ear to expose the LG.
5. Use forceps to gently loosen LG from surrounding tissue.
6. Once the LG is released from surrounding tissue, carefully remove the LG by the duct, by gripping where the duct and the epithelia surrounding the eye connect (**Figure 1**).
7. Place LGs in RNA lysis buffer for RT-PCR, or fix with 4% PFA for 20 min for immunostaining.

Representative Results

The LG develops through the process of epithelial branching morphogenesis. Brightfield images of embryonic LGs dissected at e14, e15, e16, e17, and P2 illustrate this event.

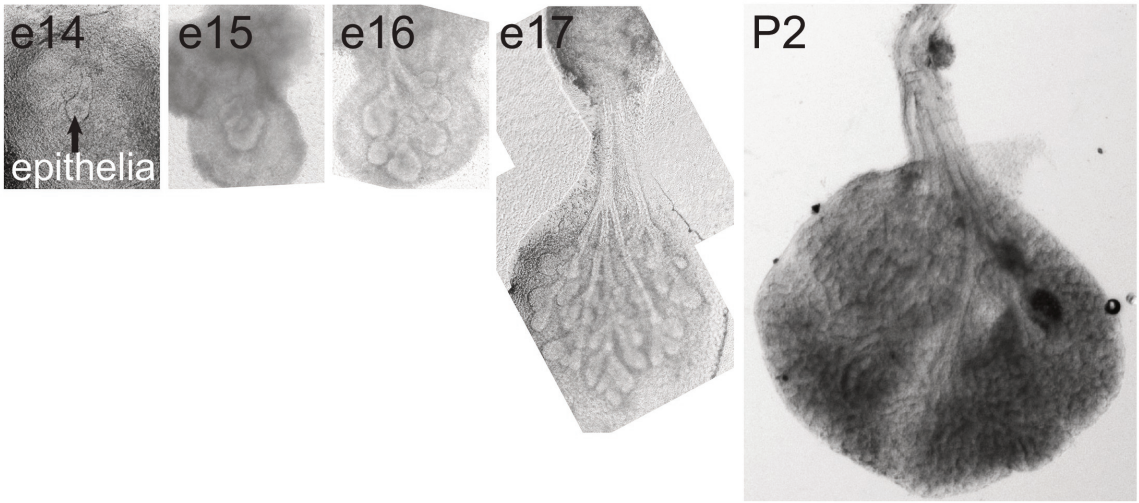

Figure 1. The LG develops through the process of epithelial branching morphogenesis. Brightfield images of embryonic LGs freshly dissected at e14, e15, e16, e17 and P2. Please click here to view a larger version of this figure.

Note that as the epithelium increases in size, the amount of mesenchyme decreases. The experimental steps for microdissection of the embryonic LGs are depicted in **Figure 2**.

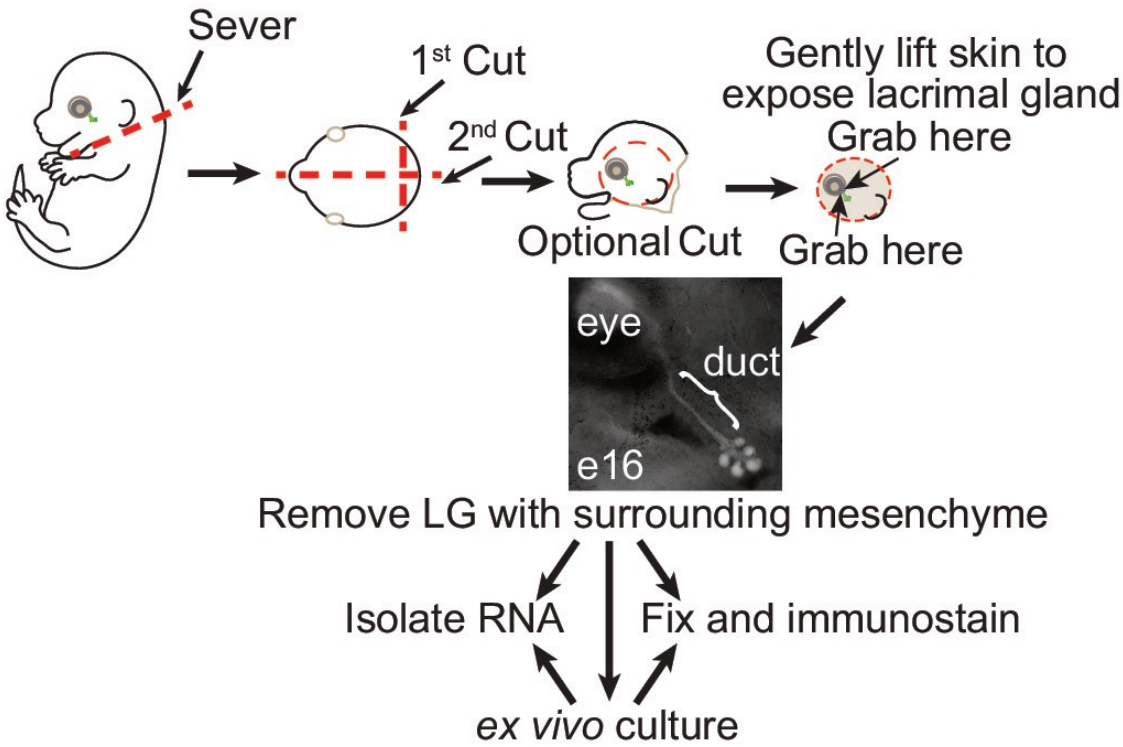

Figure 2. Schematic of steps involved in LG microdissection from mouse embryos. Schematic diagram of the dissection and removal of the LG from the embryonic mouse. Please click here to view a larger version of this figure.

For LG culture we routinely utilize e16 embryos due to the consistency in *ex vivo* growth. As shown in **Figure 3**, LG in *ex vivo* culture conditions develop in a similar manner to the *in vivo* gland (compare to **Figure 1**).

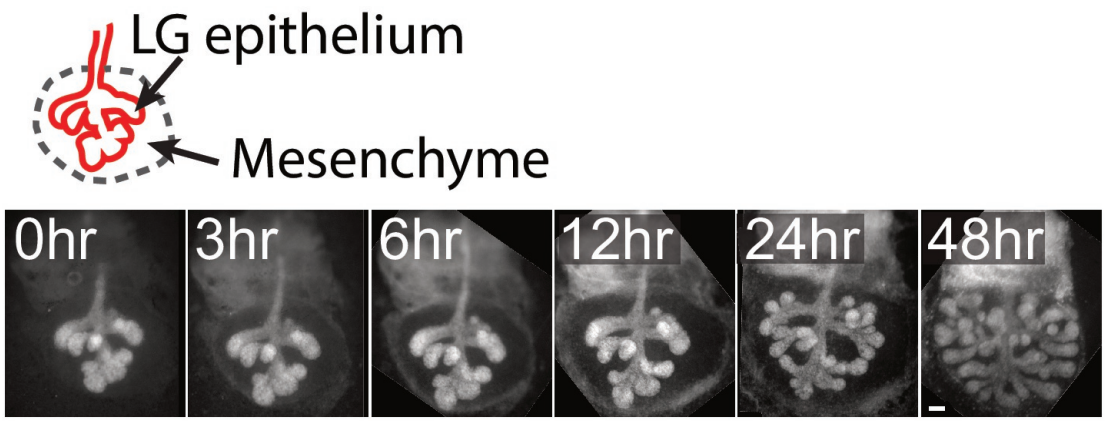

Figure 3. *Ex vivo* culture of e16 LGs recapitulates *in vivo* morphogenesis. e16 LGs were derived from Pax6-Cre, GFP embryos, where GFP is expressed by the epithelium. Consecutive fluorescent images were taken of this single gland at 0, 3, 6, 12, 24, and 48 hr. Please click here to view a larger version of this figure.

Here we employed Pax6-Cre, GFP (also called Le-Cre[5]) embryos to highlight epithelial cells, allowing us to take consecutive fluorescent images of the branching epithelium over the 48 hr culture period. Mice harboring the Pax6-Cre, GFP transgene can be obtained from JAX: Tg(Pax6-Cre, GFP)1Pgr but are not necessary for gland dissection and culture.

Cultures or freshly dissected LG can then be fixed for immunofluorescent analysis. **Figure 4** shows the visualization of 4 cellular compartments, mesenchyme, epithelium (EpCAM), nerves (Tubb3) and blood vessels (PECAM), in an e16 LG from a wild type (CD1) embryo.

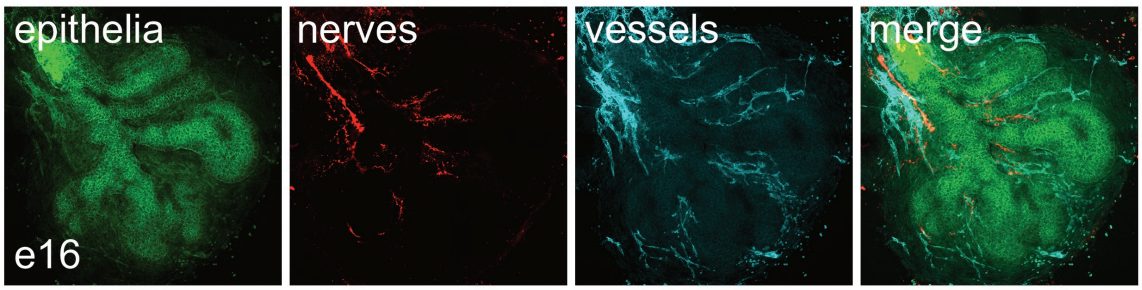

Figure 4.The LG is composed of multiple cell types including epithelial, neuronal and endothelial cells. e16 LG was immunostained for epithelial cells (Epcam, green), neurons (Tubb3, red) and endothelial cells (Pecam, cyan). Please click here to view a larger version of this figure.

For freshly dissected tissue it is easier to first embed the LG in laminin, as done for culture, so as to immobilize the gland for fixation and subsequent handling. **Figure 5** shows a schematic for dissection of the postnatal/adult LG.

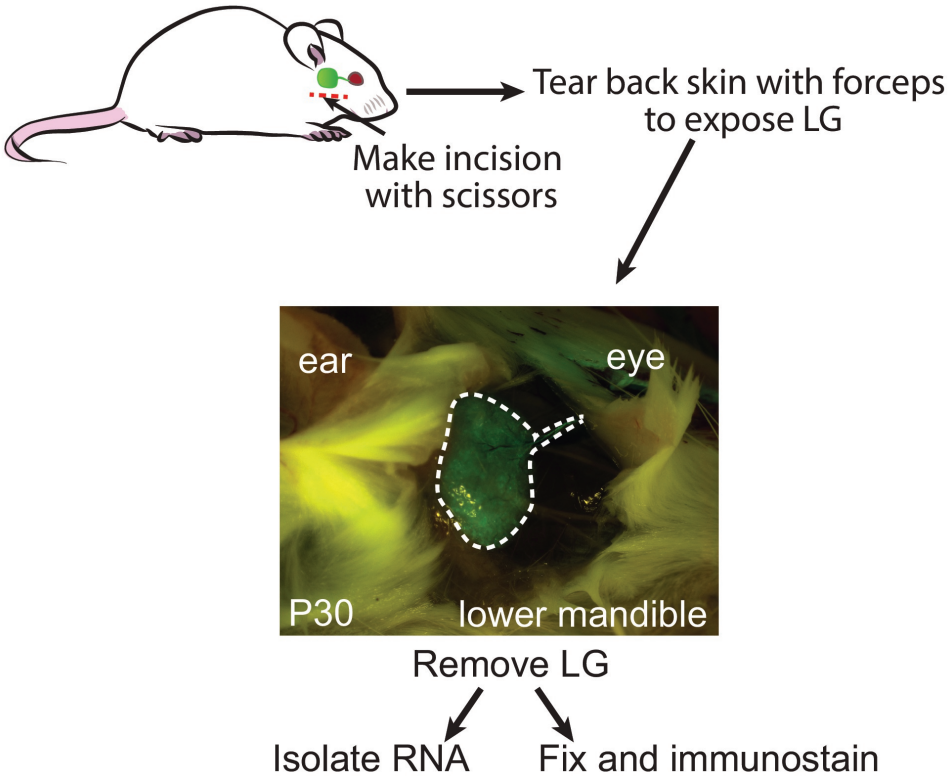

Figure 5. Schematic of steps involved in LG microdissection from post-natal and adult mouse. Schematic diagram of the dissection and removal of the LG from either post-natal or adult mouse. A Pax6-Cre,GFP post-natal day 30 mouse was used to visualize the position of the LG and associated duct. Please click here to view a larger version of this figure.

The position of the post-natal/adult LG has been visualized by using the Pax6-Cre, GFP mouse.

Discussion

The versatility to culture and manipulate the LG *ex vivo* provides significant advantages for studying its development. This includes the speed at which the researcher is able to test hypotheses and the multitude of perturbations that can be performed to assess how epithelial, neuronal, endothelial and mesenchymal cells interact to form the organ. However, there are a number of caveats when utilizing this model. First, by virtue of its isolation the gland is no longer connected to the peripheral vasculature or nervous system, which may affect the signaling pathways being tested if they work in conjunction with signals delivered by the nerves or blood vessels[9,10]. Second, the surrounding non-LG tissues may provide factors that regulate LG development[4]. To avoid this other non-mesenchymal tissue components must be removed prior to culture. Third, at present we are not able to provide the conditions necessary for complete cytodifferentiation into a fully functional tissue. This is the case for all organs cultured *ex vivo* and is likely due to multiple reasons including the absence of functional blood vessels and nerves and/or mechanical forces from surrounding tissues. Despite these caveats, mechanisms discovered in *ex vivo* culture have shown to be recapitulated in subsequent *in vivo* experiments, making this a robust system for testing new hypotheses[8,11].

It has been shown that developmental and regenerative pathways significantly overlap, indicating that the *ex vivo* culture system can also be employed to study organ regeneration[12]. Although we have not demonstrated this aspect here we, and others, have previously shown the embryonic salivary gland can be used to model the effects of therapeutic radiation on organ damage and regeneration[13]. Future studies are needed to explore the use of the LG *ex vivo* system as a model for tissue regeneration.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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