HHS Public Access

Author manuscript

Curr Eye Res. Author manuscript; available in PMC 2018 April 01.

Published in final edited form as:

Curr Eye Res. 2017 April ; 42(4): 491–497. doi:10.1080/02713683.2016.1214966.

Evaluation of Accessory Lacrimal Gland in Muller's Muscle Conjunctival Resection Specimens for Precursor Cell Markers and Biological Markers of Dry Eye Disease

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Abstract

Purpose—The accessory lacrimal glands (ALG) are an understudied component of the tear functional unit, even though they are important in the development of dry eye syndrome (DES). To advance our understanding of aging changes, regenerative potential and histologic correlates to human characteristics, we investigated human ALG tissue from surgical samples to determine the presence or absence of progenitor cell markers and lacrimal epithelial markers and to correlate marker expression to relevant patient characteristics.

Materials and Methods—ALG tissues obtained from Muller's Muscle Conjunctival Resection (MMCR) specimens were created using tissue microarrays (TMAs). Immunofluorescence staining of MMCR sections was performed using primary antibodies specific to cell protein markers. Cell marker localization in TMAs was then assessed by two blinded observers using a standardized scoring system. Patient characteristics including age, race, and status of ocular surface health were then compared against expression of stem cell markers.

Results—Human ALG expressed a number of epithelial markers, and in particular, histatin-1 was well correlated with the expression of epithelial markers and was present in most acini. In addition, we noted the presence of precursor cell markers nestin, ABCG2 and CD90 in ALG tissue. There was a decrease in precursor cell marker expression with increasing age. Finally, we noted that a negative association was present between histatin-1 expression and DES.

Conclusions—Thus, we report for the first time that human ALG tissues contain precursor marker positive cells and that this marker expression may decrease with increasing age. Moreover, histatin-1 expression may be decreased in DES. Future studies will be performed to use these cell markers to isolate and culture lacrimal epithelial cells from heterogeneous tissues, determine the relevance of histatin-1 expression to DES and isolate candidate precursor cells from ALG tissue.

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There is no conflicting commercial relationship in the form of financial support or personal financial interest to report.

Declaration of Interest

The authors report that they have no conflicts of interest.

Keywords

Accessory lacrimal gland; ocular surface infection; histatin; stem cell; dry eye syndrome

Introduction

Dry eye syndrome (DES) is a multifactorial, chronic and disabling disease often caused by decreased function of the lacrimal gland[1]. The global prevalence of dry eye is estimated to be 11% to 22%[2]. The first line of treatment for DES is lubricating eye drops or other palliative measures[3]. These treatment modalities provide only temporary relief[4]. In cases of severe dry eye, advanced therapies such as anti-inflammatory medications, and surgical interventions like punctal occlusion and salivary gland transplantation have had limited success[3]. It would be desirable for the treatment of severe DES to employ strategies that would increase the natural production of tears, maintain ocular surface integrity and reduce or eliminate the levels of existing inflammation and prevent infection[5]. With these objectives in mind, the option of cell based therapy is being increasingly explored for restoring the function of damaged lacrimal gland. Investigation into the potential of using *in vitro* cultured cells for regenerative therapy may have promising outcomes. Similar to other exocrine tissues, main lacrimal glands (MLG) contain precursor cells[6]. These precursor cells proliferate in response to inflammation and injury, but are present in the uninjured gland as well[7]. Cultures of human lacrimal gland have been shown to contain a sub-population of progenitor cells which persist in culture[6].

The accessory lacrimal gland (ALG) is a critical contributor to baseline tear production[8, 9], and limited data exist regarding ALG function and biology[10]. In addition, ALG is more readily accessible to surgical intervention than main lacrimal gland (MLG) and represents an understudied component of the tear production apparatus[9]. Numerous limitations exist in the development of a better understanding of ALG biology. One, human tissue is not frequently accessible and, when available, is limited in quantity[11]. Two, ALG and MLG cell surface markers are frequently present in other surrounding tissues, like conjunctiva[12, 13].

The lacrimal gland is a compound tubuloacinar gland consisting of acini, ducts, nerves, plasma cells and myoepithelial cells[14]. E-Cadherin is a pan-epithelial cell marker[6, 15]. Lacrimal tissue markers[10] include lactoferrin[16], lacritin[17] and aquaporin-5[18–20]. Lactoferrin is known to be produced by other ocular surface tissues, including conjunctiva[21,22]. Lacritin is a secreted protein found in tears and saliva and is mainly produced by the lacrimal gland[23]. Aquaporin-5 is a water channel protein also involved in tear generation process[24]. We recently demonstrated the utility of histatin-1 as a potential lacrimal epithelial marker, but a gold-standard lacrimal epithelial marker should be determined through the use of multiple studies and we utilize multiple markers to phenotype lacrimal epithelial cells[25].

The existence of the stem/progenitor cells in tissue may imply regenerative potential, and characterization of these cells is important for development of regenerative therapies. The murine lacrimal gland has been noted to contain a population of cells which are upregulated in response to injury and inflammation that express nestin, a multi-lineage precursor cell marker for neuronal, lacrimal tissue, salivary tissue and pancreas tissue[26, 27]. PDX-1 is a

pancreatic stem cell marker[28]. CD9, CD29, CD 44, CD81, CD 90 and CD105 are markers of salivary and pancreatic stem cells[27]. ABCG-2 is a marker of murine lacrimal stem cells[5, 29].

Various surgical approaches have been utilized to obtain human tissue for evaluation[30–32]. Muller Muscle Conjunctival Resection (MMCR) surgery is a procedure commonly used to treat blepharoptosis[30]. Both MMCR surgery and the Fasanella-Servat ptosis procedure involve the resection of the posterior layers of the eyelid in the area of the ALG[31, 32]. ALG tissue is noted in the surgical specimen in MMCR procedures approximately 50–60% of the time[31]. These surgical specimens contain conjunctiva, stromal tissue, Muller's muscle (smooth muscle), blood vessels, nerve tissue and ALG tissue. ALG tissue contains myoepithelial cells, acinar epithelium and ductal cells[6, 33–35].

In order to develop a better understanding of ALG biology, we utilized MMCR specimens to determine the localization of markers of lacrimal epithelium, the presence or absence of stem cell markers in these tissues and related their presence to relevant patient characteristics.

This study aimed to examine and assess the expression of markers for lacrimal cells and the presence of precursor marker positive cells in human ALG from MMCR specimens. In addition, we investigated whether or not histological findings combined with patient characteristics could serve as predictors for DES.

Materials and Methods

Written informed consent was obtained from patients using a consent form specifically approved for this study by the Institutional Review Board (IRB) and processed by The University of Illinois at Chicago. Completed, signed consent forms were maintained according to the university guidelines following an IRB approved protocol specific for this study.

Patient Record Evaluation

Patient samples were obtained prospectively and consented in an IRB approved study. Inclusion criteria included age greater than or equal to 18 years old, and undergoing blepharoptosis repair via Muller's Muscle Conjunctival Resection. Seventeen patients and twenty four eyelid specimens were included. Patient records were obtained and examined after IRB approval, and DES status was determined by retrospective chart review to find documentation of an existing diagnosis of DES by a practicing ophthalmologist (VKA).

Tissue Microarray Construction

A TMA was constructed from 24 MMCR patient specimens. Paraffin embedded H&E sections were analyzed to define representative ALG. The TMA block was constructed using a manual tissue array (K7 Biosystem Inc., Chicago, IL). A single 2.0 diameter cylindrical core of paraffin embedded tissue was retrieved from each paraffin block donor. Tissue cylinders were then inserted into a blank recipient paraffin block. After the array was completed and tempered in an incubator, the recipient block was cut into 3–5um sections

using an HM340E paraffin microtome (Microm International Gmbh, Walldorf, Germany). Each sample section was chosen and marked to indicate position of ALG by an ocular pathologist.

Immunofluorescence Staining and Confocal Microscopy

Single and double immunofluorescence staining of MMCR sections was performed using primary antibodies specific to stem cell protein markers: ABCG2, CD90 and nestin; a myoepithelial cell marker: α-Smooth Muscle Actin; and lacrimal epithelial markers: aquaporin-5, histatin-1, e-cadherin, lactoferrin and lacritin, which were counter stained with respective secondary antibodies. Images of stained ALG were captured with 10× and 20× microscope objectives, and analyzed using the Zeiss LSM 710 Confocal Microscope.

Immunoreactivity Evaluation and Statistical Analysis

Immunoreactivity was visually assessed by two independent observers in all 24 MMCR cores by scanning the cores with 20× microscope objective. The evaluation was performed semi-quantitatively by assigning a score based on the proportion of positively stained acini and their immediately surrounding myoepithelial cells over total number of acini (percent positivity) ranging from 0 to 100. Furthermore, the percent positivity scores were grouped into 0–30%, 31–70%, and 71–100% for data analysis. The scoring method has been found to be reproducible [36,24]. When analyzing intensity of expression of the different lacrimal and stem cell markers (stain positive acini:total acini), we categorized intensity into three categories (low, medium and high). Low was 0–30%, medium was 31–70% and high was 71–100%. Following this categorization we ran a Chi-Square test to test for significance between these categories and DED (DED positive and DED negative).” Salivary, pancreas, prostate, breast, brain and placental tissues were used as positive and negative control. Primary antibody omission was used for the negative control. The collected data were statistically analyzed and interpreted using standard methods. Patient characteristics such as age, gender, race, and status of ocular surface health were compared against semi-quantitative localization of markers for lacrimal cells, aging changes, and stem cells.

Statistical analysis was performed by analyzing scores from both observers. Pearson’s correlation coefficients, linear regression analysis and standard statistical methods were determined using SAS. Associations between patient characteristics and individual antibody staining were determined by comparing the ratio of positive acini divided by total number of acini counted by each observer.

Patients were analyzed as subgroups for age and percent positivity for localization of protein markers. With regards to age, 0–40 was one group and 41 and above was a second group. Forty years of age was used as a cut off to be consistent with aging related eye disease studies[37]. This grouping takes into account the decreasing efficacy of stem cells in middle aged and above patients, and the increasing prevalence of dry eye disease in older patients[38].

Results

Seventeen patients and twenty four MMCR cores from twenty four eyelids were included in the study. Each core contained a robust sample of ALG with appropriate histological appearance on H and E staining. Patient characteristics are noted in (Table 1). The mean age of patients was 57.7 years, 52.9% were male and 47.1% were female. 41.2% had dry eye disease and 58.8% did not have dry eye disease. H & E image of tissue microarray containing human MMCR specimen of accessory lacrimal glands is noted in Figure 1.

Localization of lacrimal and epithelial markers (e-cadherin, aquaporin-5, lacritin, lactoferrin and histatin-1) to ALG epithelial cells is shown in Figure 2. E-Cadherin localizes well to lateral and apical membranes of lacrimal epithelium, aquaporin-5 localizes to apical membranes of lacrimal epithelium, histatin-1 localizes to lacrimal epithelial cytoplasm as do lactoferrin and lacritin. Alpha smooth muscle actin localizes to peri-epithelial myo-epithelial cells.

Similarly, markers for precursor cells were localized to subpopulations of cells in ALG and immunofluorescence images are shown in Figure 3. ABCG-2 localized to apical and lateral epithelial cell membranes, as well as the cytoplasm while CD90 and nestin localized to acinar epithelial cells and periacinar cells. ABCG-2 was almost exclusively found inside acini. Immuno-localization for other precursor cell markers (PDX1, CD9, CD14, CD29, CD81 and CD105) was non-specific (data not shown).

We then examined associations among lacrimal markers (Table 2,Tables 3a and b). In particular, we found that all lacrimal markers show significant correlation with total acini, implying consistent localization in most acini of each marker. Table 3a demonstrates the correlation between aquaporin-5 lactoferrin and lacritin. Table 3b demonstrates the correlation between histatin-1 lactoferrin and lacritin. We also noted a detectable but not-strong correlation between histatin-1 and aquaporin-5 (0.35).

Linear regression analysis between total acini and aquaporin-5 revealed a beta coefficient of 1.00 after controlling for scorer, age, gender, race and dry eye syndrome ($P<0.001$). This indicates a strong, consistent localization of aquaporin-5 in most lacrimal acini. Linear regression between total acini and e-cadherin revealed a beta coefficient of 0.95 after controlling for scorer, age, gender, race and dry eye disease ($P<0.001$). These data indicate a strong, consistent localization of e-cadherin in most lacrimal acini. Thus, immuno-localization analysis of TMAs demonstrated that there is a significant and strong correlation among lacrimal cell acinar count and e-cadherin, aquaporin-5, histatin-1, lactoferrin and lacritin.

Table 4 demonstrates the cross-tabulation between the categorized ratio of histatin-1 positive to total acini staining and patient DES status. Patients with DES were less likely (76.1%) to have a high (>71%) ratio of acini with histatin-1 localization than those without DES (83.7%) ($p=0.04$). We did not find a significant association among other lacrimal markers and patient age, gender or DES.

Finally, we compared the association between precursor cell markers and patient characteristics. Table 5 demonstrates the correlation coefficients among precursor cell markers and age. Noted is a significant and negative association between age and ABCG-2. No significant associations between DES status or gender were found with precursor cell markers. Linear regression between age and ABCG-2 reveals a beta value of −0.47 after controlling for scorer, gender, race and dry eye in a significant manner (P=0.04).

Discussion

This study evaluated human ALG tissue for the presence or absence of precursor marker positive cells, and the presence or absence of lacrimal cell markers. In addition, we studied the localization of histatin-1, a protein previously thought to be primarily expressed in salivary tissues[39]. In a recent study, we demonstrated that histatin-1 is a useful marker for lacrimal epithelium[25]. We also attempted to correlate immuno-localization results to relevant patient characteristics.

Our findings in Table 2 demonstrate that the markers utilized all correlated well and significantly with the number of acini, suggesting that these markers could be useful in assessing the amount of lacrimal acini in heterogenous tissue samples. Table 3a demonstrates the significant correlation between aquaporin-5 and histatin-1, lactoferrin and lacritin. Similarly Table 3b demonstrates the significant correlation between histatin-1 and aquaporin-5, lactoferrin and lacritin. Taken together these data suggest that co-localization of lacritin and lactoferrin with either aquaporin-5 or histatin-1 could be utilized identify lacrimal epithelial cells. However, given that this study does not address all possible markers, and the presence of epithelial markers in multiple tissue types, caution should be taken with relying simply on immuno-localization. For true identification of lacrimal epithelial cells in the context of a heterogeneous sample we would recommend multiple corroborating assays.

Interestingly, we noted that the ratio of histatin-1 positive acini decreases with dry eye syndrome. We only noted a difference between DES and non-DES patients when comparing the high (>71%) percent positive ratio patients. Histatin-1 proteins have been implicated in wound healing in the mouth and also in the protection of the oral mucosa from microbial invasion, and inflammation associated with LPS[40–42]. Presence of histatin-1 in the lacrimal tissue may have implications for ocular infections, given the anti-microbial properties of histatin peptides. DES patients may have diminished ability to heal epithelial wounds, demonstrate greater ocular surface inflammation and have increased infection risk[43]. However, previous studies[44] have demonstrated only limited expression of histatin-1 on the ocular surface[45–47], and previous tear proteomic experiments have not demonstrated histatin-1 family members in the tear film [48]. These results may indicate that histatin-1 proteins are not secreted onto the ocular surface, or they may be undetected due to the limitations of body fluid proteomic analysis[48]. It may also be the case that histatins are secreted in response to environmental factors or at different times of day[49–51]. These issues underline the importance of further, larger scale studies. Furthermore, a comparison of sub-types and severity of DES and histatin-1 expression would be a useful future study.

Adult tissue stem cells have been found in numerous exocrine tissues, such as adult human pancreas[52] and salivary glands[27] and may have potential therapeutic applications. In addition, stem cells in various human glandular tissues share a similar expression profile[27]. Recently, mouse lacrimal gland tissue has been found to contain proliferating, nestin expressing cells after induction of inflammation[26]. Nestin upregulation has been observed after injury, and is thought to be a marker of multi-lineage precursor cells[26, 27].

Recent studies have shown that rat main lacrimal gland contains myoepithelial cells, which express α -smooth muscle actin, nestin, and ABCG-2, and may represent progenitor cells[7]. Other authors[6] have noted similar findings in human main lacrimal gland cultures, but have emphasized the presence of an ABCG-2 positive population of cells as representing precursors. Localization of α -smooth muscle actin was similar in human ALG compared with other studies[7], but we did find presence of ABCG-2, CD90 and nestin in acinar cells, as opposed to solely peri-acinar cells.

We found a negative correlation between precursor cell marker ABCG2 present in ALG and age. The relationship between ABCG-2 and age was statistically significant, but the relationship between nestin and age and CD90 and age, was not statistically significant. This suggests that an ABCG-2 positive cell population is present in ALG and decreases with increasing age, consistent with findings in other cell types[7, 52].

Although we chose to focus on nestin[26,27], ABCG2[29,53] and CD90[54,55] as epithelial progenitor cell markers based on our evaluation of the available literature and negative results with (CD9, CD29, CD81, CD105) there are a number of other potential markers CD44[27] and PDX-1[28] which could be investigated further. This study is the first to demonstrate the presence of a population of cells in adult ALG tissue expressing precursor markers. In a recent study, we showed the expression of a number of precursor and stem cell markers in an Affymetrix® gene array analysis of ALG[56]. However, future studies are necessary to further characterize and isolate this cell population for analysis.

Limitations of this study include small sample size, limited dry eye status assessment, semi-quantitative assessment of marker expression and staining. Future studies should be performed on a prospective basis, with detailed assessments of etiology and severity of dry eye syndrome and with more quantitative assessment of immuno-localization. In addition, larger studies with quantitative grading schema and corroborative data using protein assays such as western blotting on larger tissue samples should be performed to further characterize the role of age and other patient characteristics in cell marker expression. However, gene expression analysis performed on accessory lacrimal gland does support the expression of stem cell genes in adult accessory lacrimal gland.

Conclusion

Thus, we report that ALG tissue can be accessed and analyzed in tissue microarrays, these ALG tissues contain precursor marker positive cells, and that histatin-1 may be associated with dry eye syndrome. However, subsequent studies will be necessary to determine if these cell markers can be effectively utilized to separate and culture lacrimal epithelial cells from

heterogeneous tissues, determine the relevance of histatin-1 expression to dry eye syndrome and isolate candidate precursor cells from ALG tissue.

Acknowledgments

Sources of Funding: This work was supported by NIH-NEI Grant (K08EY024339), a seed grant from Illinois Society to Prevent Blindness, a Research Grant from Midwest Eye Banks, a Grant-in-Aid from Fight for Sight and Departmental Support through an NIH-NEI core grant (2P30EY001792-36A1) and an unrestricted grant from Research to Prevent Blindness (NY, NY).

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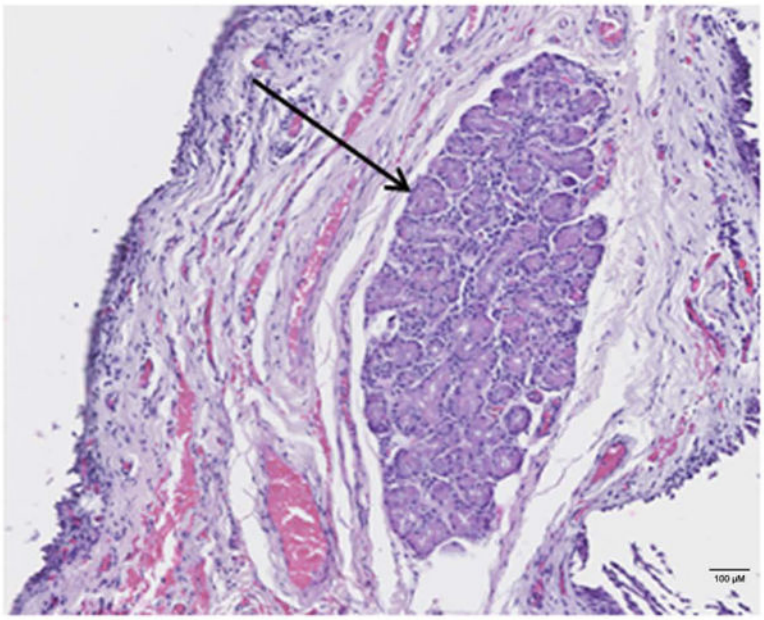

Figure 1.

H & E image of formalin fixed paraffin embedded MMCR specimen. Black arrow is indicating ALG location in heterogeneous tissue from MMCR. Scale bar is 100 µM.

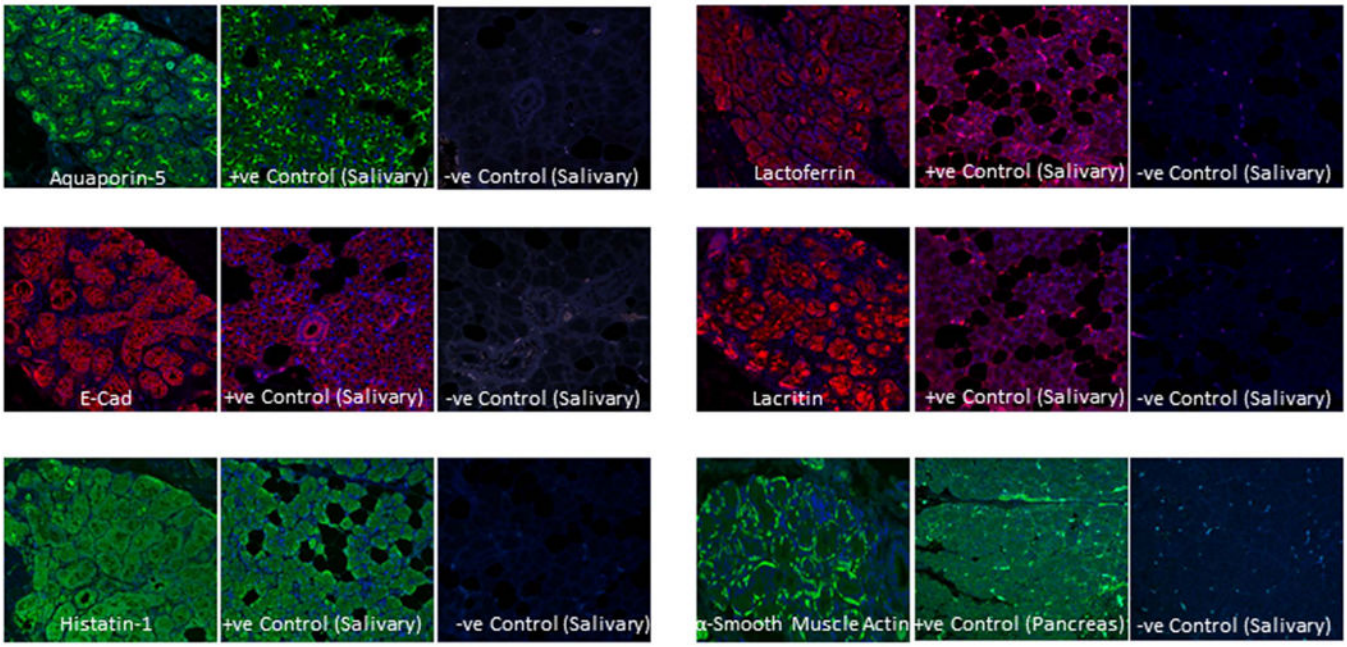

Figure 2.

Expression of lacrimal markers in ALG. Immunofluorescence imaging shows the localization of aquaporin-5, e-cad, histatin-1, lactoferrin, lacritin and α-smooth muscle actin in ALG. Sections of salivary glands were used as positive control and without primary antibodies respectively as negative control.

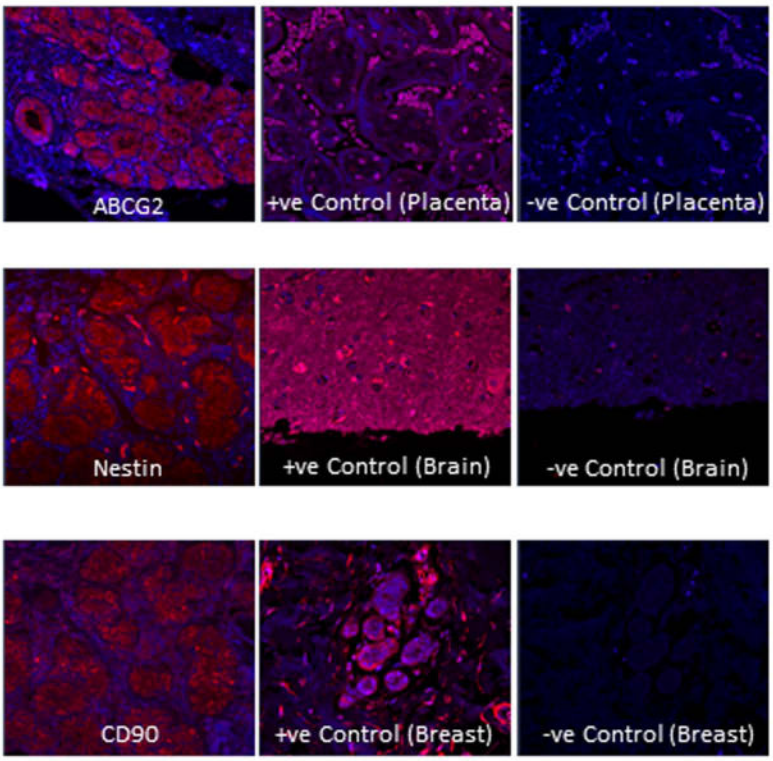

Figure 3.

Identification of stem cell markers in ALG. Immunofluorescence imaging shows the localization of the precursor cell markers ABCG2, nestin, and CD90 in ALG. Sections of placenta, brain and breasts were imaged after exposure to respective antibodies as positive control and negative control (without respective primary antibodies).

Table 1

Patient Characteristics (n=17)

Variable	Mean± SD/ n(%)
Age	57.7 ± 18.3
Gender Female Male	 8 (47.1%) 9 (52.9%)
Race White African American Hispanic Asian Other	 5 (29.4%) 5 (29.4%) 5 (29.4%) 1 (5.9%) 1 (5.9%)
Dry Eye Yes No	 7 (41.2%) 10 (58.8%)

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Table 2

Lacrimal vs Acini

Lacrimal Cell Marker	Total Acini Number r-values ($p < 0.001$)
E-Cadherin	0.99
Aquaporin-5	0.93
Lacritin	0.78
Lactoferrin	0.51
Histatin-1	0.78

Table 3a

Correlation Coefficients for Aquaporin-5

	Histatin-1	Lactoferrin	Lacritin
Aquaporin-5	0.35 (P=0.07)	0.44 (P=0.02)	0.68 (P<0.001)

Table 3b

Correlation Coefficients for Histatin-1

	Aquaporin-5	**Lactoferrin**	**Lacritin**
Histatin-1	0.35 (P=0.07)	0.55 (P=0.002)	0.55 (P=0.003)

Table 4

Categorized Ratio of Histatin-1 Positive Acini to Total Acini and Patient DES Status

Positivity (%)	Dry Eye (+)	Dry Eye (−)
Low	11.1 %	10.0 %
Medium	12.8 %	6.3 %
High	76.1 %	83.7 %

* Analysis was run by Chi-Square (P=0.04).

Table 5

Correlation of Precursor Cell Marker Positive Acini to Total Acini and Patient Age

Precursor Cell Marker	Age
ABCG2	−0.34 (P = 0.03)
Nestin	−0.24 (P = 0.11)
CD90	−0.13 (P = 0.43)