Does estrogen deficiency cause lacrimal gland inflammation and aqueous-deficient dry eye in mice?

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Abstract

Researchers have proposed that estrogen deficiency will lead to a Sjögren's syndrome (SjS)-like lacrimal gland inflammation, aqueous tear deficiency and dry eye. The purpose of this study was to determine whether this proposal is correct. Lacrimal glands were obtained from adult, age-matched wild type (WT) and aromatase knockout (ArKO) mice, in which estrogen synthesis is completely eliminated. Tissues were also obtained from autoimmune MRL/Mp-lpr/lpr (MRL/lpr) mice as inflammation controls. Tear volumes in WT and ArKO mice were measured and glands were processed for molecular biological and histological evaluation. Our results demonstrate that estrogen absence does not lead to a SjS-like inflammation in lacrimal tissue or to an aqueous-deficient dry eye. There was no upregulation of genes associated with inflammatory pathways in lacrimal glands of male or female ArKO mice. Such inflammatory activity was prominent in autoimmune MRL/lpr tissues. We also found no evidence of inflammation in lacrimal gland tissue sections of estrogen-deficient mice, and tear volumes of ArKO males were actually increased as compared to those WT controls. Interestingly, our study did show that estrogen absence influences the expression of thousands of lacrimal gland genes, and that this impact is sex- and genotype-specific. Our findings demonstrate that estrogen absence is not a risk factor for the development of SjS-like lacrimal gland inflammation or for aqueous-deficient dry eye in mice.

Keywords

lacrimal gland; mice; estrogen; male; female; sex difference; inflammation; tear volume; gene expression

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1. Introduction

The lacrimal gland is the primary source of the tear film's aqueous layer. This gland secretes proteins, electrolytes and water that collectively play a prominent role in maintaining ocular surface integrity, protecting against microbial challenge and preserving visual acuity (Sullivan, 2004). Malfunction of the lacrimal gland, in turn, leads to aqueous tear deficiency. An example is Sjögren's syndrome (SjS), an autoimmune disease characterized by extensive glandular inflammation, immune-mediated dysfunction and/or destruction of acinar and ductal epithelial cells, a precipitous decline in aqueous tear output, and consequent dry eye disease (Sullivan, 2004).

It is important to note that dry eye disease occurs predominantly in women (2007). This prevalence has been linked to estrogen status. Investigators have proposed that estrogens positively modulate the structure, function and secretion of the lacrimal gland and that estrogen deficiency leads to numerous lacrimal gland sequelae, including acinar cell disruption, apoptosis and necrosis, cellular vacuolization, DNA degradation, inflammation, glandular regression and aqueous-deficient dry eye (Azzarolo et al., 2003; Konttinen et al., 2012; Mostafa et al., 2012; Song et al., 2012; Sullivan, 2004[1]). Researchers have also posited that estrogen insufficiency promotes a SjS-like autoimmune exocrinopathy (Arakaki et al., 2010; Hayashi et al., 2004; Ishimaru et al., 2003; Ishimaru et al., 1999; Shim et al., 2004; Sullivan, 2004; Takahashi et al., 1997). Conversely, estrogen treatment purportedly corrects such defects in lacrimal gland anatomy and physiology, and promotes aqueous secretion (Affinito et al., 2003; Guaschino et al., 2003; Mostafa et al., 2012; Sullivan, 2004).

If these investigators' proposals are correct, we would predict that complete estrogen absence would induce an upregulation of inflammatory pathways in the lacrimal gland, a SjS-like inflammation in lacrimal tissue, and aqueous tear deficiency. To test this prediction, we examined molecular biological and histological attributes of the lacrimal glands, as well as tear volumes, of male and female aromatase knockout (ArKO) mice. These animals were generated by the targeted disruption of exon IX in the cyp19 gene and have no aromatase, the cytochrome P450 enzyme that controls the formation of estrogens. Consequently estrogen synthesis is completely eliminated in ArKO mice (Fisher et al., 1998).

2. Materials & Methods

2.1. Animals and tissue collections

Breeding pairs of C57BL/6J - aromatase knockout (ArKO) heterozygous mice were acquired from Dr. Orhan Oz (University of Texas Southwestern Medical Center, Dallas, TX). Animals were sent to Charles River Breeding Laboratories (Wimington, MA) for initial quarantine, health monitoring and serology, and then shipped to the Animal Facilities of the Schepens Eye Research Institute (Boston, MA). Mice were maintained and bred in constant temperature rooms with fixed light/dark intervals of 12 hours length. Offspring were genotyped by following a published protocol (Fisher et al., 1998). In brief, genomic

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[1]The "Sullivan, 2004" review contains over 40 additional references about estrogen's possible influence on the lacrimal gland. These papers were published between 1930 to 2002.

DNA was isolated from tails by utilizing a GenEluteTM Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, Saint Louis, MO). The DNA was amplified by PCR with a Hybaid OMN-E thermocycler (Thermo Electron Corp, Burlington, Ontario, Canada) by employing exon 9 gene primers (forward: GTGACAGAGACATAAAGATCG; reverse: GTAAATTCATTGGGCTTAGGG) and *neo* gene primers (forward: ATCAGGATGATCTGGACGAAGA; reverse: CCACAGTCGATGAATCCAGAA). The PCR conditions were 1 cycle (3 minutes at 94°C), 35 cycles (40 second at 94°C, 30 second at 55°C, 45 second at 72°C) and 1 cycle (5 minutes at 72°C) and amplicons were examined on 2.5% agarose gels. Band sizes equaled 220 bp from wild type (WT) mice, 170 bp from ArKO mice, and both fragments from heterozygotes. In addition to these animals, adult female and male MRL/Mp-lpr/lpr (MRL/lpr) mice were obtained from Jackson Laboratories (Bar Harbor, ME) to serve as controls.

When indicated, mice were sacrificed by CO_2 inhalation and lacrimal glands were removed for either molecular biological procedures or histology. Glandular samples were prepared by combining tissues from 5 to 6 mice/sex/group. Three different sample preparations were made for each tissue/sex/group and then processed for RNA analysis.

For histological evaluation, tissues were fixed in 10% buffered formalin, dehydrated, embedded in methacrylate, cut into 3 μm sections and stained with hematoxylin and eosin or with Periodic-acid Schiff, according to previously described techniques (Sato and Sullivan, 1994). Sections were taken from different tissue areas, all separated by minimal distances of at least 30 μm. Slides were observed with an Olympus BH-2 light microscope and micrographs were obtained with a Nikon Eclipse E800 and SPOT camera (MicroVideo Instruments Inc., Avon, MA).

All research studies with mice were approved by the Institutional Animal Care and Use Committee of The Schepens Eye Research Institute and adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Measurement of aqueous tear production

Tear volumes were measured by using a modified phenol red thread test (Zone-Quick™, Lacrimedics, Eastsound, WA) (Barabino et al., 2005). Under direct visualization with a biomicroscope, meniscal tears were removed and then threads were placed in the lateral canthus of the right eye for 30 seconds. The tear migration distance in mm was determined by using a microscope (Zeiss S4, West Germany) and a hemocytometer. Tears measurements were repeated 3 times and the results were averaged. Statistical analyses of these data were performed with Student's t-test (two-tailed, unpaired).

2.3. Molecular biological procedures

Total RNA was extracted from lacrimal glands by using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) and purified with RNAqueous spin columns (Ambion, Austin, Tx). The glandular RNA samples were treated with RNase-free DNase (Invitrogen), analyzed spectrophotometrically at 260 nm to determine concentration and assessed with a RNA 6000

Nano LabChip and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) to verify RNA integrity. The RNA samples were then stored at -80°C until processing.

Gene expression was determined by the use of two procedures. One involved the processing of RNA samples for hybridization to CodeLink UniSet Mouse 20K I Bioarrays (n ∼ 20,000 genes/array; Amersham Biosciences/GE Healthcare, Piscataway, NJ), according to reported procedures (Richards et al., 2006). In brief, cDNA was synthesized from RNA (2 μg) with a CodeLink Expression Assay Reagent Kit (Amersham) and purified with a QIAquick purification kit (Qiagen, Valencia, CA). Samples were then dried, and cRNA was generated with a CodeLink Expression Assay Reagent Kit (Amersham), recovered with an RNeasy kit (Qiagen) and quantified with an UV spectrophotometer. Fragmented and biotin-labeled cRNA was incubated and shaken at 300 rpm on a CodeLink Bioarray at 37°C for 18 hours. After this time period, the Bioarray was washed, exposed to streptavidin-Alexa 647, and scanned by utilizing ScanArray Express software and a ScanArray Express HT scanner (Packard BioScience, Meriden, CT) with the laser set at 635 nm, laser power at 100%, and photomultiplier tube voltage at 60%. Scanned image files were examined by using CodeLink image and data analysis software (Amersham), which yielded both raw and normalized hybridization signal intensities for each array spot. The intensities of the ∼ 20,000 spots on the Bioarray image were normalized to a median of 1. Standardized data, with signal intensities exceeding 0.50, were analyzed with sophisticated bioinformatic software (Geospiza, Seattle, WA). This comprehensive software also generated gene ontology, KEGG (i.e. Kyoto Encyclopedia of Genes and Genomes) pathway and z-score reports. The ontologies were organized according to the guidelines of the Gene Ontology Consortium (http://www.geneontology.org/GO.doc.html) (Ashburner et al., 2000).

The other procedure to analyze gene expression involved the hybridization of each cRNA (20 μg) sample to a GeneChip Mouse Genome 430A 2.0 Array (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol. Hybridized GeneChips were scanned with an Affymetrix Model 700 Scanner and expression data files were generated from array images using Affymetrix Microarray Suite 4.0 software. GeneChip data were standardized by selecting the default scaling in Affymetrix GeneChip Operating Software, which yields a trimmed mean intensity of 500 for each GeneChip microarray. Normalized data with a quality value of 1.0 were then evaluated with Geospiza Genesifter software.

Gene expression data were assessed without log transformation and statistical analyses of these data were performed with Student's t-test (two-tailed, unpaired) by using the GeneSifter software. Our statistical approaches were not tailored for multiple comparisons. Genes that were up- or down-regulated similarly in different groups were identified by using "Unigene" accession numbers and an intersector program (Geospiza; www.public.genesifter.net). Data from the individual CodeLink and Affymetrix arrays are accessible for free download through the National Center for Biotechnology Information's Gene Expression Omnibus (http://www.genesifter.net/web/dataCenter.html) via series accession numbers GSE5878 and GSE5876.

For results presented in this paper, we focused on data from Affymetrix GeneChips, and used the CodeLink Bioarray information for confirmation of selected gene expression. We

addressed the comparative merit of these microarrays in previous publications (e.g. Rahimi Darabad et al., 2013).

3. Results

3.1. Influence of complete estrogen absence on gene expression in male and female lacrimal glands

To determine whether complete estrogen absence induces an upregulation of inflammatory pathways in the lacrimal gland, tissues were obtained from adult, age-matched male and female WT (male = 22.7 ± 0.9 weeks; female = 21.4 ± 1.0 weeks) and ArKO (male = 21.6 ± 1.0 weeks; female = 21.7 ± 1.1 weeks) mice (n = 5/sex/group; n = 3 separate experiments). For control purposes, lacrimal glands were also collected from adult, autoimmune female and male MRL/lpr mice (n = 6/group; n = 3 experiments). Glands were pooled according to sex and group, processed for the isolation of total RNA, and evaluated for differentially expressed mRNAs by using Affymetrix GeneChips and Geospiza bioinformatics software.

Our findings demonstrate that estrogen absence does not induce an upregulation of inflammatory pathways in the lacrimal gland. There were 73 and 75 significantly upregulated biological process ontologies (n = 12 genes/ontology) in the male and female ArKO lacrimal glands, respectively (e.g. Table 1). None of these ontologies were immune-related. Similarly, there were 12 and 16 significantly upregulated KEGG pathways in the male and female ArKO tissues (e.g. Table 2). Of these, only one pathway, $F_c\gamma$ receptor-mediated phagocytosis (n = 8 genes) was significantly increased in the male. In contrast, the autoimmune female MRL/lpr lacrimal glands contained 364 significantly upregulated biological process ontologies (n = 12 genes/group), of which 81 were immune-related. Examples included those associated with the immune system process (n = 147 ↑genes; z-score [zsc] = 8.24), defense response (85 ↑genes; zsc = 6.37), leukocyte activation (67 ↑genes; zsc = 6.93), lymphocyte activation (57 ↑genes; zsc = 6.32), T cell activation (42 ↑genes; zsc = 6.03), inflammatory response (44 ↑genes; zsc = 5.12), innate immune response (40 ↑genes; zsc = 5.15) and response to cytokine stimulus (36 ↑genes; zsc = 5.15). In addition, 9 out of 22 significantly upregulated KEGG pathways in the MRL/lpr lacrimal glands had an immune basis (e.g. chemokine signaling pathway, 26 ↑genes; zsc = 2.43). Clearly, the lacrimal glands of estrogen-deficient mice were not immunologically analogous to those of the autoimmune animals.

This lack of inflammatory activity in lacrimal tissues of mice with estrogen deficiency does not reflect a simple lack of response to this change in hormonal environment. Indeed, the absence of estrogen exerted a significant impact on the expression of thousands of genes (Tables 3 and 4). Of particular interest, this influence of estrogen deficiency on lacrimal gland gene expression was sex-dependent. As shown in Table 5, less than 8.7% of the genes significantly affected by estrogen absence were the same in males and females. Further, less than 20.3% of the biological process ontologies (12 genes/ontology) were similar in both sexes (data not shown). In effect, the vast majority of gene transcripts that were significantly altered in complete estrogen absence were sex-specific.

The absence of estrogens also influenced the nature of sex-associated differences in lacrimal gland gene expression. This effect was genotype-specific. As demonstrated in Table 5, over 74% of genes upregulated in male or female tissues were unique to either WT or ArKO mice. However, despite these specific gene differences, numerous upregulated ontologies and pathways were similar in female WT and ArKO mice. Thus, 35% of the 40 highest, significant z-scores of biological process ontologies (n = 12 genes/ontology) in female ArKO lacrimal tissues were significantly increased in the WT females. In addition, 52.4% of all upregulated KEGG pathways in female ArKO lacrimal glands were also elevated in WT female tissues. These similarities were not found in male WT and ArKO mice.

To confirm in part the Affymetrix GeneChip results, gene expression was analyzed by the use of CodeLink Bioarrays. This experimental approach verified the effects of complete estrogen absence on a number of genes (Table 6). The CodeLink Biorray data also showed no evidence of any upregulation of inflammatory ontologies (n =   3 genes/ontology) in lacrimal tissues of male or female ArKO mice.

The lacrimal gland genes significantly influenced by complete estrogen absence were very different than those recently discovered in meibomian glands of ArKO mice (Darabad et al., 2013). Comparison of the meibomian gland dataset (NCBI GEO #GSE5878) to that of the present study showed that at least 92% of the meibomian and lacrimal gland genes were different (Table 7).

3.2. Effect of complete estrogen absence on the histology of male and female lacrimal glands

To examine whether complete estrogen induces an SjS-like inflammation in lacrimal tissue, glands (n = 4/sex/strain) from adult, age-matched male and female WT (male = 18.1 ± 0 weeks; female = 17.9 ± 0.3 weeks) and ArKO (male = 17.4 ± 0.7 weeks; female = 17.6 ± 0.3 weeks) mice were processed for histological analysis. As shown in Figure 1, we found no evidence of inflammation in the lacrimal glands of estrogen-deficient male or female mice. In addition, complete estrogen absence had no obvious influence on the histological appearance of any of the tissue sections.

3.3. Impact of complete estrogen absence on the tear volumes of male and female mice

To determine whether complete estrogen absence causes an aqueous tear deficiency, tear volumes were measured in WT and ArKO mice (n = 5/sex/strain; 20.2 ± 1.9 weeks old). Our results demonstrated that estrogen deficiency caused a significant ($p < 0.005$) increase in the tear volume of male mice (WT = 1.00 ± 0.03; ArKO = 1.58 ± 0.05). In contrast, this hormonal change had no effect on the tear volume of female mice (WT = 1.13 ± 0.05; ArKO = 1.19 ± 0.10). The tear volume of male ArKO mice was also greater than that of female ArKO mice ($p < 0.01$).

4. Discussion

Our study demonstrates that complete estrogen absence does not induce an upregulation of inflammatory pathways in the lacrimal gland, does not elicit a SjS-like inflammation in lacrimal tissue, and does not cause an aqueous tear deficiency in mice. Our research does

show that estrogen absence influences the expression of thousands of lacrimal gland genes, and that this impact is sex- and genotype-specific. Overall, our data demonstrate that the lacrimal glands of estrogen-deficient male and female mice are functional and appear histologically normal.

Our findings are not in agreement with those linking estrogen deficiency with lacrimal gland pathophysiology and dry eye disease (Arakaki et al., 2010; Azzarolo et al., 2003; Gagliano et al., 2014; Hayashi et al., 2004; Ishimaru et al., 2003; Ishimaru et al., 1999; Mostafa et al., 2012; Shim et al., 2004; Sullivan, 2004; Takahashi et al., 1997). This lack of agreement is not surprising. The published literature concerning estrogen influence on the lacrimal gland is replete with conflicting results. For example, a number of investigators have reported that neither estrogen insufficiency nor estrogen treatment has any impact on the size, morphology, total protein content, specific enzyme activity, lymphocyte accumulation or secretion of the lacrimal gland (Kuscu et al., 2003; Pelit et al., 2003; Sullivan, 2004). And yet other researchers have found that estrogens have a negative effect on lacrimal tissue and promote glandular regression, suppression of protein production, androgen antagonism and reduced tear output (Ishimaru et al., 1999; Sato and Sullivan, 1994; Sullivan, 2004). Some of these disparate findings regarding estrogens might be explained by differences in experimental design, hormone dosage or animal model (Sullivan, 2004; Truong et al, 2014). It is also possible that these conflicting results may be due to variations in the relative levels of progesterone, a steroid that may significantly modify estrogen effects. However, despite this lack of consensus, our data clearly demonstrate that estrogen absence does not cause an autoimmune exocrinopathy in the lacrimal gland, or an aqueous-deficient dry eye, in mice.

Our findings are also not in agreement with those of Shim et al. (2004), who proposed that ArKO mice develop an SjS phenotype. These investigators reported an extensive inflammation in salivary glands of both male and female ArKO mice between the ages of 12 to 17 months. Given that our mice were approximately 5 months of age, it may be that our different results are due to animal age. It is also possible, though, as found in SjS mouse models that the magnitude of inflammation in salivary and lacrimal glands may differ significantly. For example, male non-obese diabetic (NOD) mice have extensive inflammation in their lacrimal tissues, but essentially none in their salivary glands. Female NOD mice feature tremendous inflammation in their salivary, but not lacrimal, glands. MRL/lpr female mice have inflamed lacrimal and salivary glands, whereas males have far less, if any, immunopathology. In effect, inflammatory changes in salivary tissue are not necessarily mirrored in lacrimal glands (Toda et al., 1999).

Are estrogens, then, involved at all in the lacrimal tissue dysfunction in SjS? At present, the precise role of estrogens in the etiology of lacrimal gland inflammation and aqueous-deficient dry eye in SjS is unknown. Estrogens have been shown to accelerate (Homo-Delarche et al., 1991; Olsen and Kovacs, 1996; Roubinian et al., 1978; Talal, 1978), and anti-estrogens (Auborn et al., 2003; Nelson and Steinberg, 1987; Sthoeger et al., 2003; Wu et al., 2000) and selective estrogen receptor modulators (Apelgren et al., 1996) to retard, systemic disease processes in mouse models of SjS. Estrogens are also known to enhance the polyclonal B cell activation, autoantibody formation and tissue abnormalities encountered in SjS (Ahmed et al., 1989; Carlsten et al., 1990; Sato and Sullivan, 1994). As

further considerations, estrogens often promote inflammation (Choubey and Moudgil, 2011; Whitacre, 2001), upregulate pro-inflammatory gene expression in lacrimal tissue (Suzuki et al., 2006), stimulate the accumulation of lymphoid infiltrates in MRL/lpr lacrimal glands (Sato and Sullivan, 1994), and increase the prevalence of dry eye disease in women (Schaumberg et al., 2001). Given this background, it could be that estrogens have a deleterious impact on lacrimal gland function and reduce aqueous secretion. Consistent with this possibility are some (Sato and Sullivan, 1994; Sullivan, 2004; Toda et al., 1999), but not other (Affinito et al., 2003; Arakaki et al., 2010; Azzarolo et al., 2003; Forsblad-d'Elia et al., 2009; Guaschino et al., 2003; Hayashi et al., 2004; Ishimaru et al., 2003; Ishimaru et al., 1999; Kuscu et al., 2003; Mostafa et al., 2012; Pelit et al., 2003; Shim et al., 2004; Sullivan, 2004; Takahashi et al., 1997; Taner et al., 2004), research findings. Indeed, with the possible exception of specific genes (Suzuki et al., 2006), no consensus exists concerning the cellular targets for, or the cellular processes that may be regulated by, estrogens in lacrimal tissue (Affinito et al., 2003; Azzarolo et al., 2003; Imbert et al., 2009; Ishimaru et al., 2008; Kuscu et al., 2003; Paliwal and De, 2006; Pelit et al., 2003; Sato and Sullivan, 1994; Seamon et al., 2008; Srikantan and De, 2008; Srikantan et al., 2007; Sullivan, 2004; Suzuki et al., 2006; Taner et al., 2004; Zylberberg et al., 2007). In fact, it has not yet even been established whether estrogens have functional receptors in the lacrimal gland.

We discovered that complete estrogen absence is associated with a significant increase in the tear volume of male, but not female, mice. This male-specific effect may represent a sexually dimorphic response to the known elevations in the serum concentrations of testosterone, androstenedione, follicle-stimulating hormone, luteinizing hormone, prolactin or leptin in ArKO mice (Britt et al., 2001; Fisher et al., 1998; Jones et al., 2000; McPherson et al., 2001), or to hormone-independent processes (Isensee and Ruiz Noppinger, 2007; Rinn and Snyder, 2005), or to the influences of Y-linked genes or X inactivation in the ArKO strain (Disteche et al., 2002; Migeon, 2006; Ostrer, 2001; Xu and Disteche, 2006).

We found that complete estrogen absence impacts the expression of thousands of lacrimal gland genes, and that this effect was sex- and genotype-specific. The fact that estrogen deficiency influences the male lacrimal gland is consistent with research demonstrating that estrogen action is very important in other male tissues (Grumbach and Auchus, 1999; MacGillivray et al., 1998). Further, the sex- and genotype-specificity of the lacrimal gland response to estrogen absence is analogous to that of the meibomian gland (Darabad et al., 2013), although the affected genes are different. For comparison, other studies have shown that the nature of estrogen and/or aromatase control of the brain, liver, adipose tissue and serum leptin levels is also different in males and females (Chavez et al., 2009; Hewitt et al., 2003; Hewitt et al., 2004; Hill et al., 2004; Jones et al., 2000; Simpson et al., 2005). Moreover, we have previously demonstrated that the specific genes regulated by sex steroids in lacrimal and meibomian glands are typically not the same (Sullivan et al., 2009).

We also discovered that, although the lacrimal gland gene response to estrogen absence was genotype-specific, many of the upregulated ontologies and KEGG pathways were similar in female WT and ArKO mice. This finding would suggest that estrogens may not play a predominant role in the sex-related differences of the mouse lacrimal gland. This suggestion is consistent with our previous observation that estrogen with or without progesterone seems

to contribute very little to the known sex-associated variations in gene expression of the lacrimal gland (Richards et al., 2006; Suzuki et al., 2006).

Lastly, it is important to restate that our research was performed in mice. Though there are many similarities between the endocrine and immune systems of mice and humans, there are also many differences in both health and disease. Consequently, further research is required to determine whether the inability of estrogen deficiency to induce lacrimal gland inflammation in mice is the same response as in humans (Luu-The and Labrie, 2010; Seok et al., 2013).

5. Conclusions

Our research shows that complete estrogen absence does not induce an upregulation of inflammatory pathways in the lacrimal gland, does not elicit a SjS-like inflammation in lacrimal tissue, and does not cause an aqueous tear deficiency in mice. Our study also demonstrates that complete estrogen absence influences the expression of thousands of lacrimal gland genes, and that this impact is sex- and genotype-specific. Overall, our results show that the lacrimal glands of estrogen-deficient male and female mice are functional and appear histologically normal.

Acknowledgments

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Highlights

- investigators proposed that estrogen deficiency causes lacrimal gland inflammation

- investigators proposed that estrogen deficiency causes aqueous-deficient dry eye

- our research shows that estrogen absence does not cause lacrimal gland inflammation

- our study shows that estrogen absence does not cause aqueous-deficient dry eye

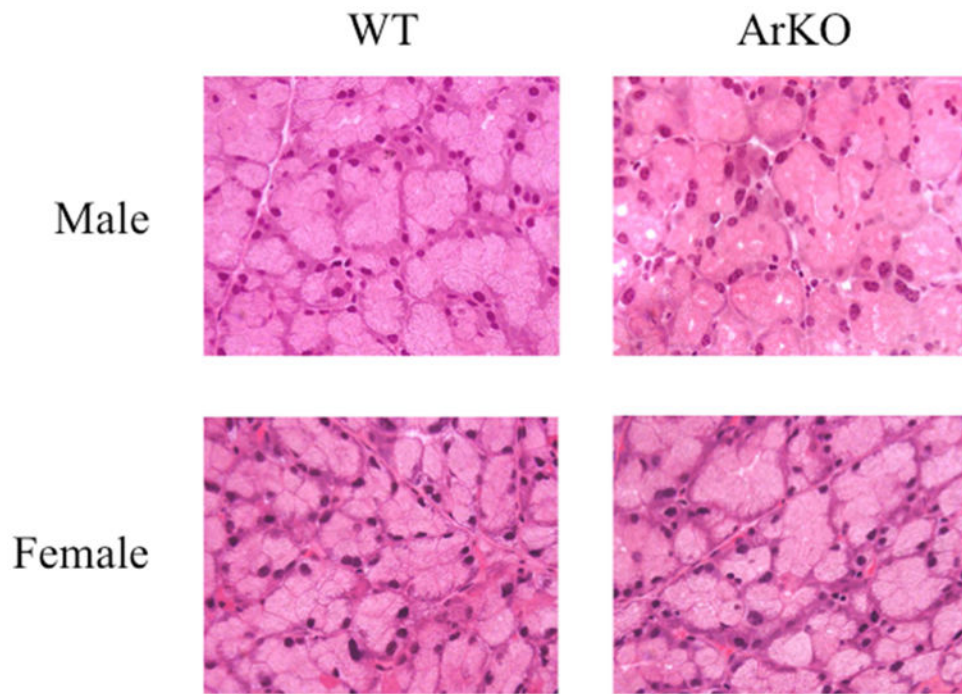- lacrimal glands of estrogen-deficient mice are functional and appear normal

Figure 1.
Representative light micrographs of male and female WT and ArKO lacrimal glands. There were no obvious histological differences between WT and ArKO glands. Sections were stained with hematoxylin and eosin. All panels are $40 \times$ magnification.

Table 1

Upregulation of biological process ontologies in the lacrimal glands of male and female mice with complete estrogen absence

Ontology	Genes↑	z-score
Female		
Monocarboxylic acid catabolic process	10	4.96
Oxidation-reduction process	56	4.6
Fatty acid catabolic process	9	4.54
Coenzyme metabolic process	20	4.42
Cellular respiration	10	4.37
Cofactor metabolic process	23	4.31
Carboxylic acid catabolic process	15	4.23
Organic acid catabolic process	15	4.23
Male		
Glutathione metabolic process	7	5.65
Sulfur compound metabolic process	12	4.16
Lipid biosynthetic process	25	3.99
Small molecule biosynthetic process	34	3.91
Peptide metabolic process	7	3.91
Small molecule metabolic process	90	3.66
Regulation of protein complex assembly	11	3.55
Regulation of protein polymerization	8	3.43

Biological process ontologies (7 genes/sex) with the highest z-scores in lacrimal glands of ArKO mice are listed. A z-score is a statistical rating of the relative expression of genes, and demonstrates how much they are over- or under-represented in a given gene list (Doniger et al., 2003). Positive z scores reflect a higher number of genes meeting the criterion than is expected by chance, whereas negative z scores represent fewer genes meeting the criterion than expected by chance (Doniger et al., 2003). Z-scores with values > 2.0 are significant. Terms: Genes↑ -number of genes up-regulated in ArKO glands; z-score - specific score for the up-regulated genes in the ArKO tissues.

Table 2

Upregulation of KEGG pathways in the lacrimal glands of male and female mice with complete estrogen absence

KEGG pathway	Genes↑	z-score
Female		
Metabolic pathways	88	4.75
Valine, leucine and isoleucine degradation	10	4.57
Alzheimer's disease	20	3.81
Peroxisome	11	3.44
Fatty acid metabolism	7	3.11
Glycerolipid metabolism	7	2.96
Oxidative phosphorylation	13	2.72
Male		
Amino sugar and nucleotide sugar metabolism	6	3.75
Metabolic pathways	57	3.49
Glycolysis / Gluconeogenesis	7	3.29
$F_c\gamma$ receptor-mediated phagocytosis	8	2.75
Fructose and mannose metabolism	4	2.64
Pyrimidine metabolism	8	2.49
Glutathione metabolism	5	2.2

KEGG pathways (7 genes/sex) with the highest z-scores in lacrimal glands of ArKO mice are listed.

Table 3

Effect of complete estrogen absence on genotype- and sex-related gene expression in female and male mouse lacrimal glands

	Genes		
Group	**WT > ArKO**	**ArKO > WT**	**Total**
Female	978	770	1,748
Male	525	542	1,067
Male + Female	516	335	851
Group	**M>F**	**F>M**	**Total**
WT	1,176	1,199	2,375
ArKO	1,258	1,278	2,536
WT + ArKO	1,590	1,585	3,175

The expression of listed genes was significantly ($p < 0.05$) upregulated between the groups. Abbreviations: WT = wild type; M = male; F = female

Table 4

Impact of complete estrogen absence on gene expression ratios in the mouse lacrimal gland

Gene Identity	Gene	Ratio	p value	Ontology
F, WT > ArKO				
Reep6	Receptor accessory protein 6	17.3	0.0002	integral to membrane
Tulp2	Tubby like protein 2	8.0	0.0047	hydrolase activity
Abcb1a	ATP-binding cassette, subfamily B, member 1A	7.9	0.0069	ATP catabolic process
Gdf5	Growth differentiation factor 5	6.5	0.0186	growth
Slc39A8	Solute carrier family 39, member 8	6.3	0.0005	transport
Snap91	Synaptosomal-associated protein 91	5.2	0.0020	synaptic transmission
F, ArKO>WT				
Ly6f	Lymphocyte antigen 6 complex, locus F	75.8	0.0031	anchored to membrane
Cyp2j13	Cytochrome P450, family 2, subfamily j, polypeptide 13	74.8	0.0137	monooxygenase activity
Mrap	Melanocortin 2 receptor accessory protein	61.3	0.0000	positive regulation of cAMP biosynthetic process
Cuzd1	CUB and zona pellucida-like domains 1	31.2	0.0051	cell cycle
AK016936	ribosomal modification protein rimK-like family member B	22.3	0.0074	protein modification process
Slc12a1	Solute carrier family 12, member 1	15.7	0.0159	transport
M, WT > ArKO				
B4galt1	UDP-Gal:betaGlcNAc beta1,4- galactosyltransferase, polypeptide 1	9.4	0.0002	epithelial cell development
Fgl2	Fibrinogen-like protein 2	3.4	0.0017	signal transduction
Cuzd1	CUB and zona pellucida-like domains 1	3.3	0.0493	cell cycle
Prlr	Prolactin receptor, mRNA	3.1	0.0100	JAK-STAT cascade
Sertad1	SERTA domain containing 1	3.1	0.0310	transcription, DNA-dependent
Cnn3	Calponin 3, acidic	3.0	0.0387	actomyosin structure organization
M, ArKO > WT				
Nmu	Neuromedin U	471.5	0.0110	gastric acid secretion
Ddc	Dopa decarboxylase	302.1	0.0024	cellular amino acid metabolic process
Smr2	Submaxillary gland androgen regulated protein 2	51.1	0.0371	response to toxin
Klk1b24	Kallikrein 1-related peptidase b24	27.9	0.0233	proteolysis
Bmp6	Bone morphogenetic protein 6	20.3	0.0045	eye development
Pcdh20	Protocadherin 20	17.9	0.0312	cell adhesion

Relative ratios were calculated from Affymetrix data by comparing the degree of gene expression in lacrimal glands from male (M), female (F), WT and ArKO groups. Genes listed had a signal intensity of > 100 in at least one group, a comparative p value (between treatments) of < 0.05 and a known identity. Gene identities are shown as gene symbols, except for ribosomal modification protein rimK-like family member B. This gene does not yet have symbol, and the gene accession number is listed.

Table 5

Effect of estrogen absence and sex on gene expression in the mouse lacrimal gland

Comparison 1	Comparison 2	Total Genes	Common Genes	Common/Total (%)
F, WT>ArKO	M, WT>ArKO	1,211	86	7.1
F, ArKO>WT	M, ArKO>WT	938	81	8.6
WT, M>F	ArKO, M>F	617	587	23.6
WT, F > M	ArKO, F>M	706	670	25.5

Common genes were identified by using the Geospiza Intersector program. The expression of genes in the "Total" column was significantly (p < 0.05) upregulated in the designated comparison group.

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Table 6

Verification of selected GeneChip gene expression results

Gene Identity	Gene	Ratio			
		WT, Affy	ArKO, Affy	WT, CL	ArKO, CL
Female > Male					
Asgr1	Asialoglycoprotein receptor 1	56.8	88.5	46.0	60.9
Cyp2a5	Cytochrome P450, family 2, subfamily a, polypeptide 4	9.2	15.2	14.0	10.1
Prlr	Prolactin receptor	4.4	10.6	2.9	5.3
Lap3	Leucine aminopeptidase 3	3.1	3.8	2.9	3.6
Ccni	Cyclin I	2.9	1.5	2.7	2.4
Gstm1	Glutathione S-transferase, μ 1	2.1	1.9	2.0	2.8
Male > Female					
Eif2s3y	Eukaryotic translationinitiation factor 2,subunit 3	385.7	183.2	23.6	17.3
Cyp2j13	Cytochrome P450, family 2, subfamily j, polypeptide 13	156.9	2.5	553.9	2.9
Pgf	Placental growth factor	22.1	2.3	10.5	2.8
Slc27a22	Solute carrier family 27 (fatty acid transporter), member 2	9.1	1.6	14.2	3.0
Ccl8	Chemokine (C-C motif) ligand 8	5.2	5.7	7.1	5.5
Anxa5	Annexin A5	2.1	1.5	1.8	1.8

The expressions of selected genes, that were significantly altered in lacrimal glands of WT and ArKO mice according to Affymetrix GeneChip analyses, were reevaluated with CodeLink Bioarrays. Numbers represent the relative increase in gene expression. Abbreviations: Imm – Immortalized; Affy – Affymetrix; CL – CodeLink

Table 7

Comparative gene expression between lacrimal and meibomian glands of WT and ArKO mice

Comparison	MeibG genes different	LG genes different	Common genes	Common genes (%)
F, WT>ArKO	745	622	80	5.5
F, ArKO>WT	779	593	45	3.2
M, WT>ArKO	706	420	52	4.4
M, ArKO>WT	438	328	24	3.0
WT, M>F	1,398	817	158	6.7
WT, F>M	923	636	98	5.9
ArKO, M>F	912	852	153	8.0
ArKO, F>M	950	698	107	6.1

Lacrimal gland ("LG") Affymetrix data were from the present study, whereas meibomian gland ("MeibG") Affymetrix data were obtained from a recently published dataset (Rahimi Darabad et al., 2013). Comparisons were made with the Geospiza Intersector program. The percentage of common genes was determined by dividing the number of common genes by the total number of lacrimal and meibomian gland genes and multiplying by 100.