The Ocular Surface Phenotype of *Muc5ac* and *Muc5b* Null Mice

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Purpose. Recent development of mice null for either Muc5ac or Muc5b mucin allows study of their specific roles at the mouse ocular surface. A recent report indicated that *Muc5ac* null mice show an ocular surface phenotype similar to that seen in dry eye syndrome. The purpose of our study was to determine the effect of lack of Muc5ac or Muc5b on the ocular surface, and to determine if environmental desiccating stress exacerbated a phenotype.

Methods. *Muc5ac* null and *Muc5b* null mice, and their wild-type controls were examined for ocular surface defects by fluorescein staining. The number of goblet cells per area of conjunctival epithelium was counted, and levels of mucin gene expression and genes associated with epithelial stress, keratinization, and differentiation, known to be altered in dry eye syndrome, were assayed. To determine if the null mice would respond more to desiccating stress than their wild-type controls, they were challenged in a controlled environment chamber (CEC) and assessed for changes in fluorescein staining, tear volume, and inflammatory cells within the conjunctival and corneal epithelia.

Results. Unlike the previous study, we found no ocular surface phenotype in the *Muc5ac* null mice, even after exposure to desiccating environmental stress. Similarly, no ocular surface phenotype was present in the *Muc5b* null mice, either before or after exposure to a dry environment in the CEC.

Conclusions. Our results indicate that deleting either the *Muc5ac* or *Muc5b* gene is insufficient to create an observable dry eye phenotype on the ocular surface of these mice.

Keywords: mucins, dry eye, animal models

Goblet cells embedded within the mucosal epithelium or present in submucosal glands secrete mucin onto the surface of the epithelia, where it is moved over the epithelium by ciliary action, peristalsis, or other movements. At the ocular surface, the blinking of the eyelid moves the conjunctival goblet cell–derived mucin over the surface of the eye. Movement of the secreted mucin is thought to clean and trap debris and pathogens, and to remove the material from the surface of the epithelium. In the case of the ocular surface epithelium, the debris is moved away through the punctum. Due to their hydrophilicity, imparted by glycans O-linked to their protein core, mucins also are thought to lubricate and maintain hydration on the epithelial surface (see the reviews of Gipson and Argueso,[1] and Gipson[2]). At the ocular surface of mice, goblet cells embedded in the conjunctival epithelium reach maximum numbers in the fornicial region of the conjunctiva,[3] while in humans, goblet cells are prevalent in the fornix, reaching their maximum in the inferior nasal fornix.[4] Cicatrizing and drying eye diseases show diminution of numbers of goblet cells in the conjunctiva in early disease, and total loss of these cells with end-stage keratinization.[5] From these later studies, in which numbers of goblet cells were inversely proportional to the severity of disease, the concept of the importance of the goblet cell to ocular surface health has been proposed. To test the function of goblet cells in maintenance of ocular surface health, we recently studied mice null for a member of the E26 transformation–specific (ETS) transcription factor family, sterile α motif pointed domain epithelia specific transcription factor (SPDEF), which regulates goblet cell differentiation in the conjunctiva.[6] Mice null for the *Spdef* gene lack conjunctival goblet cells. These mice show a mild dry eye phenotype with increased fluorescein staining and tear volume, and an increase in inflammatory and CD45-positive cells within the conjunctival epithelium. They also show an upregulation in genes associated with inflammation (*Il-1α*, *Il-1β*, and *Tnf-α*), and epithelial stress, differentiation, and keratinization (*Sprr2b*, *Tgm1*, and *K17*), and a downregulation in genes for goblet cell products (*Muc5ac, Muc5b,* and *Tff1*). It is not known if loss of specific goblet cell products, for example, the mucins Muc5ac or Muc5b, are responsible for the phenotype seen in the *Spdef* null mice.

By definition, mucins, the glycoproteins produced by all wet-surfaced mucosal epithelia, have two common features. Firstly, they have tandem repeats of amino acids in their protein backbone. These variable number of tandem repeats (VNTRs) are rich in serine, threonine, and proline. The presence of serine and threonine gives rise to the second defining characteristic of mucins, that of heavy O-glycosylation. Serine and threonine serve as the sites of O-glycan addition.[7,8] To date, 21 mucin genes have been identified; they are named in order

of characterization as MUCs 1, 2, and so forth (mouse homologues are designated Mucs 1, 2, and so forth) and they fall into two categories: secreted mucins and membrane-associated or membrane-tethered mucins (see the reviews of Gendler and Spicer,[7] Moniaux et al.,[8] and Hollingsworth and Swanson[9]). Of the secreted mucins, MUCs 2, 5AC, 5B, and 6 are the exceptionally large "gel-forming" mucins expressed by goblet cells or glandular cells of the ocular surface, as well as respiratory, gastrointestinal, and reproductive tract epithelia. Initially, MUC5 was reported to be a single mucin; however, subsequent studies suggested that MUC5 was, in fact, three distinct mucins; thus, MUC5A, MUC5B, and MUC5C were named. Upon more complete sequencing, MUC5A and MUC5C were found to be from the same gene, thus the current nomenclature designates MUC5AC and MUC5B as two distinct secreted mucins.[10] These large, secreted mucins are among the largest-known glycoproteins, with molecular weights of approximately 600 kDa.[8] Their size after secretion is even larger (~40 MDa), as they contain cysteine-rich domains in the N and C regions that facilitate homomultimerization, allowing for the formation of viscous mucus (see the review of Gipson and Argueso[1]). As demonstrated by in situ hybridization and immunohistochemistry, goblet cells of the human conjunctival epithelium express the large secretory mucin MUC5AC.[11,12] Recently, data suggest that the mouse conjunctival goblet cells express Muc5b in addition to Muc5ac, albeit at much lower levels.[6,13] It is not clear whether Muc5ac and Muc5b are expressed by the same goblet cell within the conjunctival epithelium or by different goblet cells.

Direct testing of the function of specific secreted mucins produced by conjunctival goblet cells has not been feasible, but the recent development of mice null for either *Muc5ac* or *Muc5b* allows study of their specific roles at the mouse ocular surface. A recent study of mice null for the *Muc5ac* gene reports that lack of the major secretory mucin Muc5ac results in functional and structural changes of the ocular surface in these mice.[14] Furthermore, this report suggests that *Muc5ac* null mice have a major ocular surface phenotype similar to that seen in dry eye syndrome, with decreased tear breakup time (TBUT) and increased corneal fluorescein staining observed. Additionally, corneal opacification of varying severity and location was observed in some of the null mice. Using the same *Muc5ac* null mice, as well as mice null for *Muc5b*, we examined the ocular surface for the presence of a dry eye phenotype. Results from our study differed greatly from those reported by Floyd et al.,[14] as we found no major ocular surface phenotype in the *Muc5ac* null mice; in fact, no identifiable ocular surface phenotype was observed, even after exposure to desiccating environmental stress. Moreover, our results showed no ocular surface phenotype present in the *Muc5b* null mice, either before or following exposure to a dry environment in the controlled environment chamber (CEC). Taken together, our results indicated that deletion of either the *Muc5ac* or *Muc5b* gene is not sufficient to create an observable dry eye phenotype on the ocular surface of these mice.

Methods

Mouse Models

Mice null for the goblet cell-derived, secreted mucins Muc5ac or Muc5b were developed at the University of Texas, MD Anderson Cancer Center, Houston, Texas, and generously provided by Christopher Evans, PhD. The generation of the *Muc5ac* null mouse has been described previously in detail.[14,15] Briefly, the Muc5ac locus was targeted by insertion of LoxP sites into the 5′ flanking region and intron 1 in CJ7 embryonic stem cells. Null mice then were produced by mating founder animals with Zp3-Cre transgenic (C57BL/6-Tg(Zp3-cre)93Knw/J) mice and subsequently crossing progeny with C57BL/6J mice. Mice were backcrossed onto a C57BL/6J lineage, and saturation of the C57BL/6J genome was confirmed using microsatellite marker-assisted congenic analysis at the University of Texas, MD Anderson Cancer Center Genetic Service Facility. For this study, DNA extracted from ear punches was screened by short-range PCR to identify *Muc5ac* null (–/–) animals and to confirm knockout of the *Muc5ac* gene. Age-matched wild-type C57BL/6 animals were purchased from Charles River Laboratories (Wilmington, MA) to serve as experimental controls.

The development of the *Muc5b* mouse has been described in detail.[16] Briefly, *Muc5b* null mice were generated using a targeting allele with LoxP sites flanking the exon. The targeting allele was linearized with NotI and electroporated into CJ7 embryonic stem cells at the MD Anderson Cancer Center Genetically Engineered Mouse Facility. The CJ7 cells are derived from a 129S1 mouse with an agouti coat color. Homologous insertion of the targeting allele was confirmed by long-range PCR, restriction digest, and DNA sequencing. One targeting allele-positive cell line was expanded and inserted into albino C57BL/6J blastocysts to generate chimeric founders. Chimeric males were mated with C57BL/6J females, and agouti progeny were screened for targeting allele transmission. Positive mice subsequently were bred with Rosa26-Flpe recombinase knock-in animals to remove the neomycin selection cassette. Progeny were crossed with CMV-Cre transgenic mice to generate full null animals, resulting in animals that were heterozygous mosaics for Muc5b ($Muc5b^{\Delta/+}$) in all tissues, including the germline. Germline transmission was confirmed by breeding Cre-positive $Muc5b^{\Delta/+}$ and wild type C57BL/6J mice. Thus, the Muc5b recombinant progeny were identified as $Muc5b^{+/-}$. The F1 $Muc5b^{+/-}$ mice were intercrossed, and resulting litters were viable with typical mendelian inheritance distributions. Mice were backcrossed continuously by breeding heterozygotes with wild-type C56BL/6J mice for >5 generations using strain-specific microsatellite marker–facilitated analysis (speed congenics) to confirm congenic status (>99%). For this study, DNA extracted from ear punches was screened by short-range PCR to identify *Muc5b* null (–/–) animals and to confirm knockout of the *Muc5b* gene. Age-matched *Muc5b* null mice and their wild-type controls were used for experiments.

All animal protocols were approved by the Schepens Eye Research Institute Institutional Animal Care and Use Committee (IACUC), and adhere to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Tissue Collection and Histology

Heads from *Muc5ac* and *Muc5b* null and wild-type mice were submersed in 10% formalin for fixation; eyes with intact lids then were excised, embedded in methacrylate, sectioned, and stained with PAS to determine presence of goblet cells. Also, unfixed eyes with intact eyelids were dissected and embedded in optimal cutting temperature (OCT), frozen on dry ice, and stored at –80°C until use for immunofluorescence microscopy and laser capture microdissection to harvest mRNA from conjunctiva for subsequent assay of expression levels of mucins and stress-related proteins.

Goblet Cell Density

Goblet cell density within the conjunctival epithelium was determined in PAS–stained methacrylate cross-sections of

conjunctival epithelium collected from midsagittal regions of the anterior segment in *Muc5ac*$^{-/-}$ ($n = 4$), *Muc5ac*$^{+/+}$ ($n = 4$), *Muc5b*$^{-/-}$ ($n = 4$), and *Muc5b*$^{+/+}$ ($n = 6$) mice. The total conjunctival area (from lid margin to limbus and from apical surface to epithelial basal lamina) was measured using Spot RT Software v3.1 (Spot Diagnostic Instruments, Inc., Sterling Heights, MI). Results are expressed as the number of goblet cells per 0.1 mm^2. Goblet cell density measurements were performed in a blind manner, with the genotype unknown to two independent examiners. Counts from the two blind examiners were averaged for data analysis.

CD45-Positive Inflammatory Cell Counts

The CD45-positive cells within the conjunctival epithelium, corneal epithelium, and corneal stroma of *Muc5ac* and *Muc5b* null and wild-type mice were identified and quantified using methods described previously.[6,17] Briefly, cryostat sections were incubated with Alexa Fluor 488 anti-mouse CD45 antibody (1:250 dilution; BioLegend, San Diego, CA) and the number of CD45-positive cells was counted in a blinded manner by two independent observers. Results are expressed as the number of CD45-positive cells per 1 mm linear length of conjunctival epithelial basal lamina.

Muc5ac and *Muc5b* Immunofluorescence Microscopy

Immunolocalization of Muc5ac was done on cryosections fixed in 2% paraformaldehyde with 0.1% glutaraldehyde for 15 minutes, and blocked in 5% normal donkey serum plus 1% BSA. Sections then were incubated for 1 hour at room temperature with a mouse monoclonal anti-MUC5AC antibody, 45M1 (1:50; gift from Jacques Bara, GHU Pierre et Marie Curie, Hôpital St-Antoine, Paris, France).[18,19] For Muc5b immunofluorescence, sections were left unfixed, rehydrated in PBS, and blocked in 5% normal donkey serum plus 1% BSA before incubation with chicken polyclonal anti-MUC5B antibody, 799 (1:500 dilution).[20] Sections then were incubated with either FITC donkey anti-mouse IgG secondary antibody (1:50 dilution) or FITC donkey anti-chicken IgG (1:50 dilution; Jackson ImmunoReseach Laboratories, Inc., West Grove, PA) for 1 hour, at room temperature. To determine if Muc5ac and Muc5b mucins are expressed by the same goblet cells, double labeling immunofluorescence microscopy was performed on frozen, unfixed sections. Sections were incubated for 1 hour in a mixture of the MUC5AC and MUC5B antibodies before incubation with a mixture of the same secondary antibodies described above except that the MUC5AC secondary antibody was a rhodamine-labeled (TRITC)– rather than a FITC-labeled antibody. All sections were coverslipped in Vectashield mounting medium (Vector Labs, Burlingame, CA) and then viewed on a Zeiss Photoscope III fluorescence microscope Carl Zeiss, Thornwood, NY).

Laser Capture Microscopy of Conjunctival Epithelium

Eyes with intact eyelids from *Muc5ac*$^{-/-}$, *Muc5b*$^{-/-}$, and their respective wild-type mice were cryostat-sectioned at −20°C and 6 μm sections were collected on PEN Membrane glass slides (Life Technologies, Grand Island, NY) for laser capture microdissection as described previously.[6] Briefly, sections were stained with hematoxylin and eosin (H&E) under RNase-free conditions, and 40% of the conjunctival epithelium, as measured from the deepest point of the fornicial cul-de-sac, was collected for RNA isolation.

Quantitative RT-PCR

Total RNA was isolated from laser-captured sections of conjunctival epithelium using a Qiagen RNeasy Micro Isolation Kit (Qiagen, Valencia, CA). The RNA integrity and concentration were determined using the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA) before and after the pooling of one eye from one male and one female animal into a single sample. The qRT-PCR was performed as described previously[21,22] using the Roche LightCycler 480 (Roche Applied Science, Indianapolis, IN) with RT^2 SYBR Green qPCR Mastermix chemistry (Qiagen). Prevalidated primer sets for *Muc5ac, Muc5b, Muc1, Muc4, K17, Tgm1,* and *Sprr2b* (SABiosciences, Frederick, MD) were used, with 18S RNA used as the endogenous control gene. Relative levels of mRNA were calculated for each gene examined using the ΔΔCt method described in the Qiagen RT2 qPCR Primer Assay Handbook, with the mean of the wild-type sample as the calibrator.

CEC Exposure

Muc5ac$^{-/-}$ ($n = 15$), *Muc5ac*$^{+/+}$ ($n = 17$), *Muc5b*$^{-/-}$ ($n = 9$), and *Muc5b*$^{+/+}$ ($n = 8$) mice were exposed to desiccating environmental stress in a CEC (XDry Corporation, Las Vegas, NV) for 15 days, with an average temperature of 20.6 + 0.6°C and an average relative humidity of 12.8 ± 2.2%. Before entering the CEC, *Muc5ac* and *Muc5b* null and wild-type control mice were examined for gross ocular surface and/or eyelid phenotype. Corneal fluorescein staining and tear volume measurements were obtained and analyzed as described previously[6] before entering the CEC and, subsequently, every three days. Briefly, photographs of corneal fluorescein staining were taken 3 minutes following application of 1 μL 2.5% sodium fluorescein to the corneal surface. Two independent examiners scored the corneal fluorescein staining in each image (with genotype and time in the CEC unknown) using a standardized National Eye Institute grading system.[23–25] To measure aqueous tear volume, a cotton thread with a phenol red indicator was placed into the lateral canthus of the conjunctival fornix and held in place for 30 seconds.[26] Wetting of the thread was measured in millimeters, using the scale on the thread box under a light microscope. Animals were euthanized following 15 days in the CEC (experimental day 16), and eyes with intact eyelids were excised and frozen or fixed for further assay.

Data Analysis and Statistics

Statistical analyses were performed using GraphPad InStat version 3.1a (GraphPad Software, Inc., La Jolla, CA). Mann-Whitney tests were used to evaluate differences in tear volume, fluorescein staining, and the number of inflammatory cells between groups of mice, based on genotype or time in the CEC, with $P < 0.05$ considered statistically significant. Student's *t*-tests were used to evaluate differences in relative expression of the genes of interest in all real-time qRT-PCR experiments, with $P < 0.05$ considered statistically significant.

Results

Muc5ac and *Muc5b* Null Mice Do Not Have an Altered Ocular Surface Phenotype

Mice null for either the *Muc5ac* or *Muc5b* gene were examined to determine if any alterations in ocular surface phenotype could be observed. Gross assessment of the appearance of the eyes and eyelids of *Muc5ac* and *Muc5b* null mice showed no significant changes, as they were indistinguishable from their

respective wild-type mice (Fig. 1). The *Muc5ac* and *Muc5b* null mouse colonies had been maintained in the Schepens Eye Research Institute vivarium for over two years, and no gross ocular surface abnormalities have been observed. However, some respiratory problems have been identified in the *Muc5b* null mice, beginning at approximately 5 months of age. Histologic examination of the conjunctival epithelium in *Muc5ac* and *Muc5b* null mice also showed no major detectable defects. Goblet cells were present within the conjunctival epithelium in both *Muc5ac*$^{-/-}$ and *Muc5b*$^{-/-}$ mice, and were similar in appearance to wild-type controls (Fig. 2A). Surprisingly, counts of goblet cells per unit area of conjunctival epithelium in either *Muc5ac* or *Muc5b* null mice were not statistically different from those of *Muc5ac* and *Muc5b* wild-type control mice (Fig. 2B), or from each other, despite short-range PCR verification that the *Muc5ac* and *Muc5b* genes, indeed, have been knocked out in *Muc5ac*$^{-/-}$ and *Muc5b*$^{-/-}$ mice, respectively.

Muc5ac and *Muc5b* Null Mice Show No Signs of Inflammation Within the Epithelia of the Ocular Surface

As inflammation is known to accompany dry eye disease, we assessed the number of CD45-positive inflammatory cells within the conjunctival epithelium (Fig. 3A), corneal epithelium (Fig. 3B), and corneal stroma (Fig. 3C) in *Muc5ac* and *Muc5b* null and wild-type mice. While some CD45-positive cells were observed within the conjunctival epithelium of *Muc5ac* and *Muc5b* null mice, very few CD45-positive cells were observed within the corneal epithelium or the corneal stroma (note the change in scale between Figs. 3B, 3C). The number of CD45-positive inflammatory cells were not statistically different in *Muc5ac* or *Muc5b* null mice compared to those of wild-type mice in any of the ocular surface tissues assayed.

Localization of Muc5ac and Muc5b in Conjunctival Goblet Cells

Mouse conjunctival goblet cells are known to secrete the mucins Muc5ac and Muc5b, with *Muc5ac* expression levels 3.5 times greater than that of *Muc5b*.[6,13] As expected, more mouse conjunctival goblet cells produced Muc5ac protein (Fig. 4A), while fewer conjunctival goblet cells produced Muc5b protein (Fig. 4C). The Muc5ac (Fig. 4B) and Muc5b (Fig. 4D) antibody

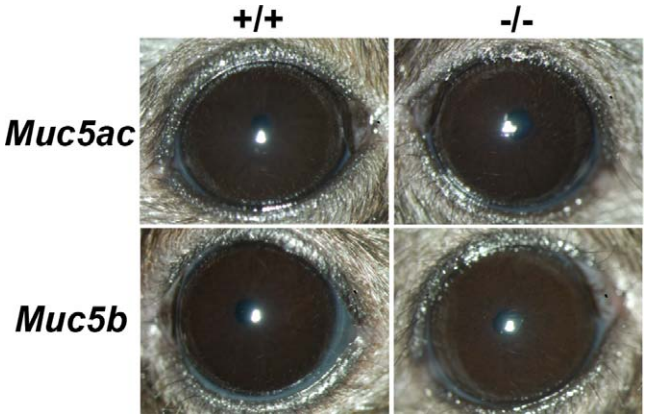

FIGURE 1. Examination of the eye and eyelids of *Muc5ac* and *Muc5b* null mice. No obvious changes or defects in the gross appearance of the cornea or eyelids were observed in either *Muc5ac* (*top panels*) or *Muc5b* (*bottom panels*) null mice compared to wild-type control mice.

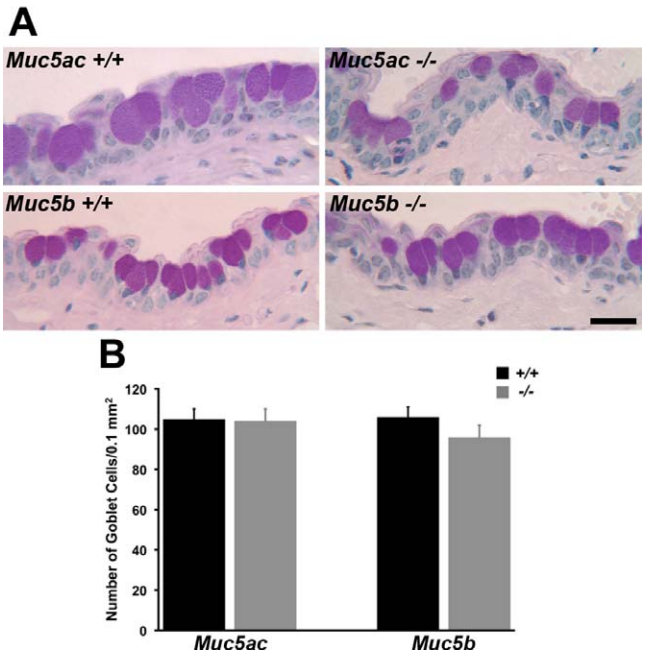

FIGURE 2. Goblet cells do not differ in morphology or number in *Muc5ac* and *Muc5b* null mice. (**A**) Goblet cells are present within the conjunctival epithelium in *Muc5ac* (*top panels*) and *Muc5b* (*bottom panels*) null mice, and are indistinguishable from those in *Muc5ac* and *Muc5b* wild-type mice. *Scale bar*: 10 μm. (**B**) No significant differences were observed in the number of goblet cells within the conjunctival epithelium in either *Muc5ac* or *Muc5b* null mice compared to their respective wild-type control mice, nor in comparison to each other. *Error bars* represent ± SEM.

binding was absent in *Muc5ac* null and *Muc5b* null mice, respectively, showing both knockout of the gene and antibody specificity. Immunohistochemical double labeling on sections from wild-type mouse conjunctival epithelium showed localization of Muc5ac and Muc5b protein in separate conjunctival goblet cells (Fig. 4E). Additionally, an increase in Muc5b labeling in conjunctival goblet cells of the *Muc5ac* null mice compared to *Muc5ac* wild-type (Figs. 4F, 4G) was observed.

Expression Level of the Mucin Muc5b Is Increased in *Muc5ac* Null Mice

To evaluate changes in gene expression in *Muc5ac*$^{-/-}$ and *Muc5b*$^{-/-}$ mice, quantitative qRT-PCR was performed on RNA isolated by laser capture microdissection from samples of the fornicial conjunctival epithelia of *Muc5ac*$^{-/-}$ and *Muc5b*$^{-/-}$ mice, as well as their respective wild types. Genes assayed for changes in expression patterns included mucin genes, both membrane tethered (*Muc1* and *Muc4*) and secreted (*Muc5b* and *Muc5ac*), and the epithelial stress, differentiation, and keratinization genes, *Sprr2b*, *Tgm1*, and *K17*, respectively, which are known to be upregulated in dry eye syndrome and in mice lacking goblet cells.[6] No changes in expression levels of the membrane-tethered mucins (Fig. 5A), nor in the epithelial stress, differentiation, nor keratinization genes (Fig. 5B), were observed in either *Muc5ac*$^{-/-}$ or *Muc5b*$^{-/-}$ mice as compared to their respective wild-type mice. However, a significant increase in the secreted mucin Muc5b was observed in *Muc5ac*$^{-/-}$ mice (Fig. 5A), corroborating the observation of increased Muc5b labeling in *Muc5ac* null mice. Interestingly, no change in the expression level of the secreted mucin *Muc5ac* was observed in *Muc5b*$^{-/-}$ mice (Fig. 5A). These data indicated that when the major secretory mucin Muc5ac is

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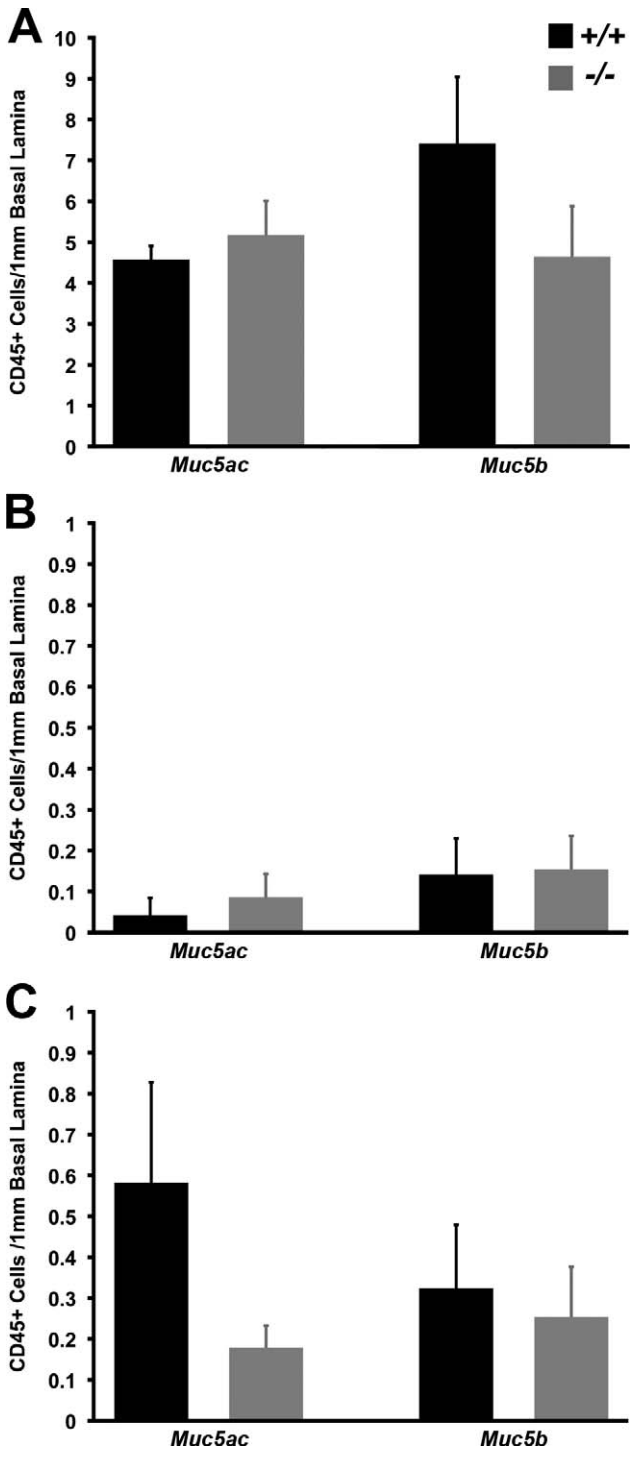

FIGURE 3. *Muc5ac* and *Muc5b* null mice do not display evidence of ocular surface inflammation. The number of CD45-positive cells within the conjunctival epithelium (**A**), corneal epithelium (**B**), and corneal stroma (**C**) of *Muc5ac* and *Muc5b* null mice were not statistically different than those of wild-type mice or of each other. Note the decrease in scale for (**B**, **C**), as very few CD45-positive cells were found in either the corneal epithelium or stroma. *Error bars* represent ± SEM.

missing, there is a compensatory upregulation in the minor secretory mucin Muc5b to compensate for the Muc5ac loss. However, when the minor secretory mucin Muc5b is missing, there is no need to compensate for Muc5b loss, as the major secretory mucin Muc5ac is still produced at levels similar to those seen in wild-type mice.

Exposure of *Muc5ac* and *Muc5b* Null Mice to Desiccating Environmental Stress Does Not Induce an Ocular Surface Phenotype

To test the effects of desiccating environmental stress on the ocular surface, *Muc5ac*$^{-/-}$, *Muc5b*$^{-/-}$, and age-matched wild-type mice were exposed to a low-humidity environment in a CEC for 15 days. Before entering the CEC (Day 0), neither *Muc5ac*$^{-/-}$ (Fig. 6A) nor *Muc5b*$^{-/-}$ (Fig. 6C) mice had fluorescein staining scores distinguishable from those of wild-type mice. Exposure to desiccating environmental stress had no effect on fluorescein staining, as scores of neither *Muc5ac* nor *Muc5b* null mice were different from those of wild-type mice throughout the duration of the 15-day CEC exposure.

Aqueous tear production also was assessed in *Muc5ac*$^{-/-}$ (Fig. 6B) and *Muc5b*$^{-/-}$ (Fig. 6D) mice over the course of exposure to environmental desiccating stress in the CEC. Tear volumes of *Muc5ac* and *Muc5b* null mice were similar to those of wild-type mice before entry into the CEC (Day 0). No significant differences in tear volume were observed in *Muc5b*$^{-/-}$ mice compared to wild-type mice throughout the 15-day CEC exposure (Fig. 6D). However, *Muc5ac*$^{-/-}$ mice had significantly lower tear volumes than their age-matched wild-type mice following 12 and 15 days of exposure in the CEC (Fig. 6B). Together, these data indicated that exposure to a dry environment did not affect corneal fluorescein staining in either *Muc5ac* or *Muc5b* null mice and, although the increase in tear volume noted in wild-type in response to environmental stress was not observed in *Muc5ac*$^{-/-}$ mice, major alterations in tear volume did not occur after CEC exposure. Thus, it appears that exposure of *Muc5ac* and *Muc5b* null mice to desiccating environmental stress does not induce an ocular surface phenotype consistent with that observed in dry eye syndrome.

DISCUSSION

Data from our study indicated that loss of either of the conjunctival goblet cell mucins, Muc5ac or Muc5b, does not produce an ocular surface phenotype consistent with dry eye in mice. A recently published manuscript using the same *Muc5ac* null mice as those used in this study reported that lack of the major secretory mucin Muc5ac caused significant functional and structural changes to the ocular surface in these mice, creating an ocular surface phenotype similar to that observed in dry eye syndrome.[14] While there are many major discrepancies between our data and those reported by Floyd et al.,[14] several findings are consistent between the investigations. Neither study found a change in the aqueous tear volume of *Muc5ac* null mice compared to that of age-matched wild-type control mice. Similarly, mice null for the *Muc5b* gene also had tear volumes similar to those of wild-type control mice. In our hands, even when challenged with exposure in the CEC to desiccating environmental stress, neither *Muc5ac* nor *Muc5b* null mice showed any changes in aqueous tear volume. These data suggested that, while the mucin composition of the tear film is altered by deletion of either the *Muc5ac* or *Muc5b* gene, the tear volume itself remains unchanged, even in response to desiccating environmental stress. Interestingly, elevated tear volumes are observed in *Spdef* null mice that completely lack conjunctival goblet cells and, thus, demonstrate loss of both *Muc5ac* and *Muc5b* expression. Perhaps the absence of both the secretory mucins Muc5ac and Muc5b is necessary to stimulate a compensatory

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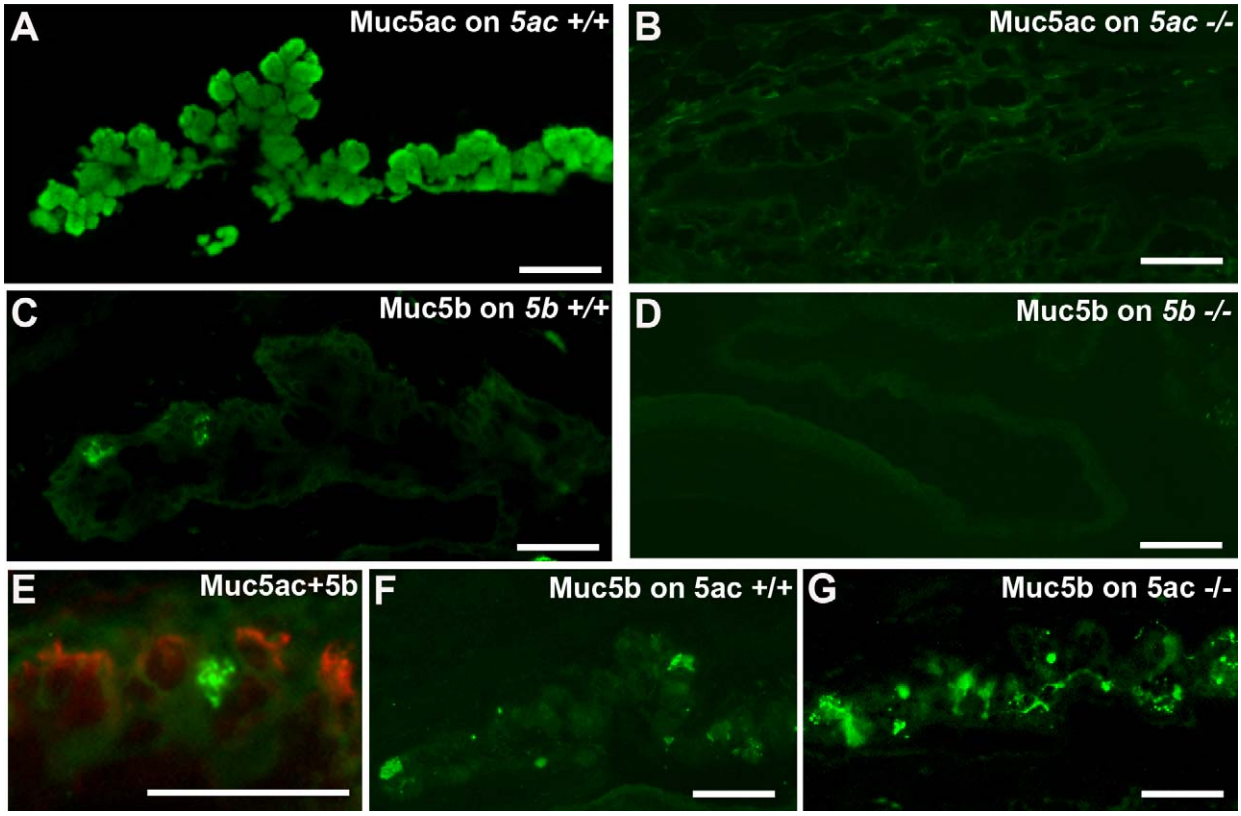

FIGURE 4. Localization of Muc5ac and Muc5b in conjunctival epithelial goblet cells of wild-type and *Muc5ac* and *Muc5b* null mice. Muc5ac protein was immunolocalized to most goblet cells within the conjunctival epithelium of wild-type (+/+) mice (**A**), whereas Muc5ac antibody binding was absent in *Muc5ac* null (–/–) mice (**B**), showing both knockout of the gene and antibody specificity. Muc5b protein was immunolocalized to only a few goblet cells within the conjunctival epithelium of wild-type mice (**C**) and Muc5b antibody binding was absent in *Muc5b* null mice (**D**) showing *Muc5b* knockout and antibody specificity. In double labeling experiments (**E**), Muc5ac (*red*) and Muc5b (*green*) localize to different goblet cells in wild-type mice, indicating that conjunctival goblet cells make one mucin exclusively. Comparison of the numbers of Muc5b-positive goblet cells in *Muc5ac* wild-type (**F**) to those in *Muc5ac* null mice (**G**) showed an increase in Muc5b localization in *Muc5ac* null mice, corroborating the finding that expression of *Muc5b* was upregulated significantly in *Muc5ac* null mice (see Fig. 6). *Scale bar*: 50 μm.

increase in tear volume; if only one mucin gene is deleted (either *Muc5ac* or *Muc5b*), the remaining mucin is able to compensate for the loss of the other one.

To add to this point, the study of Floyd et al.[14] and our study found that *Muc5ac* null mice have a significant increase in *Muc5b* mRNA expression. However, the converse was not observed, as *Muc5ac* expression levels remained unchanged in *Muc5b* null mice. This is not terribly surprising, given that findings from recent studies have suggested that, while Muc5ac and Muc5b are produced by murine conjunctival goblet cells and secreted onto the ocular surface, Muc5ac is present at much higher levels than Muc5b.[6,13] Thus, when the major secretory mucin Muc5ac is absent, we see a compensatory increase in expression of the minor secretory mucin Muc5b. However, if the minor secretory mucin Muc5b is absent, there is no need for production of additional mucin, as the major secretory mucin Muc5ac still is present.

While the study of Floyd et al.[14] and our current work both found that *Muc5ac* null mice have an aqueous tear volume similar to that of wild-type mice, and show a compensatory increase in *Muc5b* expression levels, there were many significant contradictions between the two studies. First, Floyd et al.[14] observed corneal opacification, which varied in location and severity, in a few *Muc5ac* null mice (4 of 35 animals examined), but not in wild-type control mice ($n = 15$). In our studies with the *Muc5ac* and *Muc5b* null mice, we found no obvious changes or defects in the gross appearance of the cornea or eyelids. No corneal opacity was ever noted in the *Muc5ac* or *Muc5b* null mice; in fact, the eyes of the null mice are virtually indistinguishable from those of wild-type control mice. As we have maintained a large number of *Muc5ac* (approximately 175 animals) and *Muc5b* (approximately 75 animals) null mice in the Schepens Eye Research Institute animal facility over the past 2 years with no observed ocular surface phenotype, it is possible that the corneal opacification observed within the smaller sample size used in the study of Floyd et al.[14] represents a few anomalies and not an actual phenotype. Alternatively, perhaps a difference in vivarium environments is responsible for the corneal opacification observed in the *Muc5ac* null mice by Floyd et al.[14] In fact, the investigators suggest that the low percentage of *Muc5ac* null mice with corneal opacification may reflect opportunistic injury or infection.[14] Thus, the corneal opacification noted in their study may be a secondary cause relating to infection-causing debris or pathogens and not actually the deletion of the *Muc5ac* gene. Additionally, another possibility is that the corneal opacities observed are occurring spontaneously within the mouse strain, as it has been noted that such opacities occasionally do occur in C57BL/6 mice.[27–29]

The second major discrepancy between the study of Floyd et al.[14] and ours is related to changes in conjunctival goblet cell morphology in *Muc5ac* null mice. While the other study reported that goblet cells within the conjunctival epithelium of *Muc5ac* null mice were larger and had an increased staining area compared to wild-type mice, data from our study suggested otherwise. Conjunctival goblet cells in *Muc5ac* and

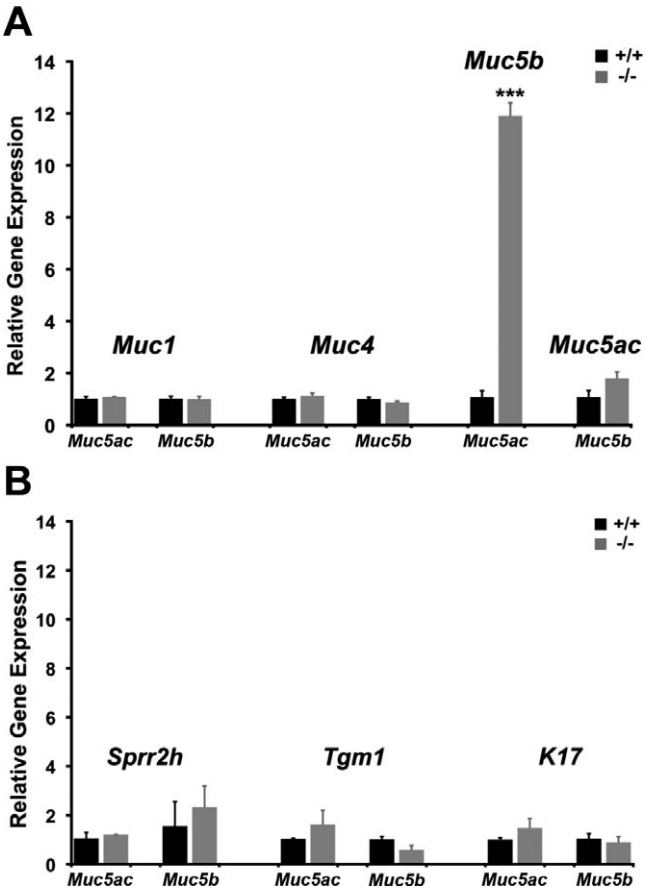

FIGURE 5. Expression levels of mucin genes (**A**), and epithelial stress, differentiation, and keratinization genes (**B**) in *Muc5ac* and *Muc5b* null mice. No significant changes in gene expression levels of *Muc1*, *Muc4*, *Sprr2h*, *Tgm1*, or *K17* were observed in either *Muc5ac* or *Muc5b* null mice compared to wild-type controls. Interestingly, while a significant increase in *Muc5b* gene expression in *Muc5ac* null mice was observed, *Muc5ac* gene expression levels in *Muc5b* null mice were not significantly different from *Muc5b* wild-type controls. *Error bars* represent ± SEM. $^{***}P < 0.001$.

Muc5b null mice were morphologically indistinguishable from those of wild-type control mice. Furthermore, no significant differences were observed in the number of goblet cells present within the conjunctival epithelium of *Muc5ac* or *Muc5b* null mice when compared to wild-type or to each other. Perhaps the contradiction in goblet cell morphology between the two studies was caused by differences in fixation or preservation methods (paraffin versus methacrylate), sectioning planes, location within the cross-section (nasal, temporal, or central), or thickness of sections.

A third difference between our current findings and those of Floyd et al.[14] was the level of corneal fluorescein staining observed in the *Muc5ac* null mice. Sodium fluorescein dye can be applied to the ocular surface to assess damage in the corneal epithelium. Increased uptake of the dye is considered to be a hallmark of dry eye syndrome and this methodology often is used in the clinic to diagnose dry eye disease.[5,23] While Floyd et al.[14] reported seeing an increase in fluorescein staining in *Muc5ac* null mice when compared to wild-type mice, we found no significant differences in fluorescein staining in either *Muc5ac* or *Muc5b* null mice compared to control mice. Furthermore, exposure to desiccating environmental stress in the CEC did not increase fluorescein staining in either *Muc5ac* or *Muc5b* null mice compared to baseline values and also to CEC-exposed wild-type control mice. Perhaps the discrepancy in the corneal fluorescein staining levels observed in the two studies was a result of different methodologies used for the diagnostic testing. The fluorescein staining method used in the two studies varied greatly. For example, the percent solutions of sodium fluorescein used, and the amount of fluorescein clearing time allowed following application and assessment were different between the two studies. Additionally, Floyd et al.[14] used HBSS rinsing and Q-tip blotting of fluorescein on the ocular surface, which were not used in our study. Moreover, two very different scoring methodologies were used to assess the levels of corneal fluorescein staining in the mice; the scoring system of Floyd et al.[14] was based on the number of spots and appearance of fluorescein plaques, whereas our scoring system was based on the NEI standard scoring system used by many other researchers assessing corneal fluorescein staining.[23–25] Thus, the differences in corneal fluorescein staining methodologies and scoring systems between the two studies could account for the discrepancy in fluorescein staining observed by Floyd et al.[14] and in our current study.

Another major difference between our findings and those of Floyd et al.[14] is that, whereas the latter investigators concluded that all goblet cells produce both Muc5ac and Muc5b, our study showed that Muc5ac and Muc5b are produced by different goblet cells, with only a few cells in the conjunctival epithelium producing Muc5b. These differences may reflect specificity of the Muc5b antibodies used in the two studies, as both studies used the same MUC5AC antibody, 45M1, which is a well-characterized antibody whose localization pattern matches mRNA production for MUC5AC. Our study used an antibody that we made to a synthetic peptide to the Human MUC5B. The specificity of the antibody was demonstrated in immunofluorescence studies that showed it bound only to tissues expressing MUC5B mRNA.[20] This antibody showed no localization to the tissues of *Muc5b* null mice, whereas it did localize to a few goblet cells in wild type and *Muc5ac* null animals, indicating its specificity.

The surprising finding of our study is that despite lack of the major secreted mucin Muc5ac, goblet cell numbers within the conjunctiva of the mice remained unchanged, suggesting that mucin content may not be the only requirement for secretory vacuole formation in the cell. The increased expression of *Muc5b* coupled with the increased number of Muc5b-expressing goblet cells in *Muc5ac* null mice may account for the similarity between the goblet cell counts in *Muc5ac* nulls and wild-type mice. However, it is unlikely that the increase in Muc5b seen in the *Muc5ac* nulls could account for the whole goblet cell population. Perhaps the goblet cell secretory granules contain other glycoproteins, previously unknown. We did find, in comparisons of array analysis of *Spdef* null mice and their wild-type, that goblet cells are producing components, particularly members of the Wnt signaling pathway, heretofore unknown.[6]

Despite a thorough examination of *Muc5ac* and *Muc5b* null mice showing no ocular surface phenotype, there are several limitations to our current study. Firstly, we are assessing the functions of the secretory mucins Muc5ac and Muc5b on the ocular surface independently of one another, not in conjunction with one another. Currently, there is no *Muc5ac* and *Muc5b* double null mouse, and creating one simply through the breeding process would be difficult as the *Muc5ac* and *Muc5b* genes are located on the same chromosome (chromosome 11p15.5 in humans),[30] chromosome 7 in mice,[31] and homologous recombination would have to occur to knock out both genes. However, our laboratory recently has characterized the ocular surface phenotype for another knockout animal, the *Spdef* null mouse, which lacks goblet cells within the conjunctival epithelium, and thus, goblet cell products, such

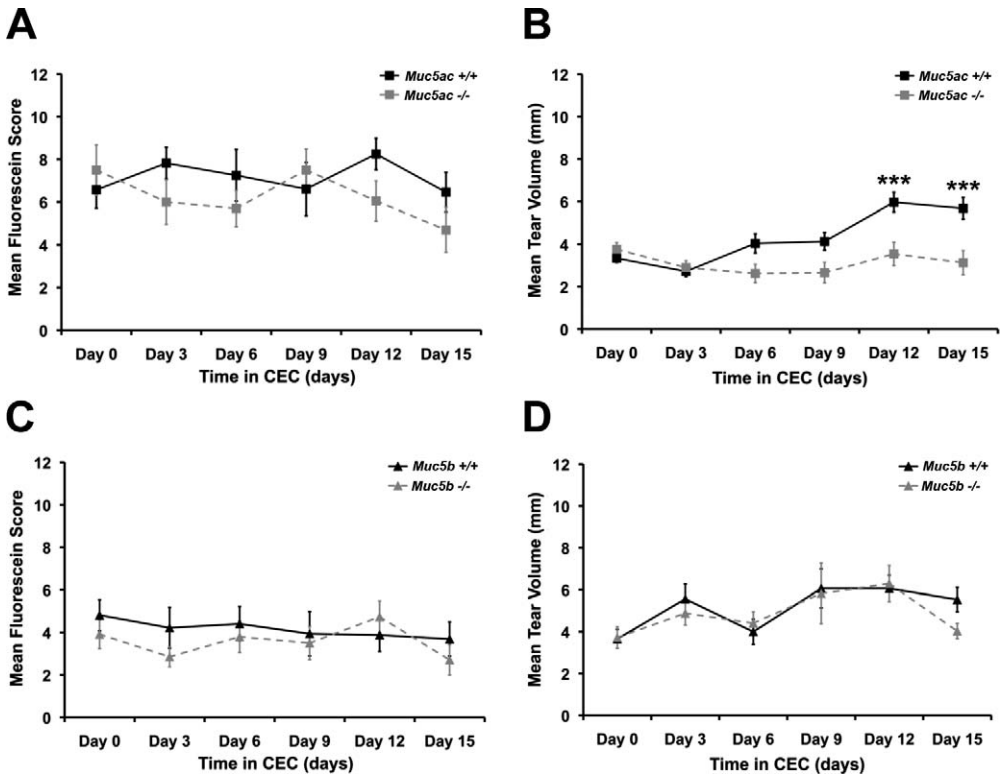

FIGURE 6. Exposure of *Muc5ac* and *Muc5b* null mice to desiccating environmental stress. (**A**) Fluorescein staining scores were not significantly different in *Muc5ac* null mice before entry into the CEC (Day 0) and were not statistically different from wild-type control mice during exposure to desiccating stress in the CEC. (**B**) Tear volume in *Muc5ac* null mice was not significantly different from wild-type mice before exposure to desiccating stress and did not change over time in the CEC; however, wild-type mice showed a significant elevation in tear production during CEC exposure at days 12 and 15. Neither fluorescein staining scores (**C**) nor tear volume (**D**) were significantly different in *Muc5b* null mice before entry into the CEC, and both fluorescein staining scores and tear volumes remained similar to wild-type mice throughout the duration of CEC exposure. *Error bars* represent ± SEM. $^{***}P < 0.001$.

as Muc5ac and Muc5b mucins.[6] The transcription factor SPDEF has been shown to be critical for goblet cell differentiation in tracheobronchial,[32,33] gastrointestinal,[34,35] and conjunctival[6,13] epithelia. In addition to the loss of conjunctival goblet cells, *Spdef* null mice demonstrated increases in corneal fluorescein staining, aqueous tear volume, and the number of inflammatory cells within the conjunctival epithelium. Furthermore, these mice had decreased expression of *Muc5ac* and *Muc5b*, and increased expression of genes associated with epithelial stress, differentiation, and keratinization (*Sprr2b*, *Tgm1*, and *K17*), which have been shown to be upregulated in dry eye disease.[36–39] Interestingly, despite completely lacking conjunctival goblet cells, and, thus, goblet cell products, such as Muc5ac and Muc5b, *Spdef* null mice only have an ocular surface phenotype consistent with early/moderate dry eye. Thus, it is not entirely surprising that mice null for only *Muc5ac* or *Muc5b* do not show evidence of a dry eye phenotype, as lack of both Muc5ac and Muc5b in the *Spdef* null mouse results in only moderate dry eye disease.

Moreover, we are limited by the fact that mouse models of gene deficiency may not predict the human condition. Mice may have compensatory mechanisms and redundancies within their ocular surface system that allows for knockout of some genes without greatly altering function or phenotype on the ocular surface. For example, conjunctival goblet cells in mice contain the secretory mucins Muc5ac and Muc5b (albeit Muc5b is expressed at lower levels), whereas human conjunctival goblet cells secrete only MUC5AC.[11,12] In mice, when the major secretory mucin Muc5ac is knocked out, as it is in the *Muc5ac* null mice, not only is the secondary secretory mucin Muc5b present, but there actually is a significant increase in *Muc5b* expression levels in these *Muc5ac* null mice. This redundancy and compensation of Muc5b within the conjunctival goblet cells of *Muc5ac* null mice is absent in humans, such that diminution or absence of MUC5AC in humans may be the cause of tear film instability and surface damage on the ocular surface.[12] Another factor that may explain the lack of phenotype in the *Muc5ac* null mice is the presence of harderian glands in mice.[40] The secretions of these glands may be protective of the ocular surface. Since humans lack harderian glands, the secretions of the goblet cells may be more critical to ocular surface health.

Finally, another limitation of our study may be the Schepens Eye Research Institute vivarium, in that it may inhibit our ability to assess the potentiality for an ocular surface phenotype in the *Muc5ac* and *Muc5b* null mice. As our animals are maintained in a clean and specific pathogen-free environment unlike the natural murine habitat, it is possible that the artificially clean environment has prevented development of an ocular surface phenotype or is masking an increased susceptibility to infection in the *Muc5ac* and *Muc5b* null animals. Although a dry eye phenotype was not induced in these null mice even in response to exposure to desiccating environmental stress in the CEC, perhaps challenging *Muc5ac* and *Muc5b* null mice with a pathogen would induce an ocular surface phenotype in these mice.

In conclusion, our examination of the ocular surface in mice, null for either *Muc5ac* or *Muc5b*, revealed no major

identifiable phenotype on the ocular surface. In fact, exposure to desiccating environmental stress did not induce an ocular surface phenotype in either *Muc5ac* or *Muc5b* null mice. Our results indicate that deletion of either the *Muc5ac* or *Muc5b* gene is not sufficient to create an observable phenotype on the ocular surface of these mice, as all hallmarks of dry eye disease, such as increased fluorescein staining; increased numbers of inflammatory cells within the conjunctival epithelium, corneal epithelium, or corneal stroma; and upregulation of epithelial stress, differentiation, and keratinization genes are absent in these mice. Thus, we concluded that *Muc5ac* and *Muc5b* null mice are poor animal models of the human dry eye condition.

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Investigative Ophthalmology & Visual Science

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