Novel Insight Into the Role of CFTR in Lacrimal Gland Duct Function in Mice

Orsolya Berczeli,[1] Eszter Vizvári,[1] Máté Katona,[2] Dénes Török,[3] László Szalay,[1] Ferenc Rárosi,[4] István Németh,[5] Zoltán Rakonczay,[2,6] Péter Hegyi,[7,8] Chuanqing Ding,[9] and Edit Tóth-Molnár[1,10]

[1]Department of Ophthalmology, University of Szeged, Szeged, Hungary
[2]1st Department of Internal Medicine, University of Szeged, Szeged, Hungary
[3]Department of Anatomy, University of Szeged, Szeged, Hungary
[4]Department of Medical Physics and Informatics, University of Szeged, Szeged, Hungary
[5]Department of Dermatology and Allergology, University of Szeged, Szeged, Hungary
[6]Department of Pathophysiology, University of Szeged, Szeged, Hungary
[7]Institute for Translational Medicine and First Department of Medicine, University of Pécs, Pécs, Hungary
[8]MTA-SZTE Translational Gastroenterology Research Group, Szeged, Hungary
[9]Pharmacology & Pharmaceutical Sciences, Ophthalmology, University of Southern California, Los Angeles, California, United States
[10]Department of Pharmacology and Pharmacotherapy, University of Szeged, Szeged, Hungary

Correspondence: Edit Tóth-Molnár, Department of Ophthalmology, University of Szeged, 6720 Szeged, 10-11 Koranyi fasor, Hungary; tme@tmedit.hu.

CD and ET-M contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Submitted: July 2, 2017
Accepted: November 27, 2017

Citation: Berczeli O, Vizvári E, Katona M, et al. Novel insight into the role of CFTR in lacrimal gland duct function in mice. *Invest Ophthalmol Vis Sci*. 2018;59:54–62. https://doi.org/10.1167/iovs.17-22533

Purpose. The role of cystic fibrosis transmembrane conductance regulator (CFTR) in lacrimal gland (LG) function has only recently received some attention, mainly from our group. In the present study, we investigated the potential changes of LG pathology, tear secretion, ocular surface integrity, and fluid secretion in isolated LG ducts from CFTR knockout (KO) mice.

Methods. Tear production and ocular surface integrity were investigated in anesthetized wild-type (WT) and KO mice using cotton threads and fluorescein staining, respectively. Immunofluorescence was used to localize CFTR protein in the LGs. Ductal fluid secretions evoked by forskolin (10 μM); cell-permeable cAMP analogue (8-bromo cAMP, 100 μM); or carbachol (100 μM) were measured in isolated LG ducts using video-microscopy. Intracellular Ca2+ homeostasis underlying carbachol stimulation was investigated with microfluorometry.

Results. Significant decrease in tear secretion and impaired ocular surface integrity were observed in KO mice. Immunofluorescence demonstrated the predominant presence of CFTR protein in the apical membranes of the duct cells from WT mice. Continuous fluid secretion was evoked by forskolin and 8-bromo cAMP in LG ducts from WT mice, while no secretory response was observed in ducts from KO mice. Carbachol caused similar secretory responses in ducts from WT and KO animals without significant differences in cytosolic Ca^{2+} signaling.

Conclusions. Our results suggest the important role of CFTR in LG ductal secretion and in the maintenance of ocular surface integrity, suggesting that CFTR may be a promising target of novel therapeutic approaches in the treatment of dry eye.

Keywords: lacrimal gland, CFTR, lacrimal gland duct, CFTR KO mouse

Dry eye is the most common ocular surface disease affecting millions of people worldwide.[1] However, so far there are only very limited treatment options available, rendering the management of this debilitating disease very challenging.[2,3] Unfortunately, our understanding of the physiologic and pathologic mechanisms of lacrimal gland (LG) secretion is limited, despite its critical importance in developing new treatment strategies. Tear secretion is a complex process with the involvement of the main and accessory LGs, corneal and conjunctival epithelial cells and the Meibomian glands, etc. LG is the main source of fluid, electrolyte and proteins in the tear, and deficiency in its secretion results in aqueous deficient dry eye.[4,5]

LG secretion is mediated by an array of ion transporters and channels including cystic fibrosis transmembrane conductance regulator (CFTR), the chloride channel that is responsible for the driving force of fluid transport in various epithelial cells.[6] CFTR plays a critical role in the transmembrane transport of chloride in many secretory epithelia including pancreas, salivary glands, sweat glands, and airway epithelium and its defect may cause cystic fibrosis (CF), the most common genetic disease among Caucasians.[7–11] However, the role of CFTR in LG secretion remains largely unknown. Recent evidences from rat and rabbit LG studies demonstrated that CFTR are localized in both acinar and ductal cells, with its predominant presence in the ducts, suggesting it may play a key role in LG ductal fluid secretion.[12–14] Indeed, several clinical studies reported dry eye symptoms in CF patients, which further strengthens the potential influence of CFTR in altered tear secretion.[15–17]

The recent availability of transgenic mouse models carrying genetic defects in CFTR allows the direct examination of its role in many tissues and organs.[18–20] Here we report our studies of the functional role of CFTR in LG duct secretion by using CFTR

Table. Composition of Solutions Used in the Experiments

Compound	Content of Solution				
	HEPES Buffered Solution	HCO_3^-/CO_2^- Buffered Solution	Isolation Solution	Storage Solution	Culture Solution
NaCl, mM	140	115			
KCl, mM	5	5			
Na-HEPES, mM	10				
$MgCl_2$, mM	1	1			
$CaCl_2$, mM	2	1			
D-Glucose, mM	10	10			
$NaHCO_3$, mM		25			
Dulbecco's modified Eagle's medium			X	X	
Collagenase, U/mL			100		
Bovine serum albumin, mg/mL			1	0,03	
McCoy's 5A tissue culture medium					X
Fetal calf serum, vol/vol %					10
Glutamine, mM					2

transgenic mice. Our data strongly suggest that CFTR plays a key role in LG ductal secretion and in the maintenance of ocular surface integrity.

Parts of the results in this manuscript have been presented in abstracts in the Annual Meeting of the Association for Research in Vision and Ophthalmology (Tóth-Molnár E, et al. *IOVS* 2016;57:ARVO E-Abstract 5222; Ding C, et al. *IOVS* 2016;57:ARVO E-Abstract 430).

Materials and Methods

Animals

CFTR KO mice used throughout our studies were congenic on the FVB/N background. The model was originally generated by Ratcliff et al.[18] and was provided as a gift from Ursula Seidler (Hannover Medical School, Hannover, Germany).[19] Genotyping was performed by RT-PCR. The animals were kept at a constant room temperature of 24°C with a 12 hour light–dark cycle and were allowed free access to specific CFTR chow (C1013, Altromin, Lage, Germany) and drinking solution. Wild-type (WT) refers to the +/+ littermates of the CFTR KO mice. The mice used in this study were 8 to 24 weeks old and 14 to 24 grams (depending on the genotype and age), the sex ratio was 1:1 for all groups.

Animals were narcotized intraperitoneally with ketamine (80 mg/kg) and xylazine (10 mg/kg) and euthanized with pentobarbital overdose (100 mg/kg).

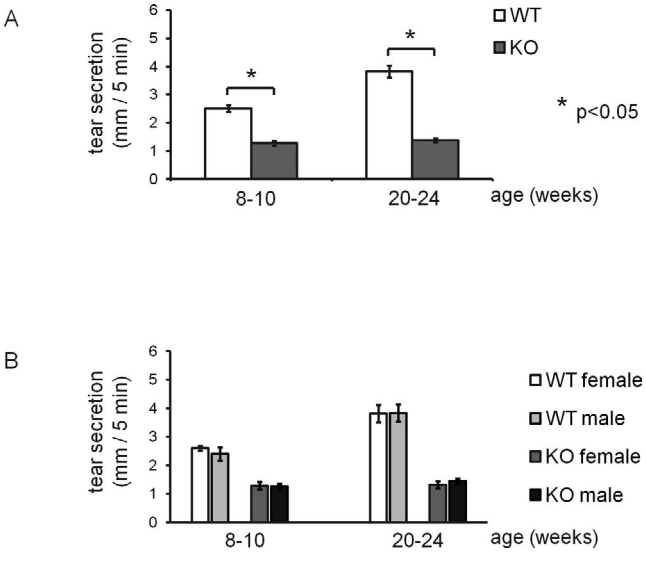

Figure 1. Tear secretions of WT and KO mice in 2 age groups. (**A**) Tear secretion was measured in two age groups in both WT and CFTR KO mice: at 8 to 10 weeks of age and at 20 to 24 weeks of age. Data from both eyes were averaged and evaluated. Tear secretion of CFTR KO mice were significantly lower compared to WTs in both age groups. (**B**) Secretion results of male and female mice were assessed and evaluated separately in WT and KO animals in both age groups. Sex-based data did not show significant difference in tear secretion either in WT or in KO mice. Data are presented as means ± SEM.

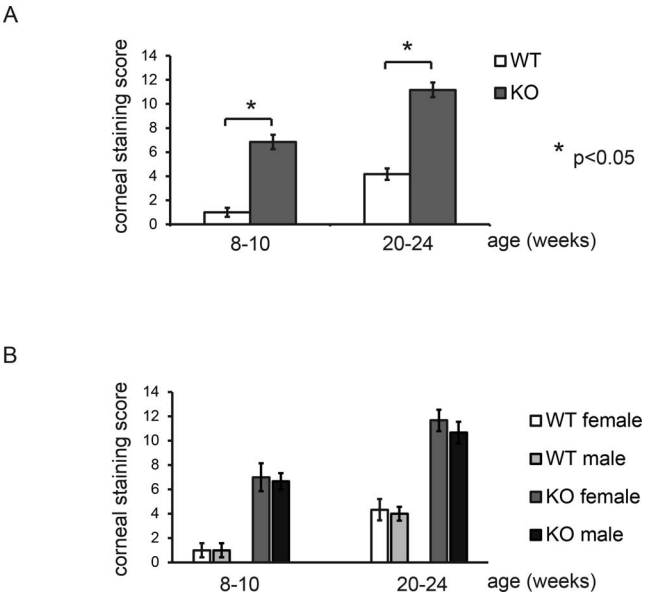

Figure 2. Corneal fluorescein staining in WT and CFTR KO mice. (**A**) Corneal fluorescein staining was measured in two age groups: at 8 to 10 weeks of age and at 20 to 24 weeks of age in both WT and CFTR KO mice. Data from both eyes were averaged and evaluated. Corneal staining scores of KO mice were significantly higher compared to WTs in both age groups. (**B**) Corneal fluorescein staining scores of male and female mice were calculated separately in WT and KO animals in both age groups. No significant differences were found between males and females either in WT or in KO mice. Data are presented as means ± SEM.

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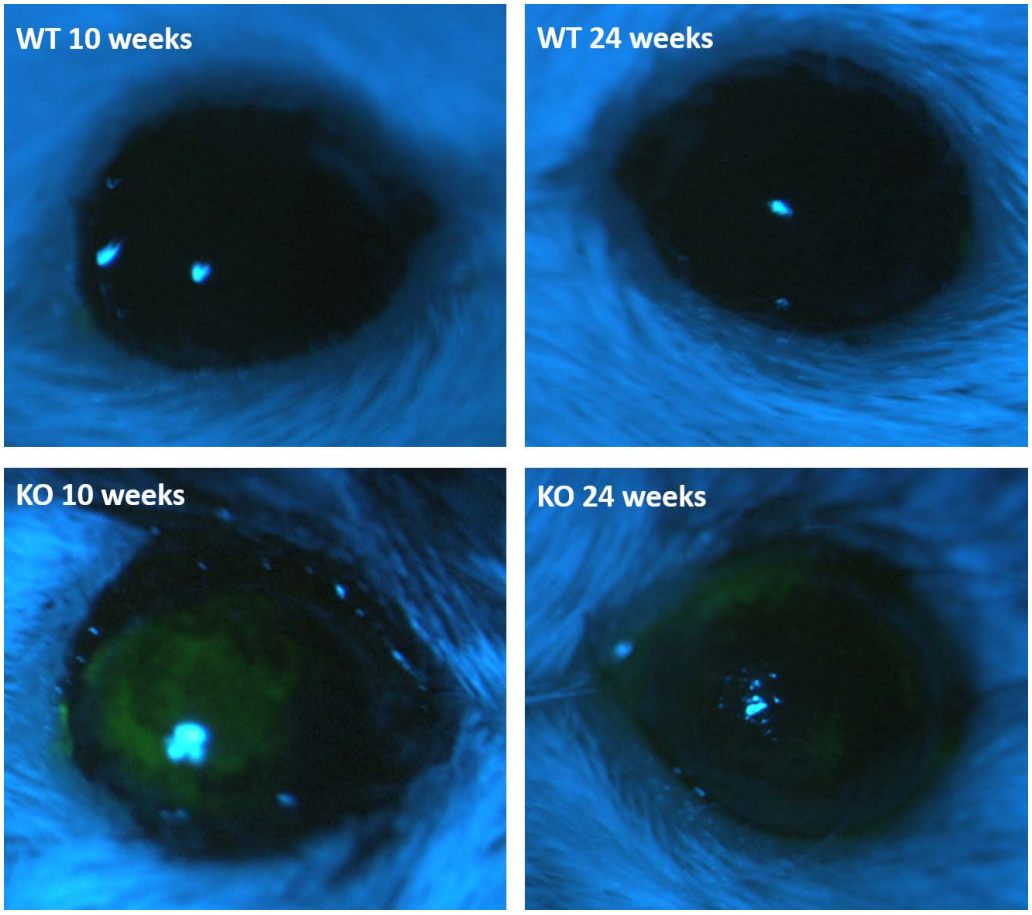

FIGURE 3. Evaluation of ocular surface integrity of WT and CFTR KO mice at 10 and 24 weeks. Virtually no fluorescence staining could be found in the corneas of WT mice, whereas profound staining were observed in the corneas from KO animals, in both age groups.

All experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol has been approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged, Szeged, Hungary and conformed to the Directive 2010/63/EU of the European Parliament.

Solutions and Chemicals

Media and its supplements (DMEM, McCoy, FCS, glutamine and BSA), carbachol (carbamoylcholine chloride), forskolin and 8-bromoadenosine-3–5-cyclic monophosphate (8-bromo cAMP) were purchased from Sigma-Aldrich Corp. (Budapest, Hun-

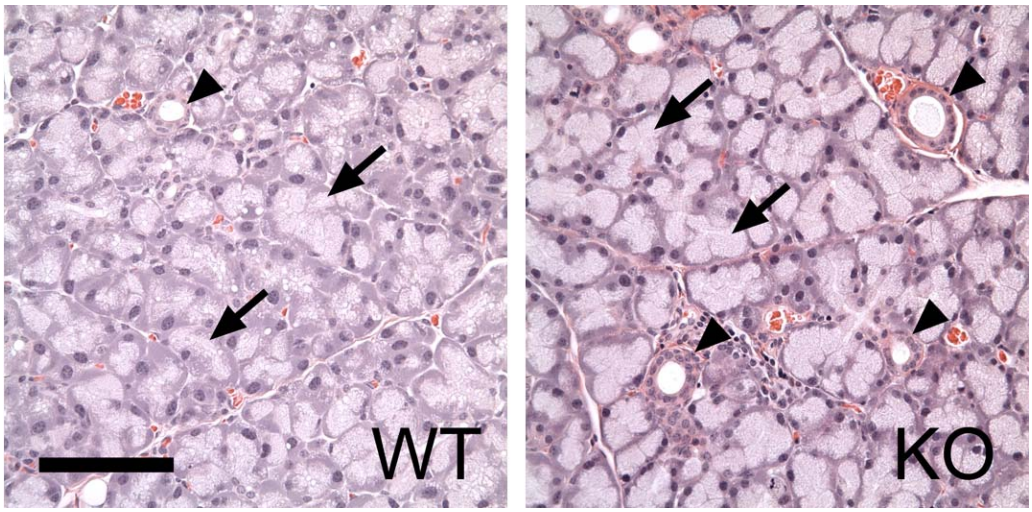

FIGURE 4. H&E staining of LG from WT and CFTR KO mice. While it appears that there are some increased luminal spaces within acinar cells (*arrows*) in WT mice, giving the image a less smooth appearance compared to that from KO mice, there are no significant morphologic changes of the LGs, including ducts (*arrowheads*). *Scale bar*: 100 μm.

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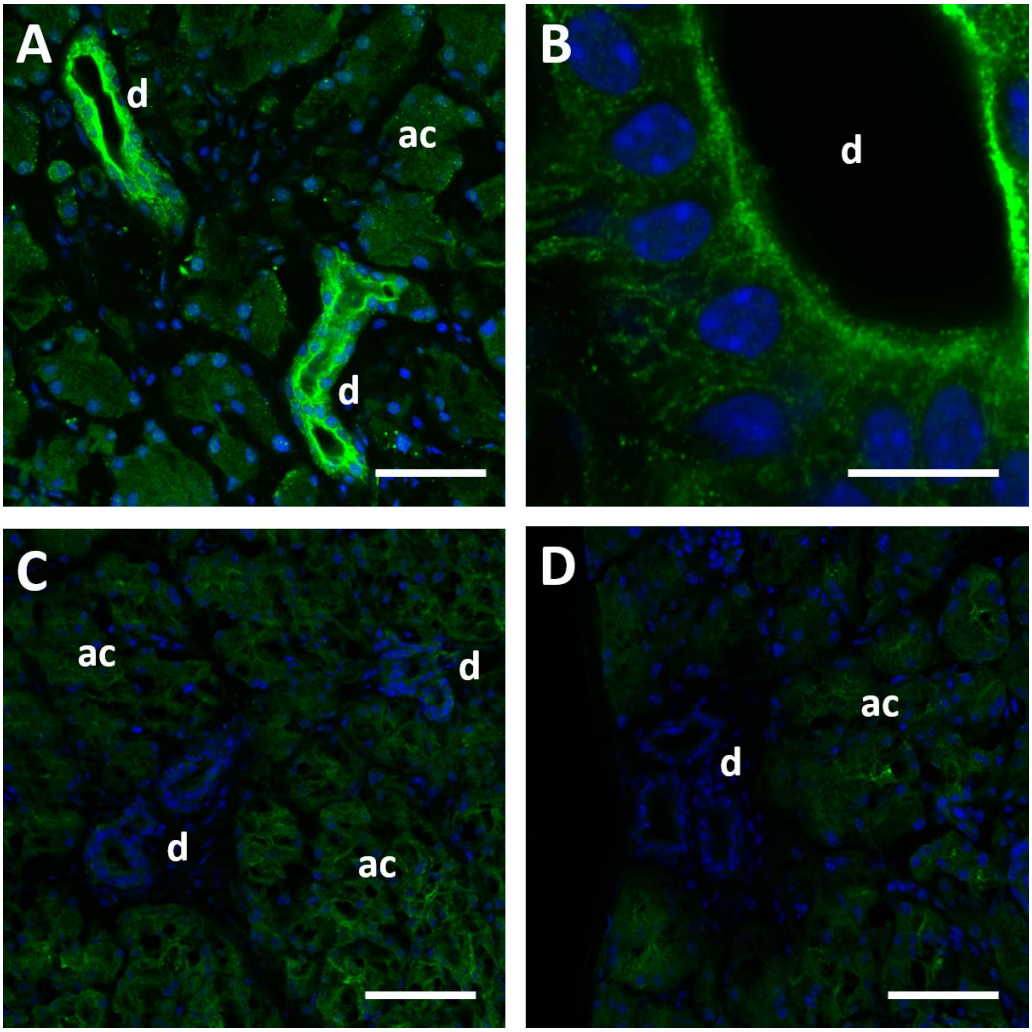

FIGURE 5. Immunofluorescence staining of CFTR in LGs from WT and CFTR KO mice. In LGs from WT mice (**A**, **B**), CFTR staining was most prominent in the apical membranes of duct cells (d), compared to acinar cells (ac). No staining could be observed in LGs from KO mice (**C**, **D**). DAPI was used to stain nuclei as blue. *Scale bar* in (**A**, **C**, **D**): 50 μm; in (**B**): 10 μm.

gary). Collagenase was purchased from Worthington Biochemical Corp. (Lakewood, NJ, USA). The composition of solutions used in our studies is summarized in the Table. The pH of standard HEPES-buffered solution was set to 7.4 with HCl at 37 °C. The standard HCO_3^-/CO_2^- buffered solution was gassed with 95% O_2/5% CO_2 at 37 °C. FURA2-AM was purchased from Invitrogen (Waltham, MA, USA).

Measurement of Tear Secretion and Corneal Fluorescein Staining

Tear production was measured in anesthetized mice using phenol red impregnated cotton threads (Zone-Quick, Showa Yakuhin Kako Ltd., Tokyo, Japan) applied into the lateral canthus of both eyes for 5 minutes. Color of the threads turns red in contact with the tears. Wetting length was measured in millimeters under a dissecting microscope.

Ocular surface integrity was evaluated by applying 0.5 μL of 5% fluorescein sodium into the conjunctival sac, followed by slit-lamp biomicroscopy (Inami L0198, Tokyo, Japan) through cobalt-blue filter. Images were captured with an anterior segment digital camera (Inami L-0541DC) attached to the slit-lamp. Staining was assessed by using the NEI grading system.[21] To quantify the staining changes, corneas were divided into five regions and staining was assessed and rated in each region from 0 to 3. Total scores from the five regions were recorded.

Hematoxylin & Eosin (H&E) Staining

Freshly dissected LG tissues, from both WT and KO mice, were fixed in 4% buffered formaldehyde and embedded in paraffin, then 3.5-μm thin serial sections were cut and stained with H&E.

Immunofluorescence

A total of 15-μm thick cryostat sections were rehydrated by washing in tris-buffered saline (TBS, 20 mM Tris-HCl, pH: 7.5, 150 mM NaCl) for 5 minutes, then fixed in 2% paraformaldehyde (PFA). After washing the sections in TBS three times for 5 minutes each, the samples were permeabilized with 0.1% Triton-X in TBS for 10 to 15 minutes. The sections were blocked with 5% FCS in TBS for 1 hour at room temperature followed by an overnight incubation with primary antibody for CFTR (1:100, Alomone Labs, Jerusalem, Israel) at 4°C. The next day the samples were incubated with secondary antibody, Alexa-488 conjugated goat anti-rabbit (1:1000, Abcam, Cambridge, UK) for 1 hour and Hoechst (1:1000, Sigma-Aldrich

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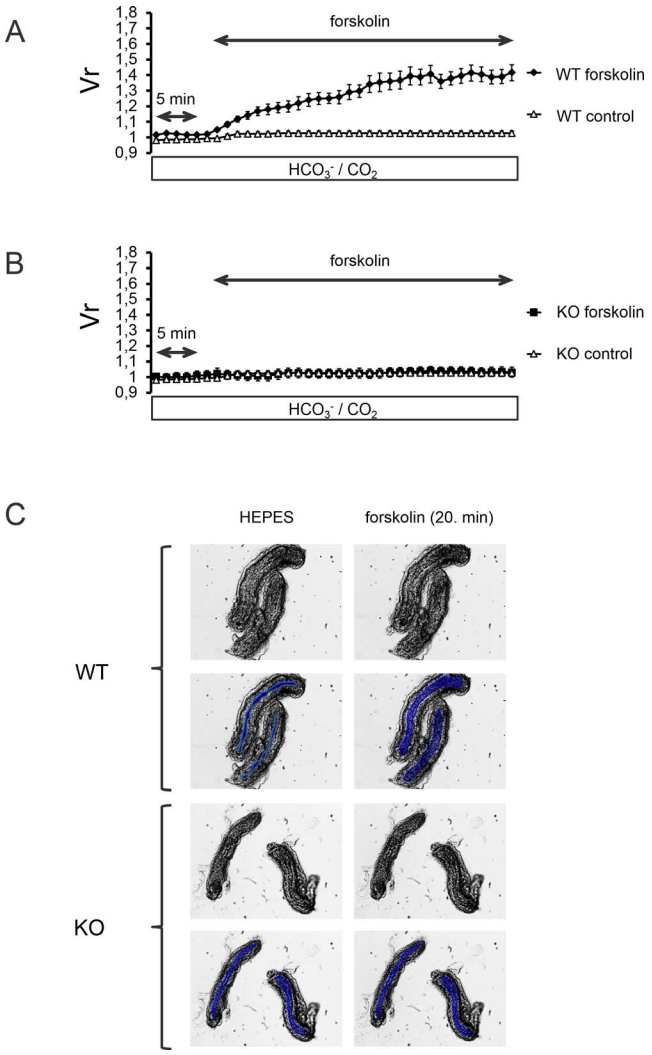

Figure 6. Effect of forskolin on fluid secretion in mouse ducts isolated from WT and CFTR KO LGs. (**A**) WT ducts were exposed either to 10 μM forskolin (filled rhombus) or to no agonist (*empty triangle*). (**B**) CFTR KO ducts were exposed either to 10 μM forskolin (*filled square*) or to no agonist (*empty triangle*). Changes in relative luminal volume (Vr) are shown. Data were obtained from six ducts isolated from three different animals in each series and are presented as means ± SEM. (**C**) Photo series of secreting isolated LG duct segments in response to forskolin stimulation shown in Supplementary Videos S2 and S3. The secretory responses observed in HEPES-buffered and in HCO_3^-/CO_2-buffered solutions were similar. The luminal space is marked with *blue* color.

Corp.) for 20 minutes at room temperature and examined under a confocal laser scanning microscope (Zeiss LSM 880; Oberkochen, Germany).

Isolation and Culture of Lacrimal Duct Segments

Interlobular and intralobar ducts from both WT and KO mice were isolated as previously described in detail by our laboratory.[22] Isolated ducts were cultured overnight in a 37°C incubator gassed with 5% CO_2.

Measurement of Ductal Fluid Secretion

Video-microscopic method was used to measure ductal fluid secretion. The method was originally developed for the measurement of pancreatic ductal fluid secretion and was modified by our laboratory for the investigation of LG duct secretion.[23,24] In brief, the ends of isolated LG ducts seal after overnight culture, forming a closed luminal space. Secretory process of the ductal epithelium results in swelling of the ducts as the luminal space fills with the secreted fluid. The change in ductal volume can be analyzed using video-microscopy. Commercial software (Scion Image; Scion Corp., Frederick, MD, USA) was used to analyze and calculate changes in the luminal space in each image.

Measurement of Intracellular Ca^{2+} Concentration

Intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ was measured using Ca^{2+}-sensitive fluorescent dye FURA 2AM (4–5 μM) as described earlier.[22] Changes in $[Ca^{2+}]_i$ were measured using an imaging system (xcellence; Olympus, Budapest, Hungary). We excited 4 to 5 small areas (region of interests: ROIs) of 5 to 10 cells in each intact duct with light at 340 and 380 nm, and the 380/340 fluorescence emission ratio was measured at 510 nm. One $[Ca^{2+}]_i$ measurement was obtained per second.

Statistics

Kruskal-Wallis test with Dunn method was used for the analysis of tear secretion. A mixed ANOVA model was applied. Effects of the stimulatory compounds (forskolin or carbachol) were taken into account as "fixed effects." The effect of the individual "duct" and the "duct and effects of forskolin, 8-bromo-cAMP or carbachol" interaction (we assumed that the value of the effect of the stimulatory compounds depend on the individual duct) were taken into account as random effects in the model. Statistical software (SigmaPlot Systat; Software, Inc., London, UK) was used to analyze the data, which were presented as means ± SEM. A value of $P < 0.05$ was regarded as significant.

Results

Tear Secretion and Corneal Fluorescein Staining

Tear secretion was measured in two age groups in both WT and KO mice, at 8 to 10 weeks of age and at 20 to 24 weeks of age. Data from both eyes were averaged and evaluated. Tear secretion result at 8 to 10 weeks of age was 2.47 ± 0.43 mm/5 min ($n = 11$) in WT mice, and 1.27 ± 0.19 mm/5 minutes ($n = 11$) in KO mice, while at 20 to 24 weeks of age, tear secretion was 3.86 ± 0.91 mm/5 minutes ($n = 11$) in the WT group and 1.36 ± 0.14 mm/5 minutes ($n = 10$) in the KO group. Tear secretion of KO animals in both age groups were significantly lower as compared to their WT littermates (8–10 weeks: $P = 0.0008$; 20–24 weeks: 0.0004). To study the effect of age (or duration of the disease in KO animals) in tear secretion, results were calculated not only vertically (i.e., separately in two different age groups, comparing WT and KO measurements, see above) but also horizontally, with the comparison of tear secretion in different age groups separately in WT and in KO animals. In WT animals, no significant difference was observed between the secretion data at the age of 8 to 10 weeks and 20 to 24 weeks. Similarly, we could not find statistically significant difference in tear secretion of KO animals measured in different age groups (Fig. 1A).

The potential difference in tear secretion between male and female mice was investigated in WT and in CFTR KO animals in both age groups. Tear secretion results calculated separately in male and in female mice did not show statistically significant difference either in WT or in KO mice (Fig. 1B).

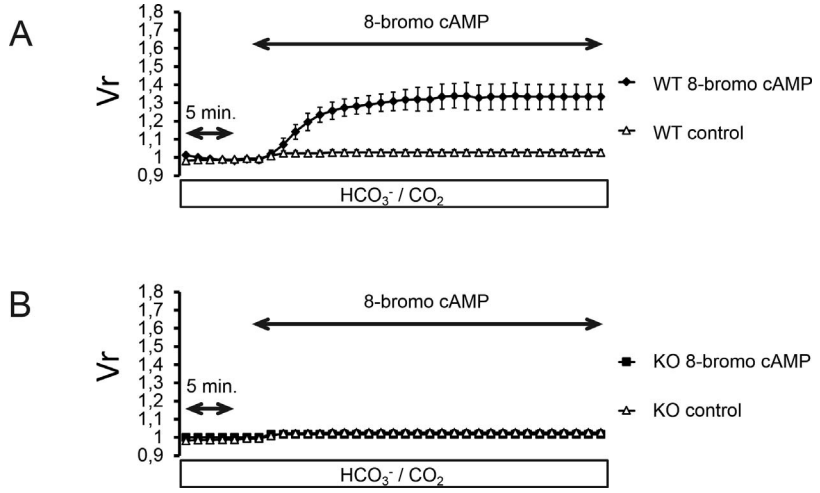

FIGURE 7. Role of 8-bromo cAMP on ductal fluid secretion isolated from WT and CFTR KO LGs. (**A**) WT ducts were exposed either to 100 μM 8-bromo cAMP (*filled rhombus*) or to no agonist (*empty triangle*). (**B**) CFTR KO ducts were exposed either to 100 μM 8-bromo cAMP (*filled square*) or to no agonist (*empty triangle*). Changes in Vr are shown. Data were obtained from six ducts isolated from three different WT animals and from six ducts isolated from two KO animals and are presented as means ± SEM.

Fluorescein staining was evaluated in both WT and KO mice at 8 to 10 weeks of age and at 20 to 24 weeks of age. Data from both eyes were pooled together. Corneal staining score was 1.0 ± 0.66 in WT mice ($n = 6$) and 6.83 ± 1.16 in KO mice ($n = 6$) in animals at 8 to 10 weeks of age, while in the 20 to 24 weeks group, the staining was 4.17 ± 0.88 in WT mice ($n = 6$) and 11.17 ± 1.16 in KO animals ($n = 6$). Corneal staining scores of KO animals in both age groups were significantly higher in KO mice as compared to their WT littermates (8 to 10 weeks: $P = 0.0002$; 20–24 weeks: 0.0004; Fig. 2A). Staining scores of female and male mice were also calculated separately. We could not find statistically significant difference in corneal fluorescein staining between female and male mice either in WTs or in KO animals (Fig. 2B). Corneal staining images of WT and KO mice at 10 weeks and at 24 weeks of age can be seen in Figure 3.

H&E Staining of LGs From WT and CFTR KO Mice

To assess the potential morphologic differences between LGs from KO and WT mice, freshly dissected LGs were processed for H&E staining. LGs of 8- to 10- and 20- to 24-week-old animals were investigated. However, no obvious structural changes have been observed in KO LG tissues as compared to their age-matched WT counterparts (Fig. 4).

Immunofluorescence

As demonstrated in Figures 5A and 5B, intense CFTR staining could be found most prominently in the apical membranes of LG duct cells from WT animals, although some diffuse staining was also found in acinar cells, mostly within the cytoplasm (Supplementary Video S1). This observation is consistent with previous reports in rat and rabbit, where the presence of CFTR mRNA and protein were demonstrated in acinar and ductal cells, with intense fluorescence staining in the apical membranes of the duct cells.[12,13,25] As anticipated, we were unable to detect the presence of CFTR protein in LGs from KO mice (Figs. 5C, 5D).

Forskolin and 8-Bromo cAMP-Induced Fluid Secretion of Isolated LG Ducts

Effects of forskolin stimulation on WT and KO LG interlobular and intralobar duct segments isolated from 14- to 24-week-old animals were investigated in HEPES- and in HCO_3^-/CO_2-buffered solutions. The secretory rates were calculated for the first 10 minutes of stimulation. Forskolin stimulation (10 μM) resulted in a continuous swelling response in WT ducts (secretory rate in HCO_3^-/CO_2-buffered solution: 176 ± 5 pl/min/mm^2). In contrast, no forskolin-evoked fluid secretion could be measured in ducts from KO animals (secretory rate in HCO_3^-/CO_2-buffered solution: 1.9 ± 5.6 pl/min/mm^2). The secretory responses observed in HEPES-buffered and in HCO_3^-/CO_2-buffered solutions were similar.

Figures 6A, 6B, and 6C show the luminal volume changes for forskolin stimulation in WT and KO ducts.

Overall, forskolin-induced fluid secretion was significantly lower in ducts from KO mice, as compared to WTs (in both HEPES and HCO_3^-/CO_2-buffered solutions: $P = 0.0006$).

Supplementary Videos S2 and S3 demonstrate the effects of forskolin on ductal fluid secretion in WT and KO ducts, respectively.

To further substantiate the secretory effect of elevated cytosolic cAMP level, cell-permeable cAMP analogue 8-bromo cAMP was used. 8-bromo cAMP resulted in a continuous secretion in WT ducts (secretory rate in HCO_3^-/CO_2-buffer in the first 10 minutes of stimulation: 141 ± 7 pl/min/mm^2) while no fluid secretory response could be observed in KO ducts (secretory rate in HCO_3^-/CO_2-buffer in the first 10 minutes of stimulation: 0.5 ± 0.4 pl/min/mm^2). Similar results were obtained in HEPES-buffered solution. 8-bromo cAMP-induced fluid secretion was significantly lower in KO ducts, as compared to WTs (in both HEPES and HCO_3^-/CO_2-buffered solutions: $P = 0.0004$; Figs. 7A, 7B). Overall, we could not find statistically significant difference between the secretory rates evoked by forskolin and 8-bromo cAMP either in WT or in KO ducts.

Cholinergic-Stimulated Fluid Secretion in Isolated LG Ducts

To examine the role of CFTR in cholinergic-evoked ductal fluid secretion, responses to muscarinic agonist carbachol were investigated in WT and KO LG duct segments isolated from 14- to 24-week-old mice. Rapid secretion could be observed in the first 4 to 5 minutes of carbachol stimulation (100 μM) followed by a slower phase both in WT ducts (secretory rate in HCO_3^-/CO_2-buffered solution in the first 10 minutes of stimulation: 133 ± 6 pl/min/mm^2) and in KO ducts (secretory rate in

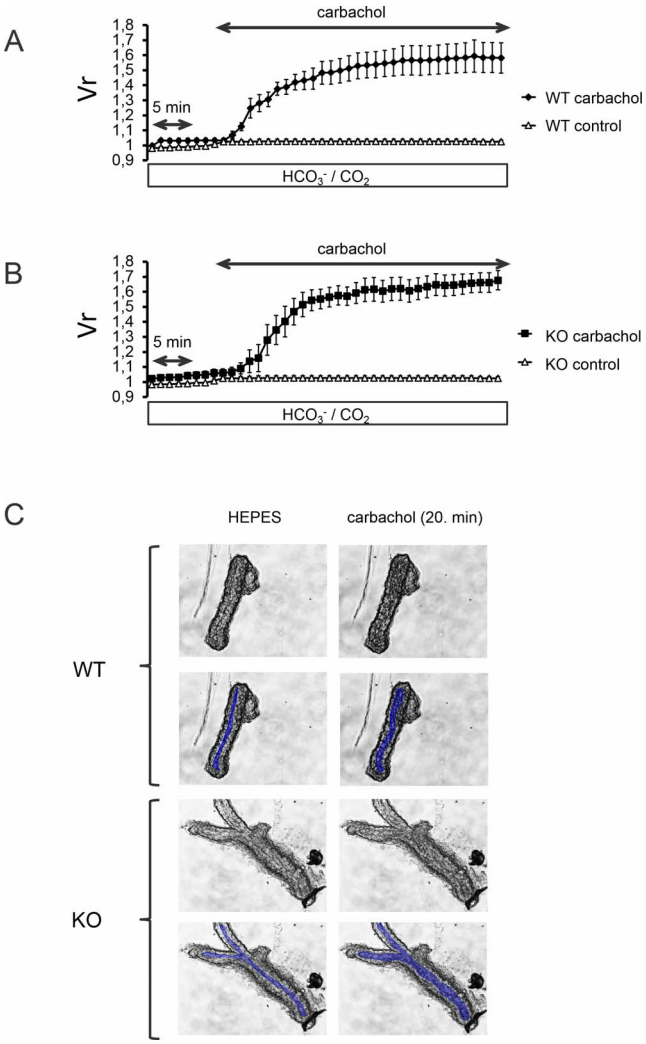

Figure 8. Effect of carbachol on ductal fluid secretion in mouse ducts isolated from WT and CFTR KO LGs. (**A**) WT ducts were exposed either to 100 μM carbachol (*filled rhombus*) or to no agonist (*empty triangle*). (**B**) KO ducts were exposed either to 100 μM carbachol (*filled square*) or to no agonist (*empty triangle*). Changes in Vr are shown. Data were obtained from six ducts isolated from three different animals in each series and are presented as means ± SEM. (**C**) Photo series of secreting isolated LG duct segments in response to carbachol stimulation were captured from Supplementary Videos S4 and S5. The secretory responses observed in HEPES-buffered and in HCO_3^-/CO_2-buffered solutions were similar. The luminal space is marked with *blue* color.

HCO_3^-/CO_2-buffered solution in the first 10 minutes of stimulation: 130 ± 5 pl/min/mm^2). Similar results were obtained in HEPES-buffered solution. Secretory rates in WT and in KO ducts did not differ significantly either in HEPES-buffered or in HCO_3^-/CO_2-buffered solutions.

Figures 8A, 8B, and 8C demonstrate the carbachol-induced luminal volume changes in WT and KO ducts.

Supplementary Videos S4 and S5 show the effect of carbachol on ductal fluid secretion in WT and KO ducts.

Carbachol-Evoked Ca^{2+} Signaling of Isolated LG Ducts

To investigate the potential difference in $[Ca^{2+}]_i$ between WT and KO ducts, carbachol-evoked elevation of $[Ca^{2+}]_i$ was measured in both groups of isolated ducts (Fig. 9). Carbachol dose-dependently elevated the $[Ca^{2+}]_i$ in both WT (F_{max}: 1 μM: 1.19 ± 0.01; 10 μM: 1.67 ± 0.05; 100 μM: 1.76 ± 0.05) and KO duct cells (F_{max}: 1 μM: 1.15 ± 0.02; 10 μM: 1.48 ± 0.03; 100 μM: 1.83 ± 0.05) without significant differences between WT and KO ducts.

Discussion

Previous reports have shown that CFTR plays an important role in epithelial secretion. Dysfunctional Cl^- and consequent fluid secretion can be observed in the pancreatic ducts, airway epithelia, and salivary glands when CFTR is absent or defective.[8–11,25,26] Unfortunately, little is known about the role CFTR may play in LG function, in contrast to the considerable attention paid to the other components of the ocular surface system, the cornea and the conjunctiva.[28–31] It has been reported, that CFTR was found on the apical membrane of conjunctival and corneal epithelial cells.[32–34] Levin and Verkman found high capacity of CFTR-facilitated Cl^- transport at the ocular surface in mice.[35]

In the present study, we demonstrated decreased tear secretion and increased corneal fluorescein staining in KO mice in two age groups studied, as compared to age-matched WT mice, suggesting CFTR plays a critical and essential role in tear secretion and in the maintenance of ocular surface integrity. Increased corneal fluorescein staining in the absence of CFTR may reflect the global dysfunction of the lacrimal functional unit including corneal, conjunctival, and LG epithelial cells. Our results show that KO mice are a useful model to investigate the role of CFTR may play in lacrimal secretion. As female sex is an established risk factor for dry eye disease, we calculated both the tear secretion results and the fluorescein staining scores separately in female and male mice.[36] We could not demonstrate the role of sex in tear secretion and ocular surface integrity which may be explained by the relatively young age of our animals.

Cystic fibrosis can cause wide range of morphologic alterations in the secretory epithelia, including lung, mucosa of the trachea, salivary glands or the pancreas.[7,10,11,26] Normal ion and water secretion is required to produce thin, free-flowing mucus. Defective chloride channels can result in reduced fluid secretion causing sticky and thick mucus formation that clogs the ducts and subsequently damages various organs. Our histologic examinations did not reveal any significant morphologic differences between the WT and KO LG tissues either in the 8- to 10- week or in the 20- to 24-week-old group. These histologic results demonstrated that functional deterioration may precede morphologic alterations during the course of disease progression. However, investigation of the duration of the disease in the alteration of LG morphology in CFTR KO mice has strong limitations as shorter life expectancies of KO mice hinder long lasting observations. Further studies are needed in order to clarify potential alterations in LG morphology later in life. In a case report by Alghadyan et al.[37] histopathologic examinations of LGs of CF patient who died of pulmonary complications revealed clogged small duct lumens and subsequent degeneration of acinar cells.

Our immunofluorescence data demonstrated the presence of CFTR in both acinar and ductal cells, with the staining most prominent in the apical membranes of ductal cells. Therefore, to further elucidate the role of CFTR in LG function, we have focused our study on the ducts by using our isolated duct segment model described earlier by our laboratory.[22] Both HEPES-buffered (nominally HCO_3^--free) and HCO_3^-/CO_2-buffered solutions were used in the experiments in order to investigate the potential role of HCO_3^- transport in the

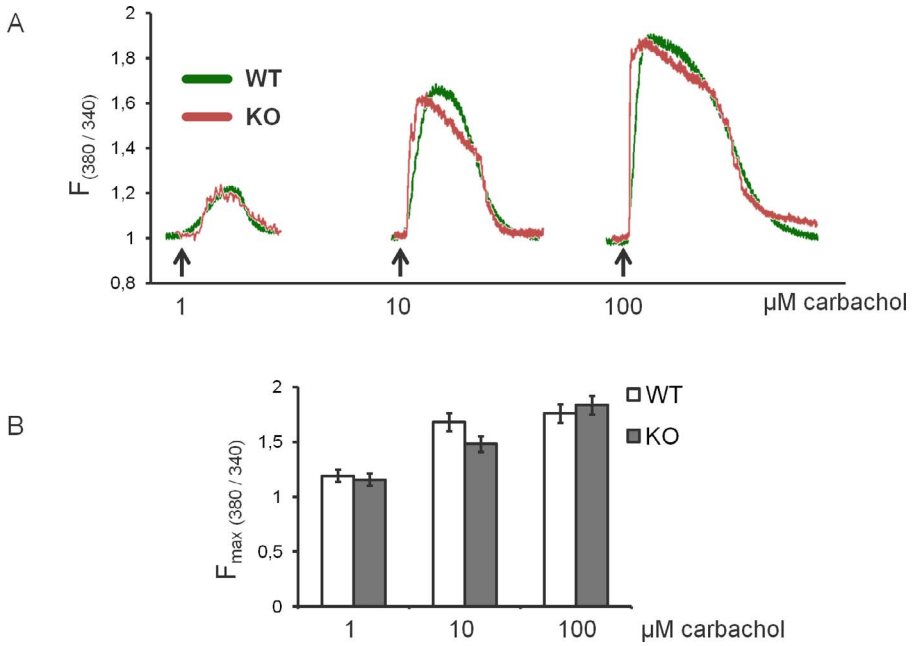

Figure 9. Effects of carbachol on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), in LG ductal cells from WT and KO mice. (**A**) Representative recordings of the microfluorescence experiments. (**B**) Carbachol dose-dependently elevated the $[Ca^{2+}]_i$, although no significant differences were found in the responses between ducts from WT and KO animals.

secretory processes. The secretory rates were independent from the buffer used (HEPES versus HCO_3^-/CO_2 buffer) suggesting the predominant role of Cl^- transport mechanisms over HCO_3^- secreting processes in mouse LG ducts. These findings are in accordance with our previous results derived from rabbit LG ducts.[24] As we have shown above, the absence of forskolin or 8-bromo cAMP-stimulated fluid secretion in ducts from KO mice suggests the important role of CFTR may play in LG duct secretion. Lack of cAMP-mediated fluid secretion in KO ducts demonstrates that CFTR may be the only cAMP-dependent transporter on the luminal surface of duct cells in mouse LG. In contrast to our findings from mouse LG ducts, pancreatic ducts isolated from CFTR KO mice has significant secretory capacity for forskolin stimulation.[38] Considering the strong predominance of CFTR protein in LG ducts, CFTR may play a key role in LG secretion through modification of LG fluids while being transported in the ducts. Cl^- secretion through CFTR may be a major contributor to the transmembrane electrochemical gradient and subsequent electrolyte and water movements and therefore defects in CFTR may significantly compromise Cl^- and water secretion from LG ducts.

Carbachol-evoked fluid secretion and cytosolic Ca^{2+} signaling in WT and CFTR KO ducts did not differ significantly, suggesting the preservation of secretory mechanisms activated by Ca^{2+} mobilizing stimuli in the absence of CFTR protein. Other Cl^- channels (i.e., voltage- and Ca^{2+}-sensitive Cl^- channels (not investigated in the present study) appeared unaffected when CFTR protein is missing.[39]

In recent studies by Flores et al. and Lee et al., small-molecule CFTR activators increased tear secretion in a LG-ablated mouse model of dry eye.[40,41] Since LG was absent in this animal model, the enhanced Cl^--driven fluid secretion could be from the conjunctival and corneal epithelial cells. Data presented here, however, suggest that CFTR affects not only corneal and conjunctival epithelial cell function, but also LG secretion.

In conclusion, our data demonstrated decreased tear secretion and impaired ocular surface integrity in CFTR KO mice, suggesting the important role of CFTR may play in LG function and in the maintenance of ocular surface integrity. Our functional studies by employing the isolated duct segment model, suggest that CFTR plays a pivotal role in the fluid secretion of LG duct system. Further studies are needed to clarify whether modification of CFTR function may serve as a potential target to stimulate LG secretion and therefore can be an option in treating aqueous deficient dry eye.

Acknowledgments

Supported by NKFIH NN 115611 (ETM); the Webb Foundation Grant (CD); EFOP-3.6.1-16-2016-00008 (ETM); NEI/NIH EY017731 (CD); and a Momentum Grant of the Hungarian Academy of Sciences LP2014-10/2014 (PH).

Disclosure: **O. Berczeli**, None; **E. Vizvári**, None; **M. Katona**, None; **D. Török**, None; **L. Szalay**, None; **F. Rárosi**, None; **I. Németh**, None; **Z. Rakonczay**, None; **P. Hegyi**, None; **C. Ding**, None; **E. Tóth-Molnár**, None

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