Corneal sensory nerves are important for both encoding pain from the ocular surface and stimulating homeostatic reflexes, such as blinking and tearing, that maintain the integrity of the ocular surface. The central nervous system reflex pathways and cellular mechanisms that mediate these diverse functions have not been fully defined. Corneal sensory nerves can be classified based on the types of stimuli that lead to activation of single nerves, including cold, mechanical, and heat stimuli, and osmoreceptors are also presumed to be important for signaling changes in the osmotic state of the ocular surface, but the molecular basis for this function has not been conclusively identified. Dry eye disease is a complex disorder that can be caused by a change in the evaporative state of the ocular surface, reducing tear production and leading to a sensation of discomfort. However, many individuals have ocular pain and a perception of dryness without any measurable changes in tear production. These findings suggest that the mechanisms underlying ocular sensation and homeostatic maintenance of the ocular surface may be distinct.

We previously reported a novel laboratory model in which we are able to cause divergent changes in the sensory and homeostatic functions in a rodent model of dry eye. We lesioned the nerves in the lacrimal gland, which we expected to reduce tear production, but in fact we did not see any changes in tear volume. We did find a reduction in the response of the rats to ocular stimulation with a noxious dose of menthol, but no changes in responses to capsaicin. This sensory deficit did not lead to any changes in tear volume or sensory nerve density in the eye. We speculated that these rats may have a deficit in TRPM8 function, which is thought to be the molecular basis for cold sensation, responses to menthol, and possible osmosensation. Recent studies implicate the TRPM8 receptor in mediating homeostatic blinking behavior. In the present study, we examined both spontaneous eye closure behaviors in rats that had saporin toxin denervation of the lacrimal gland to determine if both homeostatic and nociceptive responses are altered after this treatment. We also tested nociceptive responses 8 weeks after lacrimal gland denervation to determine if the deficits in evoked pain in response to menthol are chronic. Finally, we examined tear proteins in saporin-denervated rats to determine if changes in the tear proteome may provide clues to mechanisms that might alter sensory nerve function.
Behavioral Measurement of Ocular Sensation

Awake rats were lightly restrained while 10 μL 50 mM menthol (Sigma-Aldrich, St. Louis, MO, USA) was applied to the ocular surface. Ipsilateral eye wipes with the forelimb were counted for 3 minutes. Facial grooming behaviors or hind paw scratches were not included, as these are not validated measures of eye pain. Rats were assessed 8 weeks after lacrimal gland injections. We tested rats only once because we have found that repeated ocular applications of menthol cause sensitization of nociceptive responses (Aicher S, Hegarty D, unpublished observations, 2015).

Statistical Analyses

Two-way ANOVAs were used to compare eye closures and grooming bouts over time for saporin and control groups. Paired t-tests were used for within-animal comparisons of phenol thread measurements taken before treatment (baseline) and at the end of the study (8 weeks) for both saporin and control animals. Changes in eye wipe behavior and lacrimal gland weights were compared using t-tests.

Collection of Tear Proteins

Tear proteins from saporin-treated rats and control rats were collected using phenol red threads (see above). Although this allowed simultaneous estimation of the volume of tears collected, the presence of the phenol red interfered with quantification of the amount of protein collected. However, based on the collection of approximately 1.5 μL of tears per sample, and the reported 6 to 10 mg/mL protein concentration of tears, each sample contained from 9 to 15 μg protein. On collection, the threads were cut into 2- to 3-mm sections and placed in 50 μL of 50 mM ammonium bicarbonate buffer and frozen at –80°C until processing.

Tear Protein Digestion and Tandem Mass Tag (TMT) Labeling

Frozen threads were thawed, and 33.5 μL 100-mM triethyl ammonium bicarbonate (TEAB), 2 μL 1% ProteaseMax detergent (Promega, Madison, WI, USA), and 1 μL 0.5 M dithiothreitol were added. Following vortexing, the samples were heated at 56°C for 20 minutes, then alkylated by addition of 2.7 μL 0.55 M iodoacetamide and incubated in the dark at room temperature for 15 minutes. Proteins were then digested by addition of 1 μL 1% ProteaseMax detergent and 10 μL 0.1 μg/μL trypsin (Pierce, MS Grade; Thermo Scientific, Waltham, MA, USA). After shaking at 37°C overnight, the solution was removed from the threads by transferring to another 0.5-mL centrifuge tube, and 5 μL 10% trifluoroacetic acid added. The samples were then stored at room temperature for 1 hour, spun at 16,000g for 5 minutes, and the supernatant removed. Peptides were then solid phase extracted using MicroSpin columns (The Nest Group, Southborough, MA, USA) and dried by vacuum centrifugation. Each digest was then dissolved by vigorous shaking for 10 minutes in 20 μL 100 mM TEAB, and 200 μg TMT 10-plex reagent (Thermo Scientific) dissolved in 12 μL anhydrous acetonitrile was added. Samples were then incubated for 1 hour at room temperature and 2 μL 5% hydroxylamine added followed by an additional 15 minutes incubation.

Mass Spectrometric Analysis

Then, 2 μL of each reaction mixture were mixed, dried by vacuum centrifugation, dissolved in 5% formic acid, and
approximately 2 μg of peptide was analyzed, as previously described, to normalize the total reporter ion intensity of each multiplexed sample and check labeling efficiency. Based on these results, the remaining samples were combined with volumes adjusted to produce equal total reporter ion intensities and samples dried down in preparation for two-dimensional liquid chromatography–mass spectrometry (LC-MS) analysis. One-half of the multiplexed samples, corresponding to approximately 60 μg of peptides, were separated using an automated two-dimensional (2D) nano–reverse phase/reverse phase chromatography system and Orbitrap Fusion mass spectrometer (Thermo Scientific) using synchronous precursor isolation for MS3-based reporter ion measurement as previously described, except using 14%, 17%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 35%, 40%, 50%, and 90% acetonitrile elution steps during the first dimensional separation. Due to re-equilibration times after each LC-MS run, the entire 2D LC-MS/MS method required 34 hours of instrument time.

Data Analysis

Raw instrument files from the 17 fractions were simultaneously searched with the SEQUEST HT search engine within Proteome Discoverer (PD) version 1.4.1.14 (Thermo Scientific). A manually annotated rat UniprotKB/Swiss-Prot database containing 79,256 entries and common contaminants was used to minimize the number of shared peptides from homologous proteins. SEQUEST HT search parameters were identical to those previously used and used a reversed-sequence decoy strategy to control peptide false discovery, followed by validation using Percolator software within PD. Search results and TMT reporter ion intensities were exported from PD as text files and processed using in-house Python scripts. Only peptide spectral matches (PSMs) uniquely matching a single protein entry with q scores < 0.05, accurate masses within 10 parts per million, and trimmed average reporter ion intensity peak heights greater than 600 were used for quantification. TMT reporter ions with zero values were replaced with intensities of 150 to avoid artifacts. The individual reporter ion intensities from all PSMs were summed to create total protein intensities. Differential protein abundances between groups were determined by comparing the total reporter ion intensities using the Bioconductor package edgeR as previously described. Additional data normalizations, multiple testing corrections, and calculation of false discovery rates (FDRs) were performed within edgeR. Only results with P < 0.05 and FDR < 0.1 were considered significant. Multidimensional scaling plots generated within edgeR were used to exclude one 3-week posttreatment sample that differed greatly from other samples, presumably due to sample contamination during collection or processing. Final results listing all identified proteins, TMT reporter ion intensities, and edgeR analysis results are found in Supplementary File S1.

RESULTS

Saporin Toxin-induced Denervation Reduced Eye Closure Behavior

To assess the impact of saporin-induced lacrimal gland denervation, we examined spontaneous eye closure behavior. Saporin-treated rats received a left side denervation of the lacrimal gland (n = 4), and the control rats received anesthesia, incision, and injection of vehicle into the lacrimal gland (n = 4). Saporin injection into the left lacrimal gland produced a significant 15.4% decrease in lacrimal gland weight (treated side: 120.5 ± 6.7 mg, contralateral: 142.3 ± 8.5 mg, n = 4; t-test, P = 0.04), similar to the decrease seen in our previous study. Figure 1 shows eye closure measures from the left eye (treated side) assessed weekly for 8 weeks in saporin and control groups. Eye closures (Fig. 1A) included both bilateral (blinks) and unilateral (winks) behaviors that occurred spontaneously during a 5-minute test period. Overall, saporin-treated rats showed fewer eye closure behaviors over time compared with controls (2-way ANOVA with Holm-Sidak post hoc test, treatment group factor; P = 0.014) (Fig. 1A). There were no differences in the time or interaction factors, indicating that there was no progression in eye closure behavior over time.

Because we denervated the lacrimal gland on only one side in each animal, we wanted to determine if there were changes in eye closure behavior that were specific to the treated side. Thus, we separately examined only “wink” behaviors, which are closures of only one eye at a time. The left (treated) and right (untreated) eyes were analyzed separately. Figure 1B shows that winks were reduced on the left side in saporin-
denervated animals, which was ipsilateral to the treated lacrimal gland (2-way ANOVA treatment factor, \(P = 0.01\); no effect of time or interaction between treatment and time). In contrast, there was no change in wink behavior for the right eye (Fig. 1C; 2-way ANOVA treatment factor, \(P = 0.79\)), which was on the untreated side for each animal. These results show that the eye closure behavior can be selectively altered by lacrimal gland denervation and restricted to the treated side. Recent studies implicate TRPM8 channels in mediating blink behavior,\(^8\) supporting our hypothesis that TRPM8 is a potential molecule that may be altered in the corneal afferents of our saporin animals.

### Saporin-treated Rats Maintain Normal Tear Production

In our previous study, we found that despite substantial loss of cholinergic innervation of the lacrimal gland after saporin treatment, rats continue to have normal tear volumes 3 weeks after saporin treatment.\(^6\) In the present study, we examined rats at 8 weeks post-denervation and we found that even at this long-term point, tear volume as assessed by phenol thread test is normal (Fig. 2A). Statistical comparisons were made as paired \(t\)-tests on raw phenol thread measures from the treated (left) side and compared within each treatment group (saporin, baseline: 12.0 ± 0.9 mm, saporin 8 weeks: 14.0 ± 2.3 mm paired \(t\)-test, \(P = 0.56\); control, baseline: 10.0 ± 1.0 mm, control 8 weeks: 10.8 ± 1.9 mm paired \(t\)-test, \(P = 0.70\)). Despite changes in eye closure frequency, there was not a significant change in tear production, suggesting that mechanisms regulating lacrimal gland secretion and eye closure reflexes are distinct.

### Saporin Rats Show Reduced Responses to Noxious Menthol

We previously showed that saporin-denervated rats were less responsive to application of noxious menthol to the corneal surface, which was manifest by fewer eye wipe behaviors.\(^5\) In this study, we monitored rats after a much longer period to see if the changes evoked by denervation would be stable over time. We found reduced responses to menthol at 8 weeks following saporin injection (Fig. 2B) compared with controls (\(t\)-test, \(P = 0.06\)), demonstrating the stability of impaired sensory responding over time.

### Saporin Treatment Does Not Alter Facial Grooming Behavior

Facial grooming behaviors that are included in the stereotypic rodent cephalocaudal grooming sequence\(^1\) are distinct from nociceptive eye wipe responses; both types of behavior were assessed each week to determine if saporin denervation of the lacrimal gland may have altered behavior more generally. No changes in the number of grooming bouts were seen based on treatment group (2-way ANOVA, \(P = 0.39\) for treatment factor). There was a significant effect of time (\(P = 0.006\)) with all rats showing more grooming behavior over the 8-week study period, suggesting greater acclimation to the testing situation for all rats in the study. These findings show that saporin denervation of the lacrimal gland did not alter facial grooming behavior, supporting the idea that the saporin toxin did not alter innervation to regions other than the lacrimal gland.

### Topical Corneal Application of Saporin Does Not Contribute to Behavioral Alterations

To ensure that 192-IgG-saporin was not having a direct effect on the corneal surface itself, we applied 192-IgG-saporin topically to the corneal surface (without removal of tear film barrier) in a separate group of rats and measured eye wipe responses, tear production, and eye closures 3 weeks later. Rats that received topical saporin did not show any alterations in eye wipe responses to 50 mM menthol (topical saporin: 9.6 ± 1.5 eye wipes, \(n = 5\); control: 7.3 ± 1.2 eye wipes, \(n = 4\); \(t\)-test, \(P = 0.28\)). The data from the control animals were recently published as part of another study.\(^10\) There were no changes in tear production 3 weeks after topical saporin application (topical saporin baseline: 10.7 ± 0.9 mm; 3-week endpoint: 12.7 ± 2.6 mm, \(n = 3\); paired \(t\)-test, \(P = 0.44\)). Eye closures were also assessed 3 weeks after topical saporin and compared with naive control animals. There were no changes in total eye closures (blinks and winks) on the treated (left) side (topical saporin baseline: 6.0 ± 3.5 eye closures, 3-week endpoint: 6.7 ± 2.9 eye closures, \(n = 3\); control baseline: 3.8 ± 1.9 eye closures, control 3 weeks: 5.0 ± 0.7 eye closures, \(n = 4\); 2-way ANOVA, \(P = 0.4\)). Unilateral winks on the treated side were also not altered when topical saporin was compared with controls (topical saporin baseline: 2.3 ± 1.9 winks, 3-week endpoint: 2.0 ± 0.6 winks, \(n = 3\); control baseline: 1.5 ± 0.9 winks, 3-week endpoint: 4.3 ± 1.4 winks, \(n = 4\); 2-way ANOVA, \(P = 0.6\)). There were no changes in lacrimal gland weight after topical saporin (left: 134.5 ± 13.5 mg, right: 132.7 ± 10.8 mg, \(n = 5\); \(t\)-test, \(P = 0.9\)). These findings demonstrate that the alterations after saporin-induced denervation of the lacrimal gland were not due to a direct effect of the saporin toxin on the corneal surface.

### Saporin-induced Denervation Alters Tear Proteins

Studies from other groups have demonstrated that the TRPM8 receptor is crucial for both nociceptive responses to menthol, as well as spontaneous blinking.\(^2\)\(^2\)\(^2\)\(^2\)\(^2\) Our saporin-treated rats showed reductions in both nociceptive responses to menthol and deficits in eye blink behaviors, both of which are consistent with reduced function of the TRPM8 receptor. In our prior studies we found no changes in corneal nerve density that could account for the functional changes we have seen,\(^6\) but it is possible that denervation of the lacrimal gland could alter the protein composition of tears and thereby alter the
function of TRPM8 and other molecules within the corneal sensory nerves. We conducted extensive proteomic analysis on tears from saporin-treated rats.

A 2D LC separation of TMT-labeled peptides and mass spectral analysis identified a total of 1490 rat tear proteins in naïve control animals (n = 4) following the removal of common contaminant proteins (Supplementary Table S1; n = 5 baseline tear samples; n = 4 tear samples from saporin-denervated animals). Based on numbers of PSMs, Table 1 lists the 20 proteins having the highest overall abundance in rat tears. Some proteins from related families, particularly the keratins, are grouped together in Table 1, and their overall abundance estimated by averaging their PSM numbers reported. A full list of identified proteins appears in Supplementary File S1.

| Identified Protein                                | Accession | Description                                                                 | PSMs |
|--------------------------------------------------|-----------|-----------------------------------------------------------------------------|------|
| Secretoglobin family 2A member 2                 | P02780    | Member of a family of secreted proteins that is increased in certain cancers as well as in dry eye. 50 | 1166 |
| Cystatin-related protein 1 and 2                 | P22282 and P22283 | Proteins synthesized and secreted in rat lacrimal gland under androgen control. 53 | 564* |
| Polymeric immunoglobulin receptor                | P15083    | Receptor expressed in lacrimal acinar cells that transports antibodies involved in immune responses. 40 This protein is decreased in a rabbit model of Sjögren syndrome-associated dry eye 32 and patients with dry eye. 34 | 499 |
| Aldehyde dehydrogenase, dimeric NADP-preferring Serotransferrin | P11883 | Enzyme that is thought to be an ocular antioxidant. 42 Upregulated in dry eye patients. 41 | 432 |
| Keratins                                         | Q6P6Q2, Q6IFV4, Q6IG00, Q4FZU2, Q6IFV3, Q6IG05, Q6IFW5, Q6IFW6, Q6IMF3, Q10758 | A large family of proteins that are essential structural components of epithelial cells. Keratins have been found in human conjunctiva and may also be minor components of cornea. 45–49 | 296* |
| Alpha-actinin-4                                   | Q9QXQ0    | F-actin cross-linking protein that is thought to anchor actin to a variety of intracellular structures. | 288 |
| Thioredoxin                                      | P11232    | Catalyzes dithiol-disulfide exchange reactions and plays a role in the reversible S-nitrosylation. | 285 |
| Actins                                           | P60711, P68035 | Cytoskeletal filament. Has role in exocytosis of secretory vesicles from lacrimal acini. 46 | 283* |
| Major urinary protein                            | P02761    | Binding and release of pheromones. | 240 |
| Plectin                                          | P30427    | Interlinks intermediate filaments with microtubules and microfilaments. | 239 |
| Annexin A1 and A2                                | P07150, Q07956 | Calcium and phospholipid binding protein that inhibits phospholipase A. Thought to have anti-inflammatory activity 47 and is overexpressed in dry eye patients. 48 | 231* |
| Serum albumin                                    | P02770    | Abundant protein found in the blood that has been shown to be increased in a rabbit model of Sjögren syndrome-associated dry eye. 32 | 224 |
| Furin                                            | P23377    | Processes newly synthesized precursor proteins into biologically active proteins. The furin gene was found to have reduced expression in a rat model of severe dry eye. 49 | 221 |
| Prolactin-inducible protein homolog               | O70417    | Inhibits T-lymphocyte programmed cell death. 50 This protein is decreased in a rabbit model of Sjögren syndrome-associated dry eye 32 and in patients with mild dry eye. 41 | 193 |
| Myosin-9                                         | Q62812    | Cellular myosin that appears to play a role in cytokinesis, cell shape, and specialized functions. | 168 |
| Guanine deaminase                                | Q9WTT6    | Catalyzes the hydrolytic deamination of guanine, producing xanthine and ammonia. | 146 |
| Heat shock 70 kDa protein 1A                      | P0DMW0    | Molecular chaperone. | 144 |
| Lactadherin                                      | P70490    | Contributes to phagocytic removal of apoptotic cells in many tissues. | 136 |
| Elongation factor 1-alpha 1                      | P62630    | Promotes the GTP-dependent binding of aminocytotRNA. | 126 |

* These family members were combined and an average of their PSM numbers reported. A full list of identified proteins appears in Supplementary File S1.
that were different in abundance between baseline and 3 weeks post-saporin treatment (Table 2). Most differentially abundant proteins showed decreases, whereas 2 proteins (syntxin 8 and Proteasome subunit beta type-10) increased in saporin-treated rats. The potential functional significance of the changes in tear protein composition are described in the Discussion.

**DISCUSSION**

**Behavioral Consequences of Lacrimal Gland Denervation**

Consistent with our previous study,9 we find that partial denervation of the lacrimal gland with saporin toxin impairs menthol nociception. In the present study, we show that this deficit is still present 8 weeks after treatment. The deficit seems to be modality selective because capsaicin responses were not altered by saporin treatment. In the present study, we also show that spontaneous eye closure behavior is also reduced following saporin denervation of the lacrimal gland. These behavioral findings are consistent with a loss of function of TRPM8 receptors in corneal afferents, which have been shown to at least partially mediate responses to menthol23 as well as spontaneously blinking.9 Because we show that TRPM8 receptors in corneal afferents can be denervated by saporin treatment, 8 we now also show that spontaneous eye closure behavior is reduced due to a decrease in the ERGIC transport receptor, it could lead to the insertion of unglycosylated TRPM8 channels such as TRPV1.31 Therefore, if expression of the enzyme that converts AA to its metabolites is decreased, AA would activate the TRPV1 channel, which mediates the response to capsaicin. In addition to protein changes that may relate to TRPM8 function, we also saw changes in proteins that are related to homeostatic maintenance of the ocular surface. We detected a reduction in serotonin, a glycocyte protein expressed in secretions that has antimicrobial, antiviral, antiparasitic, and anti-inflammatory activity and is decreased in a rabbit model of dry eye.52,53 Thus, despite normal tear production in our saporin-treated rats, some protein changes are consistent with other dry eye models. We also found a reduction in beta-defensin-1, an inducible antimicrobial peptide produced in epithelial cells and constitutively expressed on the ocular surface.52,53 The reduction in this protein would be expected to make the cornea more susceptible to infection in our saporin-treated rats.

**Overall Abundance of Tear Proteins**

Using the numbers of PSMs, which are roughly correlated with the abundance of different proteins,24 we identified the 20 most abundant proteins or protein families in the rat tear (Table 1). Our findings are in partial agreement with recent studies.25 A full list of all 1490 proteins identified in rat tears are listed in Supplementary File S1. The recovery of so many tear proteins from the minute volume collected on each phenol thread emphasizes the sensitivity of the current methods.

**Changes in Abundance of Tear Proteins After Saporin Denervation of the Lacrimal Gland**

In our analysis, we identified 11 proteins that were decreased after saporin treatment (Table 2), some of which may have a role in TRPM8 channel function. Protein ERGIC-53 (endoplasmic reticulum [ER]-Golgi intermediate compartment) is a transport receptor of glycoproteins from the ER to the Golgi apparatus.20 The TRPM8 channel undergoes posttranslational glycosylation, which is important in establishing the sensitivity of this channel to cold and other stimuli in sensory neurons.27,28 Unglycosylated TRPM8 has been shown to have reduced responses to menthol in cultured trigeminal neurons.57 In our saporin model, if transport of glycoproteins is reduced due to a decrease in the ERGIC transport receptor, it could lead to the insertion of unglycosylated TRPM8 channels into the membrane, resulting in the reduced response to menthol that we observed.57 We also saw a decrease in arachidonic 15-lipoxygenase B, which is the enzyme that converts arachidonic acid (AA) into its metabolites and is expressed in human corneal epithelial cells.29,30 A previous study demonstrated that AA has a direct inhibitory effect on TRPM8 channel activation by menthol, but activates other TRP channels such as TRPV1.31 Therefore, if expression of the enzyme that converts AA to its metabolites is decreased, AA may accumulate, which could inhibit TRPM8 activation. This would also explain why capsaicin responses were not reduced in saporin-treated rats,8 because AA would activate the TRPV1 channel, which mediates the response to capsaicin.

**Table 2. Tear Proteins That Changed After Saporin Denervation of Lacrimal Gland Compared With Baseline**

| Protein Name                                      | Fold Change | Change | FDR  |
|--------------------------------------------------|-------------|--------|------|
| Phenylalanine-tRNA ligase, mitochondrial          | 5.2         | 0.098  |
| Procollagen-lysine-2-oxoglutarate 5-dioxygenase 3| 3.8         | 0.001  |
| Beta-defensin 1                                  | 3.8         | 0.004  |
| Syntxin-8                                        | 5.7         | 0.049  |
| 45kDa calcium-binding protein                    | 2.6         | 0.041  |
| Protein ERGIC-53                                 | 2.6         | 0.069  |
| Arachidonate 15-lipoxygenase B                   | 2.6         | 0.008  |
| Proteasome subunit beta type-10                  | 2.5         | 0.044  |
| Protein-glutamine gamma-glutamyltransferase E    | 2.3         | 0.084  |
| Serotransferrin                                  | 2.0         | 0.069  |
| Dual-specificity mitogen-activated protein kinase 5| 1.9      | 0.016  |
| Filamin-A-interacting protein 1                  | 1.8         | 0.052  |
| Cofilin-1                                        | 1.6         | 0.019  |

Most protein levels decreased after saporin treatment; those that increased are listed in bold type.
Sensation and Proteins Altered by Loss of Lacrimal Nerves

CONCLUSIONS

Our studies show that saporin toxin denervation of the lacrimal gland leads to impaired homeostatic blinking behavior, including fewer ipsilateral eye closures, as well as reduced responses to application of noxious menthol to the ocular surface. These behavioral changes implicate impaired function of TRPM8 receptors in corneal sensory nerves. We show that highly sensitive approaches are able to analyze the proteome in extremely small volumes of tears collected via the phenol thread test, including low-abundance proteins. Proteomic analysis suggests that downregulation of key proteins may contribute to the impaired sensory function seen in our rats and other proteins suggest a compromised ocular surface.

Acknowledgments

The authors thank Sam M. Hermes for technical assistance and Phillip Wilmarth and Ashok Reddy for assistance with proteomic analysis. Supported by grants from the NIH: DE12640 (SAA, DMH), P50 NS061800 (SA), and P50 EY010572 (John Morrison), S10 OD012246 (LLD), as well as the Medical Research Foundation of Oregon, OHSU Presidential Bridge Funds, and OHSU School of Medicine Core support.

Disclosure: D.M. Hegarty, None; L.L. David, None; S.A. Aicher, None.

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