Secreted Protein Acidic and Rich in Cysteine (SPARC)-Null Mice Exhibit More Uniform Outflow

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PURPOSE. Secreted protein acidic and rich in cysteine (SPARC) is a matricellular protein known to regulate extracellular matrix (ECM) in many tissues and is highly expressed in trabecular meshwork (TM). SPARC-null mice have a 15% to 20% decrease in intraocular pressure (IOP) compared to wild-type (WT) mice. We hypothesized that mouse aqueous outflow is segmental, and that transgenic deletion of SPARC causes a more uniform pattern that correlates with IOP and TM morphology.

METHODS. Eyes of C57BL/6-SV129 WT and SPARC-null mice were injected with fluorescent microbeads, which were also passively exposed to freshly enucleated eyes. Confocal and electron microscopy were performed. Percentage effective filtration length (PEFL) was calculated as $PEFL = FL/TL \times 100\%$, where TL = total length and FL = filtration length. IOP was measured by rebound tonometry.

RESULTS. Passive microbead affinity for WT and SPARC-null ECM did not differ. Segmental flow was observed in the mouse eye. SPARC-null mice had a 23% decrease in IOP. PEFL increased in SPARC-null ($70.61 \pm 11.36\%$) versus WT mice ($54.68 \pm 9.95\%$, $P < 0.005$; $n = 11$ pairs), and PEFL and IOP were negatively correlated ($R^2 = 0.72$, $n = 10$ pairs). Morphologically, TM of high-tracer regions had increased separation between beams compared to low-tracer regions. Collagen fibril diameter decreased in SPARC-null (28.272 nm) versus WT tissue (34.961 nm, $P < 0.0005$; $n = 3$ pairs).

CONCLUSIONS. Aqueous outflow in mice is segmental. SPARC-null mice demonstrated a more uniform outflow pattern and decreased collagen fibril diameter. Areas of high flow had less compact juxtacanalicular connective tissue ECM, and IOP was inversely correlated with PEFL. Our data show a correlation between morphology, aqueous outflow, and IOP, indicating a modulatory role of SPARC in IOP regulation. (*Invest Ophthalmol Vis Sci.* 2013;54:2035–2047) DOI:10.1167/iovs.12-10950

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Glaucoma is a major cause of blindness, affecting over 67 million people worldwide.¹ In the United States, the prevalence is approximately 5% in Caucasians and 10% in African Americans.^{2,3} Elevated intraocular pressure (IOP) is the greatest risk factor contributing to primary open-angle glaucoma (POAG).⁴ The elevated IOP of POAG is due to impaired outflow through the trabecular meshwork.⁵ Within the trabecular meshwork (TM), the juxtacanalicular connective tissue (JCT) region, in association with the inner wall endothelium of Schlemm's canal, is the anatomic location of the majority (46%–74%) of outflow resistance.^{5–8} Regulation of extracellular matrix (ECM) within the JCT region has been shown to alter IOP.^{9–13} Furthermore, abnormal accumulations of ECM within the JCT have been identified as a primary (i.e., not secondary to chronic treatment) pathophysiologic event in eyes with POAG.¹² The molecular mechanisms regulating ECM turnover within the JCT region are not fully elucidated. However, we believe that proteins that affect ECM in other tissues will affect ECM within the JCT region.

Secreted protein acidic and rich in cysteine (SPARC) is a matricellular glycoprotein that mediates ECM organization and turnover in many human tissues. In the eye, SPARC is found within several tissues and the aqueous humor, and is one of the most highly expressed gene products in the TM.¹⁴ SPARC expression is significantly increased when TM cells are stretched, a physiologic and pathophysiologic consequence of increasing IOP.¹⁵ In addition, SPARC has been implicated in the formation of cataract and corneal repair.^{16–19} Our lab has demonstrated that SPARC is highly expressed in the human TM,²⁰ and that SPARC knockout (KO) mice demonstrate a significant decrease in IOP of 15% to 20%.²¹ The drainage pathway of mice is remarkably similar to that of humans, including Schlemm's canal, collector channels, and episcleral venous drainage.^{22,23} Thus, the mechanism of how SPARC may modulate IOP and potentially contribute to glaucoma pathogenesis warrants further study.

The synergistic interactions between the JCT cells, the inner wall endothelial cells of Schlemm's canal, and the associated fenestrated basement membrane are believed to play an important role in modulating outflow resistance and IOP.^{7,8,24,25} The funneling hypothesis states that the aqueous humor flows or funnels through a finite number of pores in the Schlemm's canal (SC) endothelium. Thus, aqueous humor does not exit the anterior chamber uniformly throughout the 360° of the iridocorneal angle. Rather, drainage occurs preferentially at points where there is a more sizable separation between the JCT and SC endothelium, as well as near collector channel ostia where there is a higher concentration of pathways leading to episcleral veins.²⁶ This concept is referred to as segmental flow. Cell-ECM interactions in the JCT region have been found to mediate resistance generation, segmental flow, and IOP.^{27–30}

The use of microbead tracer quantifies the degree of segmental flow and correlates with IOP and outflow resistance. Zhang et al. used argon laser photocoagulation

of the TM in monkeys to induce a 3-fold increase in IOP but a 6-fold decrease in the total TM area utilized for outflow.³¹ Using microbeads, they demonstrated that laser damage reduced the available area for outflow and elevated IOP. Lu et al. similarly used microbeads in perfused bovine and monkey eyes and discovered that control eyes demonstrated a heterogeneous or segmental pattern of outflow, while eyes treated with the Rho-kinase inhibitor Y-27632 had a more uniform or homogeneous pattern.^{27,32} Treated eyes had a 3-fold increase in the total TM area utilized for outflow. Additionally, Lindsey et al.^{33–36} and Camelo et al.³⁷ injected fluorescent dextrans intracamerally into the rat and mouse eye to study aqueous humor outflow. The uveoscleral outflow pathway in mice was identified with this technique, as well as several other characteristics of the various outflow pathways and associated anatomy.

We hypothesize that segmental flow occurs in the mouse, and that the lower IOP seen in SPARC-null mice is due to a more uniform outflow profile from an alteration of ECM in the JCT region. We also hypothesize that there will be an inverse correlation between the IOP of a mouse eye and the area involved in active outflow.

METHODS

Animal Husbandry

All experiments were completed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Local Institutional Animal Care and Use Committee (IACUC) approval was obtained. SPARC-null and corresponding wild-type mice were originally obtained by generous donation from E. Helene Sage of the Benaroya Research Institute at Virginia Mason (Seattle, WA). Heterozygotes were bred together, and their offspring were subsequently genotyped in order to identify homozygous SPARC-null mice, which were used in these experiments. Animals used in these experiments were housed in the animal facilities of the Massachusetts Eye and Ear Infirmary and Schepens Eye Research Institute.

Passive Binding Assay

To ensure that the affinity for the fluorescent microbead tracer (FluoSpheres carboxylate-modified 20 nm microspheres; Invitrogen, Eugene, OR) utilized in these studies did not differ between the TMs of wild-type (WT) and SPARC-null tissue, WT and KO eyes were passively bathed in a microbead solution. Age and temporally matched C57BL/6-SV129 WT and SPARC KO mice were sacrificed, and their freshly enucleated eyes were placed in Dulbecco's Modified Eagle Medium (DMEM) solution. The eyes were dissected, with the removal of the posterior segment and lens. The anterior segment was then incubated in a 1:50 solution of microbeads in 1× Dulbecco's Phosphate Buffered Saline (DPBS)–/– (Invitrogen) (identical to the injected solution in later studies) for 4 hours in a 37°C water bath on a shaker, or overnight at 4°C on a shaker. The tissue was fixed in Karnovsky's fixative (KII) and sectioned into six to eight sections. Fluorescopy of the tissue en face was completed using an Olympus FSX100 fluorescence microscope (Olympus, Center Valley, PA) with a 1/55th-second exposure time. Images were stitched together using CellSens Imaging Software (Olympus), and fluorescence was quantified using ImageJ software (National Institutes of Health, Bethesda, MD). Total corrected fluorescence (TCF) was calculated for each section as $TCF = \text{integrated density} - (\text{area of TM section} \times \text{mean background fluorescence})$, akin to calculations in various publications.^{38,39} Mean background fluorescence was averaged from four different areas adjacent to the TM. TCF values for the various sections were averaged to determine the mean TCF value for that eye. Tissue was then analyzed by confocal and electron microscopy.

IOP Measurement

Age and temporally matched WT and SPARC KO mice were used. Each mouse was anesthetized with an intraperitoneal injection of 5 µL/g anesthesia (90 mg/kg ketamine + 9 mg/kg xylazine; Phoenix Pharmaceutica, St. Joseph, MO). A rebound tonometer (TonoLab; Colonial Medical Supply, Franconia, NH) was used to measure the IOP of the right eye six times between 4 and 7 minutes after the anesthetic injection. This time period was selected due to prior studies indicating that the IOP is stable during this time.^{40,41} This was repeated three times, and the mode of each set was calculated and averaged, which was recorded as the IOP for that mouse. The tonometer (TonoLab; Colonial Medical Supply) was fixed horizontally, and a pedal was used to initiate measurements to eliminate potential artifact caused by handling of the device. The probe tip was approximately 2 to 3 mm from the eye at resting state, and the mouse was positioned to allow the probe to contact the central cornea perpendicularly. The accuracy of the rebound tonometer (TonoLab; Colonial Medical Supply) in measuring IOP has been previously validated in our species of WT and KO mice.²¹ All measurements and injection experiments were conducted between 11 AM and 3 PM to minimize any potential artifact from circadian variability.²⁰

Microbead Injection

Mice were stabilized on a mounting stage (Stoelting Mouse and Neonatal Rat Adaptor; Stoelting Co., Wood Dale, IL) under a zoom dissecting microscope. The animal was positioned such that the eye was as horizontal as possible (i.e., iris plane parallel to the stage) in order to ensure even distribution of tracer throughout the anterior chamber. A 10 µL Hamilton microsyringe (Nanofil; World Precision Instruments, Sarasota, FL) was loaded with 1 µL 1:50 solution of microbead tracer diluted in 1× DPBS–/–, as well as 2 µL KII solution separated by a 0.1 µL air bubble. Smaller 20 nm microbeads were utilized to ensure that the IOP did not artificially elevate as a result of mechanical blockage from the beads themselves. Multiple rinses with the tracer were completed in order to prevent contamination of the tracer volume in the syringe with KII solution. A 35G needle (NF35BL-2; World Precision Instruments) connected to this syringe was then inserted into the right eye anterior chamber centrally to optimize uniform distribution. The tracer volume was delivered at 4 nL/s by a microprocessor-based microsyringe pump controller (Micro4; World Precision Instruments). Lubricating eye drops (Nature's Tears [hyalomellose 0.4%]; Rugby Laboratories, Duluth, GA) were applied to the cornea to prevent dehydration. The 12 o'clock position of the eye was marked using tissue marker dye (TMD Tissue Marking Dye; Triangle Biomedical Sciences, Durham, NC) to provide orientation. At 30 minutes, half of the original dose of anesthesia was administered in order to maintain the mouse at the appropriate level of sedation.

A time-course experiment was first performed to determine the optimal incubation time for tracer to migrate through the anterior chamber and strongly penetrate the TM and SC. This was found to be 45 minutes, which was used for all further experiments. The needle remained in the eye during this period, and 2 µL KII solution was subsequently injected into the anterior chamber of each eye. KII solution was also simultaneously applied to the exterior of the eye multiple times using a plastic dropper. After 30 minutes of fixation, the mouse was sacrificed by anesthetic overdose (4× the original dose), and the eyes were enucleated using a lateral canthotomy technique to reduce trauma to the globe and placed in KII solution at 4°C overnight. If precipitated tracer was observed within the syringe or if the needle made contact with the lens, the experiment was abandoned due to the reduction in effective tracer concentration.

Tissue Handling for Confocal Microscopy

After overnight fixation, each eye was dissected, with the posterior cup and lens removed and the anterior portion partially dissected into six

FIGURE 1. (A) Sunflower-like pattern of an enucleated eye dissected into eight pieces. Iris and Schlemm's canal (SC) are demarcated. Analysis of one of these eight sections is illustrated in the following panels. The yellow surface represents the side of the tissue seen in the en face view (B), radial section (C), and frontal section (D). The frontal section plane was usually cut posterior to the iris, as shown here.

to eight sections in a sunflower-like pattern. The eye was mounted on a slide; the orientation of the tissue was maintained by orienting it with the superior portion placed superiorly on the slide. Sections were viewed en face, and radial and frontal sections were prepared and examined (Fig. 1).

Although autofluorescence of the iris was noted, this tissue was not removed due to the potential loss or disruption of the TM during that process. Fluorescence in the sample was visualized using a confocal microscope (Carl Zeiss 510 Axiovert M100 Laser Scanning Microscope; Carl Zeiss, Heidelberg, Germany) with a 10× objective and a 169 μm pinhole in order to capture the entire fluorescence throughout the thickness of the tissue. The tissue was imaged through the corneal side in order to visualize the fluorescent tracer in the TM. Compiling a set of Z-stack images and quantifying the fluorescence confirmed the same intensity and overall pattern as seen with the large pinhole setting. Images of each of the six to eight sections of the tissue were captured and analyzed using the Zeiss LSM 510 operating software (Carl Zeiss).

The tissue was subsequently sectioned in both radial and frontal planes. Sections were stained with 1:1000 TO-PRO-3 (Invitrogen) for 30 minutes, followed by three 10-minute washes using 1× DPBS. Radial sections were evaluated using confocal microscopy to identify TM and SC, as well as episcleral veins. Frontal sections were prepared with the guidance of the previously observed fluorescence.

Analysis of Percentage Effective Filtration Length

Images were analyzed to quantify the overall outflow area in the eye. In each section, "total length" of the TM (TL) and "filtration length" of TM containing tracer (FL) were quantified. Percentage effective filtration length ($PEFL = FL/TL \times 100\%$) was subsequently calculated as performed in previous studies.^{27,31} Tracer-containing TM was included in the measurement only if the fluorescence created a clear banded pattern and if confocal microscopy confirmed microbead deposition along the JCT and inner wall of SC; irregular, hazy points of fluorescence were not included (Fig. 2). If a specific tissue section required two images, a landmark within the tissue was used to prevent measurement overlap. Overall PEFL for the entire eye was calculated by averaging all PEFL measurements. A masked second investigator

reviewed the quantification process to ensure its validity ($n = 10$ images). If any disagreement arose, a third party determined the accuracy of the calculations. The investigators then reviewed the data points in question together and identified the reason for the discrepancy in order to prevent future disagreements. Statistical analyses comparing PEFL and IOP of WT and KO mice were completed using a two-tailed paired Student's *t*-test. Correlation between PEFL and IOP and the associated coefficient of determination (R^2 value) was calculated using Microsoft Excel (Microsoft, Redmond, WA). SAS software (SAS Software 9.3; SAS Institute, Cary, NC) was used to run a regression diagnostic; the studentized residual test was used to identify any outliers within the linear regression data. An outlier was defined as any residual point with the standard deviation absolute value greater than 3. Prism 5 (GraphPad Software, La Jolla, CA) was used to create all graphs.

Light and Electron Microscopy

Sections containing TM and SC that displayed a high concentration of fluorescent tracer ("high-tracer") or absent tracer ("low-tracer") by confocal microscopy in both WT and KO eyes were processed for light and electron microscopy. Sections were postfixed with 2% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA) in 1.5% potassium ferrocyanide (Fisher Scientific Company, Fair Lawn, NJ) for 2 hours. Sections were dehydrated using a graded series of ethanol and subsequently embedded in Epon-Araldite mixture (Electron Microscopy Sciences). Semithin sections (2 μm) were cut and analyzed using light microscopy with an Olympus BX40 microscope (Olympus). Sections containing regions of interest were then prepared for electron microscopy; thin sections (90 nm) were produced, stained with uranyl acetate (Fisher Scientific Company), and examined using transmission electron microscopes (Philips EM 300; Philips, Eindhoven, Netherlands; and JEOL JEM-1011; EOL, Peabody, MA). Images were taken along SC at varying magnifications. Additional details regarding the processing can be found in previous publications.²⁸ For collagen fibril diameter measurements, EM images at ×40,000 magnification were used. A total of 150 fibrils counted from five different areas within the JCT were used to compute an average diameter for each subtype (e.g.,

FIGURE 2. Representative images of (A) confocal microscopy of a radial section of injected wild-type (WT) tissue. Schlemm's canal (SC) and trabecular meshwork (TM) are labeled. Microbeads are present throughout the TM and along the inner wall of SC. Cells were stained with TO-PRO-3 (blue), while the fluorescent microbead tracer appears green. Part of the ciliary body (CB) can be seen. (B) FL (blue line) and TL (red line) measurements from basic fluoroscopy of this flat mount en face image. Tracer within the TM can be observed. In terms of orientation, the tissue in (A) is being viewed on the orthogonal in (B). PEFL for this specific tissue section can be calculated from FL and TL (PEFL = $808.30/1196.18 \times 100\%$ = 72.59%). The fluorescence seen in the iris and ciliary body (CB) is due to autofluorescence. Tracer can also be seen in the episcleral vein, indicating that the microbeads traverse the normal outflow pattern. Orientation figures as seen in Figure 1 demonstrate which surface is being viewed.

WT high-tracer, WT low-tracer). Thus, a total of 300 fibers were counted for each eye. Only fibers that were clearly cut in cross section were included in order to ensure accurate measurement of fibril diameter. ImageJ (National Institutes of Health) was utilized to measure the collagen fibril diameter. Microsoft Excel (Microsoft) was used to perform statistical analyses, and Prism 5 (GraphPad Software) was used to create graphs.

RESULTS

No Significant Difference in WT and KO Affinity for Microbeads

Mean TCF of microbeads binding to the TM in passively bathed WT and KO eyes did not differ, with WT TCF = $6.05 \times 10^9 \pm 4.37 \times 10^8$ and KO TCF = $6.19 \times 10^9 \pm 3.67 \times 10^8$ at 37°C ($n = 5$ pairs, $P = 0.817$; Fig. 3A). Tracer was present within the TM uniformly in all regions of both WT and KO eyes. To assess if temperature had an effect on passive binding, the experiment was repeated overnight at 4°C , which again confirmed no significant difference in TCF (WT TCF = $9.48 \times 10^9 \pm 5.78 \times 10^8$, KO TCF = $9.59 \times 10^9 \pm 6.85 \times 10^8$; $n = 4$ pairs, $P = 0.906$). Confocal microscopy demonstrated that microbeads mostly collected between the uveal beams within the TM in both WT and KO tissue, occasionally penetrating as far as the middle of the TM (Fig. 3C). Electron microscopy also showed no significant difference in the binding of microbeads between WT and KO. In both tissues, microbeads were observed in large, open spaces between trabecular beams (Fig. 4). Microbeads were not found within the JCT or SC.

Outflow Drainage Pattern Is More Continuous in KO Mice and Correlated with a Decrease of IOP

We confirmed the previous findings of a lower IOP in SPARC KO mice, with a 22.7% reduction compared to WT mice. The mean IOP was 16.3 ± 2.4 mm Hg and 12.6 ± 2.5 mm Hg for WT and KO mice, respectively ($P < 0.005$, $n = 11$ pairs).

Segmental flow was discovered in both WT and SPARC KO mice, but to a much lesser degree in the latter. WT mice had tracer deposition only in specific regions of the eye (based on gross fluorescence); however, no consistent pattern was present among the WT eyes. The deposition pattern was much more continuous in KO mice (Figs. 5A, 5B). While outflow appeared almost always on the lateral aspects of the WT eye, outflow was more variable in the superior and inferior portions of the eye. Thus, it was not possible to attribute the outflow pattern to the presence of specific venous structures in certain regions. In contrast to data from the passive binding assay, confocal microscopy of high-tracer regions demonstrated the strong presence of microbeads throughout the TM, extending into deeper portions along the inner wall of SC; tracer could also be observed within the canal (Figs. 5C, 5D). When viewed en face, high-tracer regions often demonstrated the presence of microbeads within episcleral veins, reflecting the full penetration of microbeads through the TM and SC and into the venous system (Fig. 5E). In contrast, low-tracer regions demonstrated minimal tracer within the TM; no microbeads were found in SC or episcleral veins.

The increase in PEFL reflects a significant increase in the area of TM utilized for outflow in the KO eye. PEFL significantly increased in SPARC KO mice ($70.61\% \pm 11.36\%$) compared to WT mice ($54.68\% \pm 9.95\%$; $n = 11$ pairs, $P < 0.005$; Fig. 5F).

FIGURE 3. Representative en face images of (A) WT and (B) KO eyes bathed in the microbead solution. Due to the significant amount of background from microbeads binding the cornea and sclera, the central and peripheral portions of the images were removed. (C, D) Frontal confocal sections of WT and KO tissue, respectively, with sclera, trabecular meshwork (TM), Schlemm's canal (SC), and ciliary body (CB) labeled. Both images demonstrate the accumulation of tracer mostly among the superficial-intermediate portions of the TM, rarely approaching SC.

FIGURE 4. (A, B) Electron microscopy of WT tissue TM with Schlemm's canal (SC), juxtacanalicular connective tissue (JCT), and intertrabecular space (ITS) labeled. The region encased in the *black box* in (A) is magnified in (B). Microbeads (*) were found in the open spaces in the ITS. (C, D) KO tissue TM with SC, JCT, and ITS labeled. The *black box* region in (C) is magnified in (D). Microbeads (*) were again found in open spaces between trabecular beams.

FIGURE 5. (A, B) Representative pair of matched WT and KO eyes. Fluorescence microscopy was completed en face through the corneal side. Tracer bead distribution in the TM of WT eyes was heterogeneous, with large sections having little staining. Greater fluorescence in a more homogeneous pattern was seen in SPARC KO eyes, reflecting more uniform outflow. *Gray* portions of these images are from the projection of the brightfield lamp on the tissue and medium. Central portions of these images were removed due to iris autofluorescence. (C, D) Representative confocal microscopy of frontal sections of WT and KO high-tracer sections, respectively. Sclera, trabecular meshwork (TM), Schlemm's canal (SC), ciliary body (CB), collector channel (CC), and episcleral vein (EV) are labeled. Tracer is strongly present throughout the TM. Tracer can also be noted within SC. (E) En face image of a high-tracer section demonstrating microbeads within the TM and along the walls of episcleral veins. (F) Percentage effective filtration length (PEFL) values in WT and KO eyes with SEM error bars. KO PEFL was significantly higher (*asterisk*) than WT PEFL ($n = 11$ pairs, $P < 0.005$).

FIGURE 6. (A) IOP-PEFL correlation ($n = 22$). The outlier (circle) and its matched point (arrow) are identified. (B) IOP-PEFL correlation with these points removed ($n = 20$, $P < 0.0001$). R^2 increases significantly.

Thus, it appears that the transgenic deletion of SPARC significantly affects the aqueous humor outflow pattern.

A negative correlation between IOP and PEFL was observed (Fig. 6); PEFL decreased as IOP increased. This suggests that a decrease in the area of bead deposition corresponded to an overall decrease in outflow, linked to a relatively elevated IOP. Conversely, greater bead deposition represented increased outflow of aqueous humor, which was associated with decreased IOP. When fit with a corresponding linear regression line, an R^2 value of 0.59 was calculated ($n = 22$). This indicates a reasonable association between the two data sets. However, one data point was identified as an outlier by the studentized residual test with a standard deviation of -3.82 . When this point and its matched pair were removed from the analysis, R^2 increased to 0.72 ($n = 20$, $P < 0.0001$), indicating a more robust association between PEFL and IOP.

High-Tracer Regions Display Less Compact Morphology than Low-Tracer Regions

Upon comparing electron microscopy images at lower magnification ($\times 8000$) of high-tracer and low-tracer regions in WT and KO eyes ($n = 3$ pairs), it was apparent that high-

tracer regions had less compact morphology (Fig. 7). The JCT and TM in low-tracer images appeared to be much more compact, with the trabecular beams layered closer together. In contrast, increased space between trabecular beams was noted in high-tracer samples. The difference in morphologic data is consistent with previous findings in other animal species.^{27,31}

Collagen Fibril Diameter Is Significantly Decreased in JCT of SPARC KO Mice

No significant morphologic difference was detected between the TM of the KO and WT tissues at the light microscopy level (data not shown), consistent with our previously published report²¹; the iridocorneal angles were indistinguishable. Even at low transmission electron microscopy magnification levels, WT and KO tissues showed no morphologic difference. However, at higher magnification ($\times 40,000$), collagen fibril diameter within the JCT was found to be significantly decreased in SPARC KO mice (28.272 nm) compared to WT mice (34.961 nm, $P < 0.0005$, $n = 3$ pairs; Fig. 8). If differentiated into high-tracer versus low-tracer regions of WT and KO tissue, collagen fibril diameter was significantly decreased both in high-tracer regions of KO tissue compared to that of WT (27.232 nm and 35.459 nm, respectively, $P < 0.0005$) and in low-tracer regions of KO versus WT (29.312 nm and 34.462 nm, respectively, $P < 0.0005$).

In addition, microbeads were found in the open spaces of the JCT in high-tracer regions at the $\times 40,000$ magnification (Fig. 9). In contrast, microbeads were not identifiable in the JCT of low-tracer regions, a consistent finding given its more compact ECM (Fig. 7). More microbeads were also found in the JCT regions facing the collector channel ostia, underneath the gaps among inner wall endothelial cells.

DISCUSSION

These are the first data to demonstrate segmental flow in mice, which has previously been shown only in human, monkey, and bovine eyes. This finding has an evolutionary implication, indicating that segmental flow may be strongly conserved among mammalian species. Furthermore, it reflects yet another similarity between mouse and human outflow mechanisms, strengthening the value of the mouse as a model system for aqueous outflow studies. With respect to geographic flow patterns, we did not observe consistent differences in the outflow patterns of inferior versus superior regions of the eyes; it appears that there is an equal probability that any area of the TM will be utilized for outflow. Collector channels, which have been confirmed to be present in mice,²³ appeared without any specific pattern as well. We were not able to determine the potentially dynamic nature of flow patterns, as the outflow pattern in each mouse eye was assessed only once in one specific position before sacrifice. It is possible that a low-tracer region could become a high-tracer region at a later time, most likely dictated by positioning of the eye. Further studies would be required to determine whether such a transformation could occur.

We confirmed our previous finding of a lower IOP in SPARC-null mice and found a significant increase in PEFL in the same mice, with an overall change from segmental to more continuous outflow. The association between the PEFL and IOP is moderate, with an R^2 value of 0.59, even in the presence of an outlier. When this point and its paired point were removed, the increase in R^2 to 0.72 reflects a strong correlation ($P < 0.0001$). The increase in PEFL of KO eyes along with this association suggests that SPARC is an important modulator of outflow and thus IOP in mice. While it is tempting to suggest

FIGURE 7. Representative EM images of the four conditions with Schlemm's canal (SC) labeled. (A) TM of a WT high-tracer region. Note the giant vacuole (*) in this image. (B) TM of a KO high-tracer region. Again, a vacuole (*) can be seen. (C) TM of a WT low-tracer region. The tissue is relatively compact. (D) TM of a KO low-tracer region. The tissue features more compact morphology once again.

that changes in ECM composition increase PEFL in KO eyes, which subsequently leads to a reduction in IOP, it is not possible to determine whether IOP causes a change in PEFL or vice versa. In a previous study, IOP was identified as the causative factor leading to changes in PEFL.⁴² However, IOP was exogenously altered in bovine eyes in those experiments, which led to morphologic abnormalities such as the collapse of the aqueous plexus at high pressures. This disturbance thereby affected the area utilized for aqueous humor outflow. In our experiments, we did not observe such morphologic abnormalities in WT and KO tissue with confocal, light, or electron microscopy, and thus do not believe that baseline IOP of the mice affected PEFL in these studies. Rather, we suspect that the PEFL increase could have been due to a fundamental difference in the ECM composition caused by the lack of SPARC, such as the decrease in collagen fibril diameter that was observed. Nonetheless, we cannot conclusively identify a causative factor in the association between IOP and PEFL. Further investigation is warranted in order to establish a causal relationship.

Our passive binding studies demonstrated no difference in ECM affinity for microbeads between the TM of WT and KO mice, mirroring previous tissue bathing experiments completed with cationic ferritin.²⁴ Thus, the difference in tracer distribution we observed in the injection experiments was not due to inherent differences in ECM affinity for the microbeads. Additionally, without active pressure gradients and flow, the microbeads did not migrate into the deeper portions of the TM, but collected in large, open spaces (Figs. 3–5). Their concentration within the juxtacanalicular TM was less than that in the eyes injected *in vivo*. In the *in vivo* experiments, the natural outflow of aqueous humor maintained the pressure gradient and hence the distribution of tracer. Confocal and electron microscopy of injected high-tracer regions demonstrated microbeads within the JCT, along the SC inner wall endothelium, and within episcleral veins (Figs. 5, 9). Thus, high microbead deposition appears to represent areas of active aqueous humor outflow. We therefore believe that the concentrated presence of tracer and its penetration of the

TM reflect a high-flow region, while minimal tracer indicates a low-flow region. Previous studies corroborate this association between tracer and outflow in other animal models.^{27,31}

Our experimental model system confers two significant advantages. First, the animal model used was genetically manipulated, leading to an endogenous change in outflow. This is in contrast to other models currently used, which often compare WT eyes with eyes displaying altered outflow induced by exogenous manipulation of IOP or laser trauma. Second, rather than using enucleated *ex vivo* tissue, our model takes advantage of *in vivo* outflow mechanisms in the mouse to study the distribution of the fluorescent tracer. Studies by Lindsey, Weinreb, and others also used fluorescent tracers and *in vivo* outflow to identify specific pathways, molecular size restrictions of these pathways, and aqueous humor dynamics.^{33–37} We used fluorescent tracer distribution to characterize and compare outflow patterns and associated morphologic changes in WT and KO mouse strains.

Electron microscopy provided a structural correlation to the functional aspects of lower IOP and increased area of aqueous outflow. The difference in tracer distribution correlated with the morphologic appearance, that is, the compactness of the TM. In areas of greater tracer deposition, less compact JCT tissue was observed. In contrast, more compact TM was associated with a minimal amount of tracer in such areas. In eyes with untreated POAG, Rohen et al. found an increased amount of ECM in the JCT region and hypothesized that this inhibited outflow.¹² We demonstrate a structural and functional relationship reflecting the concept that aqueous humor appears to leave the eye in specific areas depending on the compactness of the TM and proximity to the pores of inner wall and collector channel (CC) ostia.

The significant difference in collagen fibril diameter within the JCT of WT and SPARC KO tissues reflects the importance of SPARC in ECM processing. Significant changes in collagen fibril diameter in other tissues of SPARC-null mice have been reported in previous studies.^{43,44} Bradshaw et al. showed that dermal collagen fibrils in SPARC KO mice were significantly

FIGURE 8. (A) Representative EM images of collagen fibrils in WT and KO high-tracer and low-tracer regions. Cross sections of the fibrils within the JCT can be observed. Schlemm's canal (SC) is labeled. (B) Comparison of average fibril diameter values between high- and low-tracer regions of WT and KO tissue with SEM error bars. A total of 300 fibrils per eye derived from five different sections of JCT were assessed. Three pairs of eyes were used in the calculations. Both KO high-tracer and low-tracer values were significantly decreased (*asterisks*) compared to those of WT ($P < 0.0005$).

FIGURE 9. (A) Representative image of low-tracer region. No microbeads could be identified in the JCT region. Schlemm's canal (SC) labeled. (B) Representative image of high-tracer region. Microbeads (*) are identified within the JCT. (C) High-tracer region near collector channel (CC) ostia. The JCT near the entrance to CC ostia (*) in high-tracer regions appears less compact. The region encased in the *black box* is magnified in the *next panel*. (D) Collections of microbeads (*) were found in the open spaces of the JCT underneath a pore (*arrow*) among inner wall endothelial cells. Collector canal (CC) labeled.

smaller and more uniform compared to those of WT mice.^{43,44} They also demonstrated that SPARC was essential to the maturation of collagen fibers in the dermis, highlighting the effect of SPARC on collagen processing and ECM composition.

In the eye, the decrease in fibril diameter in both high-tracer and low-tracer regions reflects the global effect of the deletion of SPARC on collagen fibrils. Thus, our data suggest a potential mechanism by which IOP is reduced in SPARC KO mice; with a significant change in collagen fibril diameter and perhaps other ECM components, more of the TM is converted into a high-tracer state and outflow subsequently increases as reflected by the PEFL data noted in this study. Our group previously reported a decrease in IOP but increased JCT collagen fibril diameter in the thrombospondin-1 and -2 (TSP1 and TSP2) KO mice.⁴⁵ The relationship between IOP and collagen fibril diameter is unclear, but it seems that the deletion of extracellular matrix proteins affects posttranslational processing of collagens.⁴⁶ Given that no significant morphologic changes were noted on light microscopy or at lower EM magnification, the physiologic changes in the SPARC KO mouse do not appear to be due to gross changes in ECM production, but rather ECM modulation such as posttranslational processing of collagen or fibrillogenesis. Fibril diameter is regulated by multiple processes such as crosslinking alteration by crosslinking enzymes (lysyl oxidase, tenascin-X, perlecan) and small leucine-rich proteoglycans (decorin, biglycan, fibromodulin); cleavage of procollagen by bone morphogenetic protein-1 (BMP-1) and other proteinases; organization of fibril assembly by fibronectin and integrins; and nucleation by collagen V and XI.^{47,48} SPARC may be involved in modulating one or more of these regulatory processes.

SPARC may affect the quality of ECM within the TM in addition to the ultrastructural anatomy. This difference may also contribute to the percentage of the entire TM that is compact, such that the lack of SPARC leads to a decreased overall amount of compact TM. Previous studies have indicated that it is not just the quantity of the ECM, which would be appreciated through electron microscopy, but the quality of the ECM that dictates outflow.⁴⁹ Others have theorized that ECM density is dependent on both collagen fibril size and strength of cell-ECM adhesion⁴⁶; thus, both fibril size and adhesive properties ought to be assessed in order to accurately gauge ECM density, which has implications on outflow. While our current study was not designed to assess ECM quality or cell-ECM adhesion, further studies to assess these factors are under way.

Limitations of this study include the inability to monitor IOP during the injection. This was not possible due to the size of the eye and physical limitations of the experimental space. An injection rate of 4 nL/s was chosen based on similar rates utilized in established studies evaluating fluorescent tracer distribution.^{33,35} However, a transient elevation of IOP may have occurred during the injection phase, given the injection rate in comparison to the endogenous aqueous humor formation rate (0.14–0.18 $\mu\text{L}/\text{min}$).^{50,51} Any such IOP elevation could have affected tracer distribution. Given a possible difference in outflow facility between WT and SPARC-null eyes,²¹ the magnitude of this IOP elevation might have differed between the two strains. Thus, the contrast in tracer distribution between WT and SPARC-null eyes could have been influenced by this potential difference in facility, in addition to baseline IOP. We were unable to separate these factors given our experimental design. However, the observed difference in tracer distribution still remains an effect of endogenous differences between WT and SPARC-null eyes.

Our study was novel in utilizing an injection system in vivo to examine one specific endogenous protein and its effect on outflow and IOP. These data provide strong evidence

implicating SPARC in the regulation of IOP and aqueous humor outflow, further defining the function of this protein in IOP regulation. Our work demonstrates for the first time that segmental flow exists in the mouse, reflecting its potential importance in mammalian homeostasis and IOP regulation. The structure-function relationship between areas of greater outflow correlating to areas of TM with less compact ECM further highlights the importance of ECM homeostasis to IOP control. SPARC appears to influence collagen fibril diameter, which may contribute to an increase in the percentage of TM in the high-flow state. Future studies are directed toward understanding the qualitative changes of JCT ECM mediated by SPARC.

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