SPARC-null Mice Exhibit Lower Intraocular Pressures

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PURPOSE. SPARC is a matricellular protein that is highly expressed in remodeling tissues, including the trabecular meshwork and ciliary body. The hypothesis for the study was that SPARC contributes to the regulation of intraocular pressure (IOP). The IOPs of SPARC-null mice, their corresponding wild-type (WT), and heterozygous animals were compared.

METHODS. Diurnal and nocturnal IOPs of C57Bl/6x129SvJ WT, SPARC-null, and heterozygous mice were measured. Fluorophotometric measurements were made to assess aqueous turnover. Central corneal thickness (CCT) was measured using histology, ultrasound biomicroscopy, and optical coherence tomography. Iridocorneal angles were examined using light microscopy (LM).

RESULTS. During the day, the mean IOP of SPARC-null mice (n = 142, 16.9 ± 2.4 mm Hg) was lower than that of both WT mice (n = 104, 19.9 ± 2.9 mm Hg; P < 10−12), and heterozygotes (n = 38, 19.3 ± 2.5 mm Hg; P < 10−4). At night, SPARC-null mice also exhibited a blunted increase in IOP in comparison to WT and heterozygous mice. CCTs were not significantly different between WT and SPARC-null mice. Heterozygous mice tended to have thicker corneas (3.4%). Fluorophotometric measurements suggest that aqueous turnover rates in SPARC-null mice are equal to if not greater than rates in WT mice. LM of the SPARC-null iridocorneal angle revealed morphology that is indistinguishable from WT.

CONCLUSIONS. SPARC-null mice have lower IOPs than do their WT counterparts with equal CCTs. The rate of aqueous turnover overs suggests that the mechanism is enhanced outflow resistance. (Invest Ophtalmol Vis Sci. 2009;50:3771–3777) DOI:10.1167/iovs.08-2489

Over the age of 60, glaucoma affects approximately 5% of white and 10% of black Americans.1–3 Elevated intraocular pressure (IOP) is a major risk factor for glaucoma.4 The relatively elevated IOP of open-angle glaucoma is caused by impaired aqueous drainage through the trabecular meshwork (TM) (conventional pathway).5 The secondary pathway for aqueous drainage, the uveoscleral pathway, also demonstrates morphologic changes in the ciliary body (CB) stroma of glaucomatous eyes, similar to those found in TM.6 The molecular events responsible for the regulation of TM and CB drainage are not known; however, extracellular matrix (ECM) turnover in these regions is at least one contributory factor.7–11 SPARC (secreted protein, acidic and rich in cysteine), a member of the matricellular family of proteins, modulates cellular interactions with ECM during embryogenesis and in adult tissues that continue to remodel.12,13 SPARC has also been implicated in several conditions in which fibrosis and altered ECM deposition are strong components of the pathophysiology, such as systemic sclerosis, hepatic fibrosis, osteoporosis, and tumor growth and metastasis in humans.14–18 Expression of SPARC is also increased in animal models of renal and hepatic fibrosis.19,20 SPARC-null mice exhibit accelerated closure of dermal wounds, attenuated collagen and connective tissue, increased adiposity, osteopenia, early-onset cataractogenesis, and a reduced foreign body reaction to implanted biomaterials.21–27 SPARC-null mice have decreased deposition of laminin and type I and IV collagens within the kidney in a diabetic neuropathy model.28 SPARC deficiency in these various tissues indicates that a primary function of this matricellular glycoprotein is ECM deposition.

SPARC is widely expressed in ocular tissues and has been implicated in cataract and corneal wound repair.29–31 The TM contains endothelial cells that express various proteinases likely to be involved in ECM remodeling.32,33 SPARC is present in aqueous humor and is produced by both TM endothelial and CB smooth muscle cells (Rhee DJ, et al. IOVS 2006;47:ARVO E-Abstract 1876).29,34,35 In TM cells, SPARC is one of the most highly expressed genes30 and, in response to mechanical stretching, is one of those most highly upregulated.37 We hypothesized that SPARC mediates ECM deposition in the TM and CB and is involved in IOP regulation. Accordingly, we compared the IOP of SPARC-null and wild-type (WT) mice to clarify the role of SPARC in these tissues.

MATERIALS AND METHODS

Animal Husbandry

All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. SPARC-null mice, which had greater than 10 backcrosses onto a C57Bl/6 background, and their WT (C57Bl/6x129/SvJ background) strain were obtained from the Benaroya Research Institute at Virginia Mason (Seattle, WA).23 They were bred independently and genotyped to confirm homozygosity. Briefly, DNA was isolated using a commercial kit including proteinase, and DNA purification reagents (QiaGen, Gaithersburg, MD). PCR was performed in 40 cycles at the following temperatures: denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and polymerization at 72°C for 1 minute. The amplified DNA products were analyzed on 1.5% of agarose gel. The following primer sequences were used, producing 450- and 300-bp bands for SPARC-null and WT genotypes, respectively. SPARC (WT allele): 5'-GATGAGGGTGTTACTGGCCCCACACATTAGGGCCCTCAG3' (forward) and 5'-CACCCACACAGCTGGGGGTATGCAGATAGGCCAG3' (reverse); SPARC (null allele): forward primer as above; 5'-GTGGTGCCAGTAGATAGCCCTCTCCACCAAG3' (reverse, located in Neo insert). The “neo insert” refers to the insertion of neomycin-resistance genes in exon 4 of the Sparc gene.
Mating pairs of male SPARC-null mice and female WT mice were used to produce SPARC-heterozygous mice, which were also genotyped to confirm heterozygosity. All animals used in this experiment were born in the Massachusetts Eye and Ear Infirmary animal facility, fed ad libitum, and housed in clear plastic rodent cages under 12/12-hour light-dark cycles (on 0700 hours, off 1900 hours) at 21°C. Animal ages at the time of experimentation were 5 to 8 weeks. The minimum age of 5 weeks was selected because the mouse iridocorneal angle and its structures reach maturity by this age.38 We studied mice up to the age of 8 weeks to avoid confounding effects of cataracts such as phacomorphic or phacolytic glaucoma, which clinically occur only in mature cataracts. SPARC-null mice develop immature cataract by 1.5 months of age and mature cataract without complications between 3.5 and 4.5 months; some animals develop complications of mature cataracts after 5 months of age.21

Measurement of IOP

The mice were anesthetized by intraperitoneal (IP) injection of a ketamine/xylazine mixture (100 and 9 mg/kg, respectively; Phoenix Pharmaceutica, St. Joseph, MO). A previously validated commercial rebound tonometer (TonoLab; Colonial Medical Supply, Franconia, NH) was used to take three sets of six measurements of IOP in each eye.39,40 Right and left eye measurement sets were alternated with the initial eye selected randomly. All measurements were taken between 4 and 7 minutes after IP injection, as prior studies have shown this to be a period with stable IOP.41,42 As indicated by the manufacturer, the tonometer was fixed horizontally for all measurements, and the tip of the probe was 2 to 3 mm from the eye. To reduce variability in measurements, the tonometer was modified to include a pedal that activated the probe without handling of the device. The probe contacted the eye perpendicularly over the central cornea. Verification of targeting was performed under direct visualization with 5.5× magnification. The average of a set of measurements was accepted only if the device indicated that there was “no significant variability” (per the protocol manual; Colonial Medical Supply). Daytime measurements were taken between 1100 and 1500 hours. Nighttime measurements were taken in a subgroup between 2100 and 2400 hours under dim red-light illumination at least 1 week after the daytime measurements. Prior studies have shown that weekly administration of this anesthesia mixture does not affect IOP.43

Validating IOP Measurement

The validation experiment was repeated to confirm that our IOP measurement technique was consistent with the commercial calibration.39 In brief, WT and SPARC-null eyes were cannulated through the temporal limbus with a 30-gauge needle attached to a water reservoir and pressure transducer.44 IOP measurements were taken at various reservoir heights between 7 and 37 mm Hg in random ascending and descending order with the open-stopcock technique. All measurements were performed by one investigator, and the IOPs were independently recorded by another investigator.

Measurement of Central Corneal Thickness (CCT)

Contact tonometry may be affected by CCT. To assess whether this confounder contributes to any observed IOP difference, CCTs of WT, heterozygous, and SPARC-null mice were measured by three modalities: optical coherence tomography (OCT), ultrasound biomicroscopy (UBM), and histology. The mice were imaged with OCT (Stratus; Carl Zeiss Meditec, Inc., Dublin, CA) under anesthesia. CCT was obtained...
by using the internal software (ver. 4.0.7), measuring the peak-to-peak amplitude distance. While under anesthesia, mice were also imaged with an ultrasound biomicroscope (UBM; P60 UBM, Paradigm Medical Ind., Salt Lake City, UT) at 35-MHz, similar to a previous report.45– 47 The CLI 1500 probe (Paradigm) contains an internal water bath and uses gel (GenTeal gel; cabosymethylcellulose 0.25%; Novartis Pharmaceuticals, East Hanover, NJ) to maintain conductivity. Images were captured and exported for analysis with commercial software (P60, ver. 3.2.0; Paradigm Medical Industries, Salt Lake City, UT). CCT was obtained from collected images measuring the distance between top and bottom layers. Mice were then killed and eyes were enucleated for histologic measurements of CCT. Processing was identical with that described in the following section, except the eyes were cut at the equator to preserve the central cornea for CCT measurement. Midsagittal sections were imaged and scaled. CCT measurements were made at three central locations with the ImageJ software (version 1.41; http://rsbweb.nih.gov/ij/download.html). The central location was determined by using sections in which the pupil was visible.

Assessment of Aqueous Humor Turnover

To further investigate the mechanism of the IOP difference observed between WT and SPARC-null mice, we noninvasively measured aqueous humor turnover, by using a modified approach to a previously published fluorophotometric technique.48 In brief, all measurements were made between 1100 and 1500 hours, to reduce potential variability related to diurnal variation of aqueous inflow or outflow. After anesthetizing each mouse with the same anesthesia mixture as that used for IOP measurement, 10 μL of 0.02% benzalkonium chloride (BAC) in saline were applied to right eye to permeabilize the cornea to fluorescein.49 After 5 minutes, the BAC solution was blotted at the lid margin without contacting the corneal epithelium and 10 μL of 0.02% fluorescein in saline were applied to the eye for 5 minutes. The eye and lids were then carefully washed with 600 μL of saline. The microscope was focused to a depth intermediate to the iris and cornea, and images were captured in 10-minute intervals thereafter for 1 hour with a camera and microscope (AxioCam ICC 1 camera and Stemi SV11 microscope; Carl Zeiss Meditec, Inc.) equipped with a GFP filter and acquisition software (AxioVision, release 4.6.3; Carl Zeiss Meditec, Inc.). Using the ImageJ software, an area with no corneal defects was selected and analyzed for average pixel intensity in the green channel. All averages were normalized to the intensity calculated for the image taken at time 0.

Histology

Eight-week-old WT and SPARC-null mice were euthanatized with CO2, and the eyes were enucleated for immediate fixation. An incision was made in the central cornea with a surgical blade (no. 11) to facilitate fixation. The globe was placed into fixative consisting of 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer with 0.08 M CaCl2 at 4°C for 24 hours, washed in 0.1 M cacodylate buffer, and

Table 1. Average Daytime and Nighttime IOPs of WT, Heterozygous, and SPARC-null Mice

<table>
<thead>
<tr>
<th></th>
<th>WT (n)</th>
<th>Heterozygous (n)</th>
<th>SPARC-null (n)</th>
<th>P (WT vs. Heterozygous)</th>
<th>P (WT vs. SPARC-null)</th>
<th>P (Heterozygous vs. SPARC-null)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daytime IOP, mm Hg</td>
<td>19.9 ± 2.9 (104)</td>
<td>19.3 ± 2.5 (38)</td>
<td>16.9 ± 2.4 (142)</td>
<td>0.26</td>
<td>10−12</td>
<td>10−4</td>
</tr>
<tr>
<td>Nighttime IOP, mm Hg</td>
<td>23.6 ± 2.2 (54)</td>
<td>21.8 ± 2.6 (38)</td>
<td>18.9 ± 2.2 (66)</td>
<td>10−3</td>
<td>10−20</td>
<td>10−7</td>
</tr>
<tr>
<td>Increase in IOP day to night, %</td>
<td>21.5 ± 19.2 (54)</td>
<td>12.7 ± 16.8 (38)</td>
<td>13.3 ± 16.7 (66)</td>
<td>0.03</td>
<td>0.01</td>
<td>0.93</td>
</tr>
</tbody>
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P is calculated from nonpaired Student’s t-tests.
postfixed for 1.5 hours in 2% aqueous OsO₄. Tissue was dehydrated in graded concentrations of ethanol, transitioned in propylene oxide, infiltrated with propylene oxide and Epon mixtures (TAAB 812 resin; Marivac, Quebec, Canada), embedded in Epon, and cured for 48 hours at 60°C. Sections (1-μm-thick) were cut on a microtome (Ultracut UCT; Leica, Wetzlar, Germany) and stained with 1% toluidine blue in 1% borate buffer for light microscopy.

**Statistics**

Daytime and nighttime IOPs, the percentage increase from day to night, and CCT measurements of WT, heterozygous, and SPARC-null mice were analyzed by the use of (paired) Student’s t-test and one-way ANOVA followed by all pair-wise multiple-comparison procedures (Student-Newman-Keuls and Bonferroni methods for IOP and CCT, respectively) to determine between-group differences. Aqueous humor turnover data were analyzed by the use of two-way repeated-measures ANOVA followed by all pair-wise multiple-comparison procedures (Student-Newman-Keuls, P < 0.05) to determine between-group differences. Aqueous humor turnover data were presented as the mean ± SD.

**RESULTS**

**Calibration and IOPs**

The IOP measurements (TonoLab; Colonial Medical Supply) were correlated linearly with the manometric pressures: WT-TonoLab IOP = 1.044 · IOP\textsubscript{manometric} + 0.449 (R² = 0.99, n = 2 eyes; Fig. 1A); SPARC-null IOP = 1.058 · IOP\textsubscript{manometric} − 0.663 (R² = 0.99, n = 2 eyes; Fig. 1B). There was also excellent agreement between the reservoir height and pressure transducer recordings.

Average daytime IOPs of WT, heterozygous, and SPARC-null mice (WT, n = 104; heterozygous, n = 38; SPARC-null, n = 142; Fig. 2, Table 1) were 19.9 ± 2.9, 19.3 ± 2.5, and 17.4 ± 2.4 mm Hg, respectively. The average IOP of SPARC-null animals was lower than WT and SPARC-heterozygote IOPs (ANOVA, P < 10⁻¹²; Student-Newman-Keuls, P < 0.05); however, WT and heterozygous IOPs were not different from each other. With these sample sizes and standard deviations of WT and heterozygous mice, our study had an 80% power to detect a difference of at least 1.5 mm Hg (6.5% of WT IOP).

Average nighttime IOPs of WT, heterozygous, and SPARC-null mice (WT, n = 54; heterozygous, n = 38; SPARC-null, n = 66; Fig. 2, Table 1) were 25.6 ± 2.2, 21.8 ± 2.6, and 18.9 ± 2.2 mm Hg, respectively. The IOPs of all three genotypes were significantly different (ANOVA, P < 10⁻¹²; Student-Newman-Keuls, P < 0.05) from each other.

Nighttime IOPs of WT, heterozygous, and SPARC-null mice increased 21.5% ± 19.2%, 12.7% ± 16.8%, 13.3% ± 16.7%, respectively, from day to night. The increase in WT mice was greater than that observed in heterozygous and SPARC-null mice (ANOVA, P < 0.0113; Student-Newman-Keuls, P < 0.05).

**Central Corneal Thickness**

The CCTs of WT, heterozygous, and SPARC-null mice were measured by OCT, UBM, and histology for the same groups of mice (Figs. 3, 4, Table 2). Average CCTs measured by OCT were 105.6 ± 4.3, 109.1 ± 3.6, and 104.5 ± 3.9 μm for WT, heterozygous, and SPARC-null mice, respectively (n = 14, 12, and 6, respectively). The CCTs of WT, heterozygous, and SPARC-null mice by OCT were significantly different by ANOVA (P < 0.05) because CCTs of heterozygous mice were trending toward being significantly larger than WT and SPARC-null CCTs (P = 0.104, 0.09, respectively). Average CCTs measured by UBM were 110.7 ± 3.9, 114.9 ± 5.1, and 106.9 ± 7.1 μm for WT, heterozygous, and SPARC-null mice, respectively (n = 14, 12, and 6, respectively). The CCTs of WT, heterozygous and SPARC-null mice by UBM were significantly different by ANOVA (P < 0.01) with the CCTs of heterozygous mice significantly larger than those of SPARC-null (P < 0.01). Average CCTs measured by histology were 74.0 ± 8.6, 78.5 ± 12.5, and 74.3 ± 6.3 μm for WT, heterozygous, and SPARC-null mice, respectively (n = 14, 12, and 6, respectively). The CCTs of the eyes from all three genotypes by histology were not significantly different, by ANOVA (P = 0.488).

**Assessment of Aqueous Humor Turnover**

Aqueous humor clearance of WT and SPARC-null mice measured by fluorophotometry between 1100 and 1500 hours (Fig. 5, 6).

**Table 2: Average CCTs of WT, Heterozygous, and SPARC-null Mice**

| Genotype     | WT (n)        | Heterozygous (n) | SPARC-null (n) | P  
|--------------|---------------|-----------------|----------------|-----
|              | (μm)          | (μm)            | (μm)           |     |
| OCT          | 105.6 ± 4.3   | 109.1 ± 3.6     | 104.5 ± 3.9    | 0.104 |
|              | 14            | 12              | (6)            | 1    | 0.089 |
| UBM          | 110.7 ± 3.9   | 114.9 ± 5.1     | 106.9 ± 7.1    | 0.125 |
|              | 14            | 12              | (6)            | 0.403 | 0.010 |
| Histology    | 74.0 ± 8.6    | 78.5 ± 12.5     | 74.3 ± 6.3     | 0.798 |
|              | 14            | (12)            | (6)            | 1    | 1    |

Data are in micrometers. P of multiple comparisons is shown.
Least-squares fit analysis yielded exponential decay constants of 0.0097%/min (r^2 = 0.97) and 0.0125%/min (r^2 = 0.97) for WT and SPARC-null data, respectively. Although all SPARC-null relative intensities were less than those in WT mice at the same time point, repeated-measures ANOVA comparison revealed no significant difference in WT and SPARC-null intensities at all other time points (P > 0.05), except at 60 minutes (P < 0.05).

Morphology

During UBM measurement of CCT, it was possible to image the entire globe. There were no retinal detachments or other posterior segment abnormalities which could have coincidentally altered our IOP measurements.

By light microscopy, the iridocorneal angles appear grossly indistinguishable with similar Schlemm’s canals, trabecular beams and cellularity, uveoscleral outflow pathway, and ciliary body location (Fig. 7).

DISCUSSION

SPARC-null mice have lower IOPs than their corresponding WT mice. To investigate the possibility of artifactual differences in rebound tonometry IOP readings, CCT was measured by using three different modalities: OCT, UBM, and histology. CCTs of heterozygous mice tended to be slightly larger; however, the largest difference between strains was 3.4%, which is unlikely to account for any significant differences in corneal biomechanics. Moreover, heterozygous mice had an intermediate IOP, which suggests that lower IOP is not a result of differing biomechanics. Mice demonstrate strain-dependent differences in CCT. Our average CCT measurements are consistent with previously reported ranges. Although the tonometer (TonoLab; Colonial Medical Supply) had been validated by other groups, we directly validated its measurements by controlling IOP manometrically and confirmed a one-to-one correlation between manometric IOP and the tonometric readings in both WT and SPARC-null mice. Furthermore, impact rebound tonometry does not seem to be subject to CCT.

FIGURE 5. Representative series of images captured from one mouse before application of fluorescein (background) and at 10-minute intervals after washing away excess fluorescein. Average intensity at each time point is normalized to intensity at time 0.

FIGURE 6. Aqueous fluorescein concentration relative to values at t = 0 are shown for WT and SPARC-null mice (n = 8 for each group). Least-squares fit for exponential decay yielded (% intensity) = 100e^{-0.0097\text{ time}} \text{ for WT} and (% intensity) = 100e^{-0.0125\text{ time}} \text{ for SPARC-null data, respectively. } * \text{Significant difference in relative intensities between WT and SPARC-null mice at 60 minutes (P < 0.05).}

FIGURE 7. Light microscopic images of iridocorneal angles of (A) WT and (B) SPARC-null mice appear grossly indistinguishable with similar Schlemm’s canals, trabecular beams and cellularity, uveoscleral outflow pathway, and ciliary body location. There is an artifactual separation of the outer TM in (B, arrowhead). Other labels are as follows: Ciliary processes (CP), anterior chamber (*), and Schlemm’s canal and TM (arrow). A 40× objective was used. Scale bar, 50 μm.
The lower IOP of SPARC-null mice can result from two mechanisms: decreased aqueous production or decreased resistance in aqueous outflow pathways. Our fluorophotometric studies indicate that the aqueous turnover of SPARC-null mice is at least as rapid as the turnover in WT mice. Given the lower IOP in the setting of equal or increased aqueous turnover, our aqueous turnover findings are most consistent with decreased outflow resistance.

Both conventional and uveoscleral outflow pathways have been identified in the mouse. At the light microscopy level, we did not find a difference between SPARC-null and WT mice in the iridocorneal angles. The lack of gross ocular differences in the anterior chamber angle under light microscopy was previously reported by other investigators as well. Future investigations may involve the use of electron microscopy and immunohistochemistry to investigate morphologic or compositional differences in outflow pathways.

At night, an even larger difference in IOP was observed between WT and SPARC-null mice. The percentage increase in IOP from day to night is also reduced in SPARC-null mice, an observation suggesting that outflow pathways with IOP-dependent facilities are disproportionately affected by the absence of SPARC. Mice heterozygous for the SPARC-null allele exhibit an intermediate phenotype. Daytime IOPs are comparable to those of WT mice, whereas the IOP increase at night is blunted as it is in SPARC-null mice. Such an intermediate phenotype has also been observed for the cataractogenic phenotype of SPARC-null mice. SPARC-null mice develop cataract as early as 1.5 months, with 100% penetrance at 5 months of age, whereas heterozygous mice develop cataract at 11 months, and WT mice have no significant cataracts at 13 months. A possible explanation for the intermediate phenotype of heterozygous mice is that the effects of SPARC are dose-dependent. In the past 10 years, investigators have found the mouse to be a useful valid model for the study of glaucoma and IOP regulation. The development of techniques to measure IOP and the availability of relevant transgenic mice provide investigators with new tools to study the mechanism of glaucoma, IOP regulation, and new therapeutics for glaucoma management. SPARC is transcribed at high levels by TM cells and is further enhanced in response to mechanical stretching, with results indicating an important role in IOP regulation. In other tissues, SPARC mediates enhanced fibrosis under physiologic and pathophysiologic conditions. ECM turnover is important for the regulation of outflow resistance in both conventional and uveoscleral pathways.

Our findings of a lower IOP and equal or greater aqueous turnover in SPARC-null mice suggest that SPARC is involved in outflow resistance. Whether the mechanism of resistance is accumulation of ECM in the JCT region, altered cellular function not involving changes in ECM, or alterations in the uveoscleral tract remains to be determined. SPARC has not yet been implicated in glaucoma; however, another member of the matricellular protein family, thrombospondin-1, is more highly expressed in glaucomatous human TM. In the future, the reduction of SPARC may have therapeutic benefits for glaucoma.

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