Mutant Fibulin-3 Causes Proteoglycan Accumulation and Impaired Diffusion Across Bruch’s Membrane

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PURPOSE. The mutation R345W in EFEMP1 (fibulin-3) causes macular degeneration. This study sought to determine whether proteoglycan content and diffusion across Bruch’s membrane are altered in Efemp11/4/8 mice carrying this mutation or in Efemp11/4/8 mice.

METHODS. Proteoglycans in mouse Bruch’s membranes were stained with Cupromeronic Blue (CB). Heparan sulfated proteoglycan (HSPG) and chondroitin/dermatan sulfate proteoglycan (C/DSPG) distributions were visualized following treatments with chondroitinase ABC (C-ABC) or nitrous acid. Total sulfated glycosaminoglycans (sGAGs) in Bruch’s membrane/choroid (BrM/Ch) were measured with dimethylmethylene blue (DMMB). Matrix metalloproteinase (MMP)-2, MMP-9, and tissue inhibitor of metalloproteinase (TIMP)-3 were examined by immunofluorescence and quantified using Image J. Molecules with different Stokes radius (Rₛ) were allowed simultaneously to diffuse through mouse BrM/Ch mounted in a modified Ussing chamber. Samples were quantified using gel exclusion chromatography.

RESULTS. HSPGs and C/DSPGs were markedly increased in Efemp11/4/8 Bruch’s membrane, and MMP-2 and MMP-9 were decreased, but TIMP-3 was increased. Diffusion across Efemp11/4/8 Bruch’s membrane was impaired. In contrast, the proteoglycan amount in Efemp11/4/8 Bruch’s membrane was not significantly different, but the size of proteoglycans was much larger. MMP-2, MMP-3, and TIMP-3 levels were similar to that of Efemp11/4/8 mice, but they were localized diffusely in retinal pigment epithelium (RPE) cells instead of Bruch’s membrane. Diffusion across Efemp11/4/8 Bruch’s membrane was enhanced.

CONCLUSIONS. Mutant fibulin-3 causes proteoglycan accumulation, reduction of MMP-2 and MMP-9, but increase of TIMP-3, and impairs diffusion across Bruch’s membrane. Fibulin-3 ablation results in altered sizes of proteoglycans, altered distributions of MMP-2, MMP-9, and TIMP-3, and enhances diffusion across Bruch’s membrane.

Keywords: age-related macular degeneration, Malattia Leventinese, EFEMP1 (fibulin-3), proteoglycans, diffusion

Bruch’s membrane is a thin layer of connective tissue interposed between the RPE and the choriocapillaris. It serves as a semipermeable filtration barrier for bidirectional diffusion of nutrients and metabolites between the outer retina and the choriocapillaris. Bruch’s membrane contains five diffusion zones: outer collagenous zone, outer lamina, inner collagenous zone, elastic fiber zone, outer collagenous zone, and the basement membrane of the endothelium of the choriocapillaris.

The ground substance of Bruch’s membrane is composed largely of sulfated proteoglycans. These anionic proteoglycans contribute to charge-selective properties of Bruch’s membrane associated with diffusion. It has been suggested that the sulfated glycosaminoglycan (sGAG) side chains of proteoglycans provide an electrolytic barrier to diffusion. Thus, alterations in the proteoglycan content of Bruch’s membrane could influence its diffusion properties, cause retention of materials in Bruch’s membrane, and ultimately disrupt the function of the RPE and outer retina leading to disease. Sub-RPE deposits between the RPE and Bruch’s membrane (basal laminar deposit [BLamD]) or within Bruch’s membrane (basal linear deposits and drusen) are a hallmark of age-related macular degeneration (AMD). The most common cause of incurable blindness in developed countries. Although it is still not clear how these deposits develop, one hypothesis is that the sub-RPE deposits may result from a change in the ability of materials to diffuse across Bruch’s membrane.

Similar to AMD, an autosomal dominant macular degeneration Malattia Leventinese/Doyne’s honeycomb retinal dystrophy (ML/DHRD) is also characterized by the presence of sub-RPE deposits. ML/DHRD is caused by the mutation R345W in fibulin-3, a basement membrane glycoprotein encoded by the gene EGF containing fibulin-like extracellular matrix protein 1 (EFEMP1). Fibulin-3 is a member of the fibulin family. Fibulins are a family of extracellular matrix (ECM) proteins that share an elongated structure containing tandem arrays of calcium-binding epidermal growth factor (EGF)-like domains and a carboxy-terminal fibulin-type
The functions of fibulins are not completely understood yet, but a common feature among them is that they have a high affinity for proteoglycans. In addition, fibulin-1 is a co-factor for proteoglycan ADAMTS-1 (a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 1-12) and an antagonist for ADAMTS-4 and 9. Fibulin-3 up-regulates tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-3, physically interacts with TIMP-3, and down-regulates matrix metalloproteinase (MMP)-2, MMP-3, and MMP-9. These MMPs have known proteoglycan activities.

In contrast, BLamD is not observed in mice that recapitulate the important histopathology of ML/DHRD. Type-1 motifs 1), 17 and an antagonist for ADAMTS-4 and fibulin-1 is a co-factor for proteoglycanase ADAMTS-1 (a membrane, and lead to retention of materials and sub-RPE alteration of sGAGs in tissues can be measured by a colorimetric tent of sGAGs in tissues can be measured by a colorimetric electron microscopy as filaments with different sizes and types of proteoglycans bound to CB can be visualized under electron microscopy of sGAGs in tissues can be measured by a colorimetric electron microscopy as filaments with different sizes and types of proteoglycans bound to CB can be visualized under electron microscopy of sGAGs in tissues can be measured by a colorimetric electron microscopy as filaments with different sizes and types of proteoglycans bound to CB can be visualized under electron microscopy of sGAGs in tissues can be measured by a colorimetric electron microscopy as filaments with different sizes and types of proteoglycans bound to CB can be visualized under electron microscopy of sGAGs in tissues can be measured by a colorimetric

In Efemp1+/+ mice that carry the R345W mutation, basement membrane-like materials accumulate between the plasma and basement membranes of the RPE to form BLamDs. Lipid-rich debris is retained within continuous sheets of BLamDs. There is also an accumulation of heterogeneous materials in a thickened Bruch’s membrane. These mice recapitulate the important histopathology of ML/DHRD. In contrast, BLamD is not observed in Efemp1−/− mice that lack fibulin-3. Thus, Efemp1+/+ and Efemp1−/− can serve as useful tools for studying the role of fibulin-3 in Bruch’s membrane and the underlying mechanism by which mutant fibulin-3 causes sub-RPE deposit formation.

The cationic dye Cupromeronic Blue (CB) binds glycosaminoglycan side chains of sulfated proteoglycans. Different types of proteoglycans bound to CB can be visualized under electron microscopy as filaments with different sizes and electron density. CB staining coupled with treatments that eliminate selective groups of proteoglycans can reveal proteoglycan distribution patterns in tissues. Bruch’s membrane mainly contains Heparan sulfated proteoglycans (HSPGs) in the basement membranes of the RPE and endothelium of choriocapillaris, and chondroitin/dermatan sulfate proteoglycans (C/DSPGs) in the fibrous layers. Treatment with nitric acid eliminates HSPGs, and treatment with chondroitinase ABC (C-ABC) removes C/DSPGs. Content of sGAGs in tissues can be measured by a colorimetric assay with the metachromatic dye dimethylmethylene blue (DMMB). In this study, we investigated whether distribution and content of sulfated proteoglycans are altered in Bruch’s membrane of Efemp1+/+ or Efemp1−/− mice by these methods.

Studies of the permeability of Bruch’s membrane using randomly coiled linear polymers (dextran) have shown that there is an age-related decline in the diffusion of linear polymers. We have previously established a method to study globular protein and small molecule diffusion across Bruch’s membrane by simultaneously measuring the flux of multiple molecules with different R, using quantitative gel exclusion chromatography. Coupling this method with a modified Ussing chamber has allowed us to study diffusion across very small pieces (1.8 mm²) of isolated Bruch’s membrane/choroid (BrM/Ch). In this study, we used this system to examine Bruch’s membrane’s diffusion properties in Efemp1+/+ and Efemp1−/− mice.

Methods

Mice

Efemp1+/+ and Efemp1−/− mice were generated previously. Efemp1+/+ and Efemp1−/− mice were fixed at 4°C in 1% formaldehyde, 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Chorioretinal tissues were dissected from fixed eyes, cut into strips, and divided into four groups for enzymatic or nitrous acid treatment: (group 1) control group without enzyme or nitrous acid treatment; (group 2) C-ABC (Sigma-Aldrich Corp., St. Louis, MO, USA) treatment; (group 3) nitrous acid (Sigma-Aldrich Corp.) treatment; and (group 4) combinational treatment with both C-ABC and nitrous acid. For C-ABC treatment, tissue strips were incubated with 1 unit/mL C-ABC in 0.25 M Tris buffer containing 0.05% BSA, 5 mM benzamidine-HCl, and 0.1 M 6-amino-capric acid, pH 8.0, at 37°C for 24 hours. For nitrous acid treatment, tissue strips were incubated in a nitric acid solution containing 5% sodium nitrite, 35% acetic acid, 5 mM benzamidine-HCl, and 0.1 M 6-amino-capric acid for 90 minutes at room temperature. Each group of tissue strips was then stained with 0.05% CB (Sigma-Aldrich Corp.) in 25 mM sodium acetate, 0.2 M MgCl2 and 2.5% glutaraldehyde, pH 5.7 overnight as described. After staining, tissue strips were processed for transmission electron microscopy. Thin sections were cut with a Reichert Ultracut microtome, stained with uranyl acetate and lead citrate, and imaged with a Philips CM-12 electron microscope equipped with an AMT CCD camera (Advanced Microscopy Techniques Corp., Danvers, MA, USA).

CB-stained proteoglycan filaments were quantified by counting the filaments from different view fields at the same magnification. Specimens from three individual mice for each genotype were included in the filament quantification. Three sections from each specimen were used, and 10 different fields from each section were counted. The numbers of filaments from Efemp1+/+ or Efemp1−/− mice were compared with those in Efemp1−/− mice using a Student’s t-test. Proteoglycan filament lengths were measured using the Adobe Photoshop ruler tool. Ten different filaments per field for each category (HSPG or C/DSPG) were measured. Due to their small size, filament lengths were measured to the nearest 5 nm.

sGAG Quantification Assay

After mice were killed with CO₂ asphyxiation, eyes from 9-month-old Efemp1+/+, Efemp1+/−/+, and Efemp1−/− mice were enucleated, and the lens and anterior segments were removed. The retina was detached using forceps, the eyecup filled with PBS buffer, and the RPE was removed using a fine camel’s hair brush. The BrM/Ch was detached from the sclera, weighed, and minced. Five sets of BrM/Ch samples from five mice per genotype (Efemp1+/+, Efemp1+/−/+ or Efemp1−/−) were analyzed. Samples were digested with 300 μg papaain (Sigma-Aldrich Corp.) in 20 μl of sodium phosphate buffer (pH 6.8) containing 1 mM EDTA and 2 mM dithiothreitol at 60°C for 2 hours. Samples were diluted with 10 mM iodoacetic acid and
50 mM Tris/HCl (pH 8.0). The sGAG content in papain-digested samples was measured by colorimetric assay with DMMB using an assay kit (Amresco, Solon, OH, USA). The manufacturer’s instructions were followed. sGAG measurements from Efemp1ki/ki and Efemp1+/- mice were compared with those in Efemp1ki/ki mice using a Student’s t test.

**Immunofluorescence**

Eyes from 9-month-old Efemp1ki/ki, Efemp1ki/ki, and Efemp1+/- mice were fixed in 4% paraformaldehyde. Immunofluorescence staining of 10-μm frozen sections of mouse eyes was performed as previously described using a rabbit polyclonal antibody against MMP-2 (Abcam, Cambridge, MA, USA), MMP-9 (Abcam), TIMP-3 (Abcam), or fibulin-3. A goat anti-rabbit IgG Alexa Fluor 488 conjugate was used as a secondary antibody (Invitrogen, Carlsbad, CA, USA). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Sections were examined and photographed using a Nikon E600 microscope (Melville, NY, USA) equipped with a CDD camera.

Quantification of immunofluorescence was performed using Image J software (National Institutes of Health, Bethesda, MD, USA). Sections from three mice (n = 3) per genotype (Efemp1ki/ki, Efemp1ki/ki, or Efemp1+/-) were used. Three images taken from three randomly selected areas in each stained section were measured. In each image, the outline of the RPE layer and Bruch’s membrane was drawn, and the area, mean fluorescence, and background readings were measured. The total fluorescence was calculated as integrated density subtracting (area of selected sections multiplying mean fluorescence of background readings). Fluorescence from Efemp1ki/ki or Efemp1+/- mice was compared with that in Efemp1ki/ki mice using a Student’s t-test.

**Diffusion Assay**

The BrM/Ch prepared from 9- or 18-month-old Efemp1+/-, Efemp1ki/ki, and Efemp1+/- mice was mounted between two pieces of X-ray film, which had corresponding 1.0-mm diameter holes and were coated with a thin film of silicon grease. Diffusion of molecules through mouse BrM/Ch was studied using a dual Ussing chamber model U2500 (Warner Instruments, Hamden, CT, USA). The BrM/Ch-X-ray film “sandwich” was mounted, separating the two chambers with only the 1.0-mm diameter circle of BrM/Ch allowing communication between them. Four molecules with different R0 were selected for their ability to separate cleanly by gel exclusion chromatography, cytokine (molecular weight [MW] = 110,141, R0 < 1.0 nm; Sigma-Aldrich Corp.), RNase A (MW = 13,700, R0 = 1.72 nm; Teknova, Hollister, CA, USA), human serum albumin (MW = 66,382, R0 = 3.5-3.9 nm; EMD Chemicals, Gibbstown, NJ, USA), and horse spleen ferritin (MW = 450,000, R0 = 6.15 nm; EMD Chemicals). A mixture of these molecules dissolved in PBS with 1 mM MgCl2 and 0.1 mM CaCl2 (PBS-CM) was added to the reservoir on the BrM side of the tissue with only PBS-CM on the Ch side.

**Flux and Permeability Coefficients**

Flux and permeability coefficients were calculated as previously described. Molecules were quantified after 48 hours using gel exclusion chromatography with a 1.6 × 60 cm Hi-Prep Sephacryl S-500HR (Amersham Biosciences, Piscataway, NJ, USA) column equilibrated with PBS-CM. The column was developed at a linear flow rate of 15 mL/h using an AKTA prime chromatography system controlled by PrimeView software (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Peaks were detected by their absorbance at 280 nm, and peak areas were determined by integration using PrimeView software. Solute concentrations were quantified by linear regression analysis using peak areas derived from known quantities of each test molecule. Flux (J) was calculated using the equation J = (C1 - C2)/t where C1 = accumulated molar concentration, A = the area (cm2) of the exposed tissue (0.000785 cm2), and t = 48 hours. Permeability coefficients (P) were calculated using Fick’s first law P = (J/L)/A where L = the thickness of the BrM/Ch preparation and A describes the difference in moles of molecules between the two compartments of the Ussing chamber.

**RESULTS**

Rod-like filaments representing sulfated proteoglycans were observed at high magnifications under electron microscopy in Bruch’s membrane of 9-month-old Efemp1ki/ki, Efemp1ki/ki, and Efemp1ki/ki mice after CB staining (Fig. 1). The size and number of filaments were different in mice with different Efemp1 genotypes (Fig. 1). In Efemp1ki/ki Bruch’s membrane, filaments with an average length of 50 nm were observed in the collagenous/elastic fibrous layers, and filaments with an average length of 40 nm were observed in the basement membranes of the RPE and endothelium of choriocapillaris (Figs. 1A, 1B, 1C1, 1C2; Table 1). Smaller sizes of filaments may represent the same type of filaments cut shorter during sectioning or only partially visible in any given microscopic viewing field. Filaments in the fibrous layers were eliminated by C-ABC treatment (Fig. 2), indicating that they were C/DSPGs. Filaments in the basement membranes were removed by nitric acid treatment (Fig. 3), indicating that those filaments were HSPGs. Combined treatment of both C-ABC and nitrous acid eliminated all the filaments from Bruch’s membrane (Fig. 4). This indicates that all the filaments observed were either HSPGs or C/DSPGs.

**Proteoglycan Accumulation in Bruch’s Membrane of Efemp1ki/ki Mice**

Markedly increased filaments were observed in Bruch’s membrane of Efemp1ki/ki mice (Figs. 1B, 1B1, 1B2). By counting the filaments from different view fields at the same magnification, we determined that there were on average three times as many filaments in Efemp1ki/ki Bruch’s membrane as those inEfemp1ki/ki Bruch’s membrane (Table 2).

After C-ABC treatment, filaments with an average length of 40 nm (Table 1) representing HSPGs remained in the basement membranes of the RPE and endothelium of choriocapillaris in Efemp1ki/ki mice (Fig. 2). There were 2.35 times as many filaments in Efemp1ki/ki basement membranes as those in Efemp1ki/ki mice (Table 2). After nitrous acid treatment, filaments with an average length of 50 nm (Table 1) representing C/DSPGs remained in the fibrous layers of Efemp1ki/ki Bruch’s membrane (Fig. 3). There were 4.15 times as many filaments in Efemp1ki/ki fibrous layers as those in Efemp1ki/ki fibrous layers (Table 2). Combined treatment of C-ABC and nitrous acid eliminated all the filaments from Efemp1ki/ki Bruch’s membrane (Fig. 4), indicating that all the filaments observed were either HSPGs or C/DSPGs.

Biochemical analysis by DMMB colorimetric assays revealed that total sGAG content in 9-month-old Efemp1ki/ki BrM/Ch was significantly higher than that of Efemp1ki/ki mice (Fig. 5). The average amount of sGAGs in Efemp1ki/ki BrM/Ch was 15.89 μg/mg wet tissue weight, comparing to 10.15 μg/mg in Efemp1ki/ki mice.
Different Sizes of Proteoglycans in Bruch's Membrane of Efemp1−/− Mice

In Efemp1−/− mice, there were slightly more filaments observed in Bruch's membrane than in Efemp1+/+ mice, but the difference was not statistically significant (Table 2). However, the size of filaments in Efemp1−/− Bruch's membrane was strikingly larger (Figs. 1C, 1c1, 1c2). After C-ABC treatment, filaments with an average length of 120 nm (Table 1) representing HSPGs were observed in Efemp1−/− basement membranes of the RPE and endothelium of choriocapillaris.

**Table 1.** Proteoglycan Sizes in Efemp1+/+, Efemp1ki/ki, or Efemp1−/− Bruch's Membrane

<table>
<thead>
<tr>
<th>PG Type</th>
<th>Efemp1+/+</th>
<th>Efemp1ki/ki</th>
<th>Efemp1−/−</th>
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<tbody>
<tr>
<td>HSPG</td>
<td>40 ± 10 nm</td>
<td>40 ± 15 nm</td>
<td>120 ± 30 nm</td>
</tr>
<tr>
<td>C/DSPG</td>
<td>50 ± 10 nm</td>
<td>50 ± 15 nm</td>
<td>105 ± 20 nm</td>
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Proteoglycan filament length = mean ± SD. n = 3 mice per genotype.

**Figure 1.** Electron micrographs of Bruch's membrane of 9-month-old mice stained by CB. (A) Efemp1+/+(+/+). a1 and a2 are higher magnification of box “a1” and “a2” areas of (A). (B) Efemp1ki/ki (ki/ki). b1 and b2 are higher magnification of box “b1” and “b2” areas of (B). (C) Efemp1−/− (−/−). c1 and c2 are higher magnification of box “c1” and “c2” areas of (C). Arrows indicate filaments representing sulfated proteoglycans in Bruch's membrane. OS, photoreceptor outer segment; RPE BI, RPE basal infolding; BrM, Bruch’s membrane. Scale bar: 2 μm for A, B, and C; 200 nm for a1 to c2.
(Fig. 2). After nitrous acid treatment, filaments with an average length of 105 nm (Table 1) representing C/DSPGs were observed in the fibrous layers of Efemp1+/− Bruch’s membrane (Fig. 3). Despite the different sizes, combined treatment of C-ABC and nitrous acid eliminated all the filaments from Efemp1+/−/ki/ki mice, but that filaments (arrows) representing HSPGs in the basement membranes of the RPE and the endothelium of choriocapillaris were preserved. BI, basal infolding; BM, basement membrane; EN, endothelium.

Results of DMB assays showed that total sGAG content in BrM/Ch from 9-month-old Efemp1+/−/+ mice was similar to that of Efemp1+/−/ki/ki mice (Fig. 5). The average amount of sGAGs in Efemp1+/−/+ Brm/Ch was 11.04 µg/mg.

Altered Levels and Distributions of MMP-2, MMP-9, and TIMP-3 in the RPE and Bruch’s Membrane of Efemp1ki/ki and Efemp1+/− Mice

Immunofluorescence revealed that MMP-2, MMP-9, and TIMP-3 were localized to the basal side of the RPE along Bruch’s membrane in Efemp1+/−/+ and Efemp1ki/ki/ki mice (Fig. 6A). TIMP-3 and fibulin-3 were co-localized together and had a complete overlap-staining pattern in these mice (Fig. 6A). However, quantification of immunofluorescence showed that the levels of both MMP-2 and MMP-9 were significantly reduced in Efemp1ki/ki/ki mice (Fig. 6B). In contrast, TIMP-3’s level was significantly increased in Efemp1ki/ki/ki mice (Fig. 6B). Without fibulin-3 in Efemp1+/−/+ mice, MMP-2, MMP-9, and TIMP-3 had a drastically different pattern of distribution. All three proteins had a diffuse distribution in RPE cells (Fig. 6A). This suggests that fibulin-3 may normally function to anchor these proteins to Bruch’s membrane. Quantification of immunofluorescence showed that the levels of all three proteins in the Efemp1+/−/+ RPE and Bruch’s membrane were slightly lower than that in Efemp1+/−/+ mice, but the difference was not statistically significant (Fig. 6B).

Different Diffusion Rates Across Mouse Bruch’s Membrane of Efemp1+/+, Efemp1ki/ki, and Efemp1+/− Mice

We previously established a system using a modified Ussing chamber coupled with quantitative gel exclusion chromatography to study the diffusion of multiple molecules with different Rg through small samples (<2 mm²) of Bruch’s membrane.41 Here we used this system to measure differences in the diffusion of four molecules through Bruch’s membrane of 9- and 18-month-old Efemp1+/+, Efemp1ki/ki, and Efemp1+/− mice in the BrM to Ch direction. We established a concentration gradient of all four molecules across BrM/Ch and applied Fick’s first law to calculate the flux and permeability coefficient of each molecule as described previously.41 The flux of each molecule remained constant, and the concentration gradient was not significantly influenced by the diffusion of molecules throughout the time course of each experiment. Flux and permeability coefficient decreased with increasing Rg (Fig. 7).

For Efemp1+/+ tissue (n = 5 mice per age), cytosine had a flux of 5.33 × 10⁴ (9 months) and 2.99 × 10⁴ mmol cm⁻² h⁻¹ (18 months), and a permeability coefficient of 0.94 (9 months)
3
3
10
3
8 for 18 months old) (Fig. 7). Cytosine had a flux of 1.13
¼
6 for 18 months old) (Fig. 7). Cytosine had a flux of 8.54 (9
Scale bar
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treated with C-ABC and nitrous acid coupled by CB staining. Note that all the filaments in Bruch’s membrane were eliminated. Black dots distinct from the filaments representing proteoglycans in +/+ and ki/ki samples were likely background staining. Bl, basal infolding; BM, basement membrane; EN, endothelium. Scale bar: 500 nm.

and 0.47 cm−2 h−1 (18 months). Ferritin had a flux of 1.32 (9
months) and 0.86 nmol cm−2 h−1 (18 months), and a
permeability coefficient of 9.25 × 10−5 (9 months) and 2.38 × 10−5 cm−2 h−1 (18 months). Flux and permeability coefficient
of each molecule were higher for 9-month-old than 18-month-
tissue (Fig. 7).

We found both flux and permeability coefficients of each
molecule were significantly (P < 0.05) decreased for BrM/Ch
samples from Efemp1ki/ki mice (n = 5 for 9 months old, and n
= 6 for 18 months old) (Fig. 7). Cytosine had a flux of 8.54 (9
months) and 6.71 nmol cm−2 h−1 (18 months), and a
permeability coefficient of 6.92 × 10−4 (9 months) and 2.24 × 10−4 cm−2 h−1 (18 months). Ferritin had a flux of 4.80 × 10−2 (9
months) and 1.47 × 10−2 nmol cm−2 h−1 (18 months), and a
permeability coefficient of 1.01 × 10−6 (9 months) and 3.13 × 10−7 cm−2 h−1 (18 months). This indicates that diffusion across
Bruch’s membrane of Efemp1ki/ki mice was markedly decreased,
with ferritin virtually impermeable at both 9 and 18
months of ages.

In contrast to Efemp1ki/ki mice, we found increased flux
and permeability coefficient of each molecule for BrM/Ch
samples from Efemp1−/−/− mice (n = 6 for 9 months old, and n
= 8 for 18 months old) (Fig. 7). Cytosine had a flux of 1.13 × 10−3 (9 months) and 4.79 × 10−4 nmol cm−2 h−1 (18 months),
and a permeability coefficient of 5.30 (9 months) and 2.93
cm−2 h−1 (18 months). Ferritin had a flux of 3.98 nmol (9
months) and 2.19 nmol cm−2 h−1 (18 months), and a
permeability coefficient of 5.56 × 10−4 (9 months) and 1.12 × 10−4 cm−2 h−1 (18 months). This indicates that diffusion
across Bruch’s membrane of Efemp1−/−/− mice was increased at
both 9 and 18 months of ages. The permeability coefficient
was similar for albumin and ferritin for BrM/Ch samples from
either Efemp1−/− or Efemp1−/−/− mice (Fig. 7B), suggesting that
the ferritin is near the physical size exclusion limit of Bruch’s
membrane for these mice.

**Table 2.** Proteoglycan Distributions in Efemp1+/−, Efemp1ki/ki, and Efemp1−/− Bruch’s Membrane

<table>
<thead>
<tr>
<th>PG Type</th>
<th>Efemp1+/−</th>
<th>Efemp1ki/ki</th>
<th>Efemp1−/−</th>
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<tbody>
<tr>
<td>Total SPG</td>
<td>304 ± 67</td>
<td>906 ± 106a</td>
<td>348 ± 57</td>
</tr>
<tr>
<td>HSPG</td>
<td>183 ± 20</td>
<td>431 ± 31a</td>
<td>197 ± 19</td>
</tr>
<tr>
<td>C/DSPG</td>
<td>96 ± 26</td>
<td>398 ± 46a</td>
<td>113 ± 22</td>
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Proteoglycan filaments per field. The number for each category is
the average of 10 fields of each section (three sections) from each
specimen (three specimens) ± SD. n = 3 mice per genotype. *P < 0.05.

**DISCUSSION**

The R345W mutation in fibulin-3 causes sub-RPE deposit
formation both in human and mouse,13,33,34 but the pathogenic
mechanism behind this remains unknown. In this study, we
have found that Efemp1ki/ki mice carrying this mutation have
markedly increased sulfated proteoglycans in their Bruch’s
membrane, although the type and size of proteoglycans are
similar to those found in the wild-type mice. Both HSPGs and
C/DSPGs are significantly increased. In Efemp1−/− mice, which
do not express fibulin-3, we have found that the sizes of HSPGs
and C/DSPGs are significantly larger than those observed in
wild-type mice, but their amount is not significantly different.
These results indicate that fibulin-3 regulates the level and type
of proteoglycans in Bruch’s membrane.

The accumulated proteoglycans in Efemp1ki/ki mice can be
due to either over-synthesis or deficient degradation. Our
data suggest that deficient degradation may be a more
important factor. MMP-2 and MMP-9, two ECM enzymes
with known proteoglycanase activities, are significantly decreased
in Efemp1ki/ki Bruch’s membrane, but TIMP-3, an inhibitor of

![Figure 4.](Image 58x598 to 554x729)  
**Figure 4.** Electron micrographs of mouse Bruch’s membrane of 9-month-old Efemp1+/− (+/+), Efemp1ki/ki (ki/ki), and Efemp1−/− (−/−) mice treated with C-ABC and nitrous acid coupled by CB staining. Note that all the filaments in Bruch’s membrane were eliminated. Black dots distinct from the filaments representing proteoglycans in +/+ and ki/ki samples were likely background staining. Bl, basal infolding; BM, basement membrane; EN, endothelium. Scale bar: 500 nm.

![Figure 5.](Image 363x95 to 507x301)  
**Figure 5.** Total amount of sGAG of BrM/Ch samples from 9-month-old mice. Error bars indicate the mean ± SD. n = 5 mice per genotype. *P < 0.05 comparing to the value of Efemp1+/− mice. +/+, Efemp1+/−; ki/ki, Efemp1ki/ki; −/−, Efemp1−/−.
a broad range of proteoglycanases, is significantly increased. Without fibulin-3 in Efemp1\(^{+/+}\) mice, MMP-2, MMP-9, and TIMP-3 are diffusely localized in RPE cells instead of Bruch’s membrane, suggesting that fibulin-3 normally activates proteoglycan turnover in Bruch’s membrane by controlling proteoglycanase activities. In the absence of fibulin-3, proteoglycans normally present in Bruch’s membrane, thus, leading to the exposure of retained lipids to the high oxidant stress environment at the RPE/Bruch’s membrane interface. Oxidative modification of lipids triggers complement activation to remove the oxidatively damaged molecules.\(^5\) The sustained supply of lipids trapped in proteoglycans can turn complement activation into chronic inflammation with the accumulation of inflammatory products to form sub-RPE deposits.

Proteoglycans affect the diffusion properties of Bruch’s membrane. Consistent with the changes in proteoglycans in Efemp1\(^{+/+}\) and Efemp1\(^{+/−}\) mice, we have found that diffusion across Bruch’s membrane is impaired in Efemp1\(^{+/−}\) mice, but enhanced in Efemp1\(^{−/−}\) mice. Both flux and permeability coefficient decreased exponentially for all four molecules with \(R_0\) ranging from <1.0 to 6.15 nm for Efemp1\(^{+/−}\) mice comparing to those for the wild-type mice. This suggests that the porosity of Bruch’s membrane is greatly reduced in Efemp1\(^{+/−}\) mice. The increased amount of proteoglycans in these mice may structurally obscure pores of Bruch’s membrane, or may increase the viscosity of the fibrous layers. Impaired diffusion would hinder the removal of metabolic wastes and other molecules secreted from the RPE, and accelerate the deposition of heterogeneous materials in Bruch’s membrane of Efemp1\(^{+/−}\) mice. For Efemp1\(^{−/−}\) mice, the flux and permeability coefficient across Bruch’s membrane are more in line with those of the wild-type mice, but the increase is significant. The substantially larger sizes of proteoglycans in Efemp1\(^{−/−}\) mice may structurally expand pore sizes of Bruch’s membrane resulting in enhanced diffusion as we observed.

In summary, our study has found that mutant fibulin-3 causes proteoglycan accumulation and impaired diffusion across Bruch’s membrane. Fibulin-3 may have specific proteoglycanase substrate(s). It would be worthwhile to investigate this in future studies. The impairment of diffusion across Bruch’s membrane would block nutrient and waste flow to and from the retina, contribute to the initiation and/or growth of sub-RPE deposits, and induce degeneration of the RPE and photoreceptors. The enhanced diffusion across Bruch’s membrane of Efemp1\(^{+/−}\) mice suggests that reduction or elimination of fibulin-3 could be a valuable strategy in developing treatments for macular degeneration.
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References


