An Atlas of Heparan Sulfate Proteoglycans in the Postnatal Rat Lens

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PURPOSE. The arrangement of lens cells is regulated by ocular growth factors. Although the effects of these inductive molecules on lens cell behavior (proliferation, survival, and fiber differentiation) are well-characterized, the precise mechanisms underlying the regulation of growth factor-mediated signaling in lens remains elusive. Increasing evidence highlights the importance of heparan sulfate proteoglycans (HSPGs) for the signaling regulation of growth factors; however, the identity of the different lens HSPGs and the specific roles they play in lens biology are still unknown.

METHODS. Semiquantitative real-time (RT)-PCR and immunolabeling were used to characterize the spatial distribution of all known HSPG core proteins and their associated glycosaminoglycans (heparan and chondroitin sulfate) in the postnatal rat lens. Fibroblast growth factor (FGF)-2-treated lens epithelial explants, cultured in the presence of Surfen (an inhibitor of heparan sulfate [HS]-growth factor binding interactions) were used to investigate the requirement for HS in FGF-2-induced proliferation, fiber differentiation, and ERK1/2-signaling.

RESULTS. The lens expresses all HSPGs. These HSPGs are differentially localized to distinct functional regions of the lens. In vitro, inhibition of HS-sulfation with Surfen blocked FGF-2-mediated ERK1/2-signaling associated with lens epithelial cell proliferation and fiber differentiation, highlighting that these cellular processes are dependent on HS.

CONCLUSIONS. These findings support a requirement for HSPGs in FGF-2 driven lens cell proliferation and fiber differentiation. The identification of specific HSPG core proteins in key functional lens regions, and the divergent expression patterns of closely related HSPGs, suggests that different HSPGs may differentially regulate growth factor signaling networks leading to specific biological events involved in lens growth and maintenance.

Keywords: lens, heparan sulfate proteoglycan (HSPG), syndecan, glypican

The ocular lens is a well-established model for studying growth factor regulation of cell behavior.[1] The lens is a relatively simple tissue, composed of two distinct cell types, with a monolayer of cuboidal epithelial cells overlying the anterior surface of elongate and precisely aligned fiber cells, that make up the lens bulk. Postnatally, lens growth is dependent on the proliferation of the epithelial cells in the germinative zone, and their subsequent differentiation into fiber cells at the transitional zone.[2,3] Fiber differentiation is characterized by distinct molecular and morphologic changes, such as exit from the cell cycle, cell elongation, loss of cytoplasmic organelles, and nuclei, as well as the accumulation of fiber-specific crystallin proteins.[4] The normal architecture, and hence transparency of the lens, depends on the maintenance of this distinctive pattern of growth.

The organization of lens cells is regulated by the ocular environment, namely, the aqueous and vitreous humors that bathe the lens. In vitro studies have shown that growth factors native to the aqueous humor promote the mitogenic activity of the lens epithelial cells,[5] whereas factors in the vitreous humor induce the differentiation of secondary lens fiber cells.[6] These native factors, including insulin-like growth factor-I (IGF-I)[7]; epidermal growth factor (EGF)[8]; platelet-derived growth factor-A (PDGF-AA)[9,10]; hepatocyte growth factor (HGF)[11]; ephrins[12,13]; and bone morphogenetic proteins (BMPs)[14] act in concert with fibroblast growth factors (FGFs)[15,16] to potentiate and regulate lens epithelial cell proliferation[5,17] and fiber differentiation.[18]

FGFs have been reported to be particularly important in the regulation of lens cell survival, proliferation, and differentiation.[15,16,19] They activate their high affinity receptor tyrosine kinases (RTKs; e.g. FGFRs) in lens in a concentration-dependent manner,[20] stimulating a proliferative and/or fiber differentiation response[21] via downstream signaling, including the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and phosphoinositide-3-kinase (PI3K/Akt) pathways.[17,18,22] Differential distribution of FGF bioavailability in the eye can regulate these lens growth processes,[21] with the proliferating lens epithelial cells exposed to lower levels of FGF activity in the aqueous, and the differentiating fiber cells exposed to higher levels of FGF activity from the vitreous.[23,24] Although the many distinct and overlapping effects of growth factors on lens cell behavior are well-characterized, the mechanisms

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underlying how they traverse the extracellular space, arrive precisely at the target cells, and transmit signaling within cells at the appropriate level, is still unclear. Recent studies in mice and other animal models have revealed the importance of cellular and extracellular matrix (ECM)-associated heparan sulfate proteoglycans (HSPGs) for the signaling and extracellular distribution of growth factors.[25]

HSPGs are complex glycoconjugates composed of a core protein backbone decorated with covalently linked linear heparan sulfate (HS) and/or chondroitin sulfation (CS) glycosaminoglycan (GAG) chains. The negatively charged sulfate groups of HS/CS chains interact with positively charged lysine/arginine-rich regions of many proteins, including growth factors and ECM molecules (e.g. integrins, laminin, fibronectin, and collagens).[26] The interactions of growth factors with native HSPGs can influence their receptor binding and signaling activation,[27,28] gradient formation,[29] modulation of their signaling activity,[30] as well as protecting cells from degradation.[31] Through modulation of the bioavailability, receptor binding, and signaling activation of mitogens and morphogens, HSPGs are positioned as master regulators of growth factor signaling in cell biology and development.[25,32]

The HSPG superfamily is divided into three major groups based on their core-protein structure. The transmembrane syndecans (1–4)[33] and glycerophosphatidylinositide (GPI)-anchored glypicans (1–6)[34] are localized to the cell surface, while the large secreted HSPGs, including perlecan, agrin, and collagen XVIII/endostatin, are primarily secreted by cells and deposited to the ECM and basement membranes.[35] Different HSPG core proteins play unique roles in tissue homeostasis, and these roles are often tissue and cell-type specific.[25] Moreover, the number, localization, and HS/CS sulfation patterns of HSPGs can modify their protein interactions, and therefore their function.[36] For instance, to date, HSPG core proteins identified in the vertebrate eye have been found to have functional roles unique to the tissue where they are expressed. HS-linked forms of perlecan are required for structural integrity of the lens capsule, but not the glomerular basement membrane,[37] whereas overexpression of agrin in the lens causes a dysgenic phenotype that is specific to the eye.[38] The localization of HSPG core proteins can hence provide valuable information pertaining to their functional roles in specific tissues.[39] Immunolabeling of HSPG core proteins syndecan-1, syndecan-4, and glypican-4 within the proliferative zone of the brain, together with in vitro evidence that HSPGs are required for FGF-stimulated proliferation of brain precursor cells, identified these HSPGs as key regulators of FGF-stimulated proliferation in brain development.[40] Earlier studies suggest that specific HSPGs may differentially regulate biological processes in the lens. For example, heparan-binding growth factors can bind electrostatically to gradients of HS proteoglycans.[41] This is observed in the extracellular matrix of the lens capsule, with complimentary antero-posterior gradients of the matrix-associated HSPG perlecan, FGF bioavailability, and signaling activity.[20,42] Other studies have also provided support for a direct role of cell-surface HSPGs, rather than basement membrane localized HSPGs, in FGF signaling.[43–46]

To determine which specific HSPG core proteins could potentially be involved in the regulation of signaling events important for lens cell biology, here, we comprehensively characterized the presence and comparative spatial localization of all known HSPG core proteins in the postnatal rat lens. We found that the lens has a unique profile of HSPG core proteins that are differentially localized to distinct functional regions of the lens. The significantly divergent expression patterns of closely related members of the one HSPG family (e.g. syndecans), suggests that different HSPG core proteins may differentially regulate different growth factors, such as FGFs, and specific lens cellular processes (e.g. proliferation, fiber cell elongation, and differentiation). Our in vitro studies also show that it is the heparan sulfation on the HSPGs and not their chondroitin sulfation that is required for FGF-2-induced ERK1/2 activity, leading to lens epithelial cell proliferation and fiber differentiation.

METHODS

Animals

All animal experimental procedures were approved by The University of Sydney Animal Ethics Committee (AEC #2017/1269) and conducted in accordance with the guidelines set by the National Health and Medical Research Council (Australia), and the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. 10-day-old albino Wistar rats (*Rattus norvegicus*) were euthanized by asphyxiation with carbon dioxide followed by cervical dislocation.

Gene Expression

Total RNA was extracted from dissected 10-day-old rat lens epithelial cells and lens fiber cells (n = pooled tissue from at least 5 animals) using the ISOLATE II RNA Micro Kit (Bioline, Eveleigh, New South Wales, Australia), according to the manufacturer's instructions. Following DNAse treatment and reverse transcription (SensiFAST cDNA Synthesis Kit, Bioline), the relative expression of transcripts encoding all known HSPG core proteins was investigated using semiquantitative reverse transcription polymerase chain reaction (RT-qPCR). RT-qPCR was performed as biological triplicates, according to the manufacturer's instructions with the QuantStudio 12K Flex Real-Time PCR System (ThermoFisher Scientific, Waltham, MA, USA) using TaqMan Gene Expression Assays for proteoglycan core proteins (accession numbers: Syndecan-1 [*Sdc1*]: Rn01469776_m1; Syndecan-2 [*Sdc2*]: Rn00565242_m1; Syndecan-3 [*Sdc3*]: Rn00696372_m1;Syndecan-4 [*Sdc4*]: Rn00664688_m1; Glypican-1 [*Gpc1*]: Rn01427370_m1; Glypican-2 [*Gpc2*]: Rn00593367_m1; Glypican-3 [*Gpc3*]: Rn00516722_m1; Glypican-4 [*Gpc4*]: Rn01457394_m1; Glypican-5 [*Gpc5*]: Rn01468174_m1; Glypican-6 [*Gpc6*]: Rn01466049_m1; Perlecan [*Hspg2*]: Rn01515780_g1; Agrin [*Agrn*]: Rn00598349_m1; Collagen XVIII alpha chain 1 [*Col18a1*]: Rn01428983_m1; and glyceraldehyde 3-phosphate dehydrogenase [*Gapdh*]: Rn01462662_g1; Life Technologies, Carlsbad, CA, USA). No-template controls were included for each assay. Relative core protein transcript expression was quantified relative to housekeeping (*Gapdh*) expression for lens epithelial cells and lens fiber cells respectively. Data were represented a *log(ΔCt)* relative to *Gapdh* ± standard error of the mean (SEM), from at least three independent experiments.

Immunolabeling of Heparan Sulfate Proteoglycans

Intact eyes or uncultured lens epithelial wholemounts were collected and fixed for 24 hours or 5 minutes, respectively, in 10% neutral buffered formalin (NBF; Sigma, Castle Hill, New South Wales, Australia) before processing for routine paraffin embedding (lens epithelial wholemounts were pre-embedded in 1% noble agar; Sigma). Paraffin-embedded tissues were serially sectioned at 5 µm. Lens epithelial wholemounts were also isolated and immediately fixed for 1 minute in cold methanol (Sigma) in preparation for histochemical staining or immunolabeling procedures. Mid-sagittal sections and lens epithelial wholemounts from at least three animals were used for staining and immunolabeling. Representative sections were also stained with periodic acid–Schiff (PAS) to highlight lens morphology.

Whole eye sections and lens epithelial wholemounts were first labelled for HS using a monoclonal antibody (HS F58-10E4, 370255; Amsbio LLC, Abingdon, UK). As some HSPGs (i.e. Syndecans −1[47] and −3,[48] agrin,[49] and collagen XVIII/endostatin[50]) may carry CS GAG chains, in addition to the predominant HS chains, we also labeled for highly sulfated forms of CS (CS-56, C8035; Sigma). As the interactions of most HSPG-binding proteins are electrostatic in nature, and rely on GAG chain sulfation, these antibodies can be used to detect all sulfated "active" HSPGs in a tissue.[51–53] When used alongside core protein-specific antibodies, a more complete picture of the HSPG profile of the lens can be determined.

As specific HSPGs can differentially regulate cell responses and behaviors, and their differential expression is reported to be spatiotemporally regulated in a cell type- and tissue-specific manner,[39,40] we characterized the spatial localization of the different HSPGs in the postnatal lens. Localization of HS and HSPG core proteins were investigated by immunolabeling 10-day-old rat lens mid-sagittal sections and lens epithelial wholemounts, using commercially available antibodies (see Supplementary Table S1). For all immunofluorescent labeling studies, cell nuclei were counter-labeled with bisbenzimide (Hoechst 33258; Sigma). We included the following controls for the immunofluorescence studies: (i) unlabeled tissue, (ii) chain-specific isotype controls, (iii) secondary antibody alone, (iv) single label, and (v) no Hoechst.

Brightfield and immunofluorescence images were acquired using either a Leica DMLB 100S microscope equipped with a DFC-450C camera using Leica Application Suite (LAS) software version 4.8.0 (Leica Microsystems); a ZEISS LSM-800 microscope with an AxioCam 506 camera using ZEN Blue Edition version 2.3 software (Carl Zeiss, Jena, Germany), or a ZEISS AxioScan.Z1 microscope (Carl Zeiss) with a Hamamatsu ORCA-flash 4.0 version 2 camera (Hamamatsu Photonics, Shizuoka, Japan) using ZEN SlideScan 2012 software (Carl Zeiss). Phase-contrast images were acquired using an Olympus CK2 microscope (Olympus, Tokyo, Japan) with a Leica DFC-280 camera (Leica Microsystems) using Leica FireCam software version 1.5 (Leica Microsystems). Images were saved as tagged information field format (TIFF) without compression at 8-bit (phase-contrast images) or 16-bit depth. Photoshop version 21.1.1 (Adobe, Inc., San Jose, CA, USA) and ImageJ (FIJI) version 2.0 (Wayne Rasband, National Institutes of Health [NIH], Bethesda, MD, USA) were used for image processing within the Mac OS X 10.15.6 (Apple Inc., Cupertino, CA, USA) and Windows 10 (Microsoft Inc., Seattle, WA, USA) operating systems.

Preparation and Treatment of Lens Epithelial Explants

Lenses were dissected from 10-day-old rat eyes and epithelial explants were prepared as previously described.[23,54] Explants were cultured in medium 199 with Earle's salts (M199) supplemented with 0.1 µg/mL L-glutamine, 50 IU/mL penicillin, 50 µg/mL streptomycin, 2.5 µg/mL Amphostat B, and 0.1% bovine serum albumin (BSA; all from Sigma, Castle Hill, New South Wales, Australia) at 37°C in 5% CO_2. Lens epithelial explants were cultured with 1 ng/mL (for up to 24 hours) or 150 ng/mL (for up to 5 days) recombinant FGF-2 (PeproTech Inc., Rocky Hill, NJ, USA) to stimulate epithelial cell proliferation or lens fiber differentiation, respectively, as previously described.[17,55] To block HS sulfation, explants were co-treated with either 30 mM sodium chlorate ($NaClO_3$, in M199; Sigma)[16,56] or with up to 10 µM Surfen (bis-2-methyl-4-amino-quinolyl-6-carbamide, in DMSO; Sigma).[57] Control explants were untreated or treated with an equal volume of DMSO vehicle (in experiments with Surfen). Cells were pretreated overnight (for $NaClO_3$)[16] or for 1 hour (for Surfen) prior to the addition of FGF-2.

Lens Epithelial Proliferation and Viability

Cell (nuclei) counts were performed on representative images of explants (at least 3 animals per treatment group) cultured with increasing doses of Surfen (1, 5, or 10 µM) for 24 hours to assess cell viability. Explants treated with Surfen (5 µM) for 1 hour were immunolabeled for HS and CS GAGs. To identify cells in S-phase of the cell cycle, 5′-2′-bromo-deoxyuridine (BrdU; 150 µg/mL) was added to explant cultures 18 hours post-treatment and cultured for an additional 6 hours prior to fixation in 10% neutral buffered formalin (10 minutes). BrdU-immunolabeling was performed as previously described[17] and explants were counterstained with Hoechst 33258 (Sigma). Representative images were taken of each explant per treatment group. Cell counts (total cells and BrdU-positive) were performed using an optimized batch processing macro using ImageJ (FIJI) software (version 2.0; Wayne Rasband, NIH, USA) from images captured using an epifluorescent microscope (Leica Microsystems, Weltzar, Germany), and averaged for each treatment group. BrdU-proliferation assays were performed on representative images of explants cultured with a proliferating dose of FGF-2 (1 ng/mL), together with increasing doses of Surfen (1, 5, or 10 µM) for 24 hours.

Measuring Secondary Lens Fiber Differentiation

To visualize early changes in downstream MAPK/ERK signaling activation, explants treated with FGF-2 (1 ng/mL for proliferation,[16] or 150 ng/mL for lens fiber differentiation[58]), with or without Surfen (5 µM), were fixed with methanol 4 hours after treatment and immunolabeled for mouse anti-phospho-ERK1/2 (p-ERK1/2, 9106; 1:100; Cell Signaling Technology). Explants treated with a differentiating dose of FGF-2 (150 ng/mL), with or without Surfen (5 µM), were fixed (10% NBF for 5 minutes) 5 days after treatment and immunolabeled for markers of fiber differentiation: rabbit anti-β-crystallin[23] and mouse

anti-Aquaporin-0/MIP[59] (1:100; Santa Cruz Biotechnology Inc., Dallas, TX, USA).

Western Blot Analysis

Lens epithelial explants (at least 5 animals per treatment group) were collected at relevant time points post-treatment, into cold protein lysis buffer containing 2.5 mM EDTA, 25 mM Tris-HCl, 0.375 M NaCl, 250 mM NaVO₃, protease and phosphatase inhibitors (Roche, Basel, Switzerland), 10 mM sodium deoxycholate, and Triton X-100 (Sigma). Sample protein concentrations were determined using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific). Lysates were prepared for electrophoresis with Laemmli sample buffer (Bio-Rad Laboratories, New South Wales, Australia) containing 5% (v/v) β-mercaptoethanol. Samples were run on 12% SDS-PAGE gels and transferred onto Immobilon-PSQ polyvinylidene fluoride (PVDF) membranes (Merck Millipore). Following transfer, membranes were blocked at room temperature for 1 hour in 2.5% (w/v) BSA blocking buffer (in Tris-buffered saline-Tween 20 [TBST]: 0.1% v/v Tween-20 in Tris-buffered saline; Sigma), and subsequently incubated overnight at 4°C with primary antibodies appropriately diluted in BSA blocking buffer. The following primary antibodies were used: mouse anti-phospho-ERK1/2 (p-ERK1/2, 9106; 1:1000; Cell Signaling Technology), rabbit anti-ERK1/2 (ERK1/2; 9102, 1:1000; Cell Signaling Technology) and mouse anti-GAPDH (G8795; 1:5000; Sigma). Following primary antibody incubation, membranes were rinsed in TBST and incubated with secondary antibodies (1:5000 in TBST) for 2 hours at room temperature. Secondary antibodies used were a horse anti-mouse IgG HRP-linked antibody (7076; Cell Signaling Technology) and a goat anti-rabbit IgG HRP-linked antibody (7074; Cell Signaling Technology). Membranes were imaged using the enhanced chemiluminescence (ECL) kit (Merck Millipore) and ChemiDoc MP imaging system (Bio-Rad Laboratories Inc.). Band densities were quantified using Image Lab software (Bio-Rad Laboratories Inc.), and normalized relative to GAPDH internal control.

Statistical Analysis

Data were presented as the mean or change in mean ± SEM from three or more independent experiments. All statistical analyses were performed using Prism 8 software (GraphPad Software Inc, San Diego, CA, USA). Unless otherwise stated, primary analyses of all dependent variable data were performed using unpaired Students *t*-test with Welch's correction, and the Holm-Šídák method when multiple comparisons were corrected. Effects were considered statistically significant if $P < 0.05$.

RESULTS

Relative Expression of HSPG Core Protein Transcript in the Lens

Transcripts for all 13 HSPG core proteins were detected in the lens (Fig. 1). *Gpc2* mRNA appeared to be the most highly expressed in lens epithelial cells, whereas most other HSPG core protein transcripts were highest in fiber cells (see Fig. 1). Moreover, mRNA encoding S*dc2*, *Sdc4*, and *Col18a1* were the lowest of any HSPG core protein transcript expressed in lens epithelial cells, whereas for fiber cells the lowest expression levels were observed for *Sdc2* and *Hspg2* (see Fig. 1).

Localization of Sulfated GAGs (HS and CS). As some HSPGs (i.e. Syndecans −1[47] and −3,[48] agrin,[49] and collagen XVIII/endostatin[50]) may carry HS or both HS/CS GAG chains, the distribution of HS- and CS-GAG labeling was compared in 10-day-old rat eye sections (Fig. 2), and lens epithelial cell wholemounts using antibodies that recognize the functionally "active" sulfated domains on HS[51,52] (see Figs. 2B1–B11) or CS[53] (see Fig. 2C1–C11) GAG chains. HS-GAG labeling was prominent in the lens capsule (see Figs. 2B2, 2B3, 2B5), the cytoplasm of lens epithelial
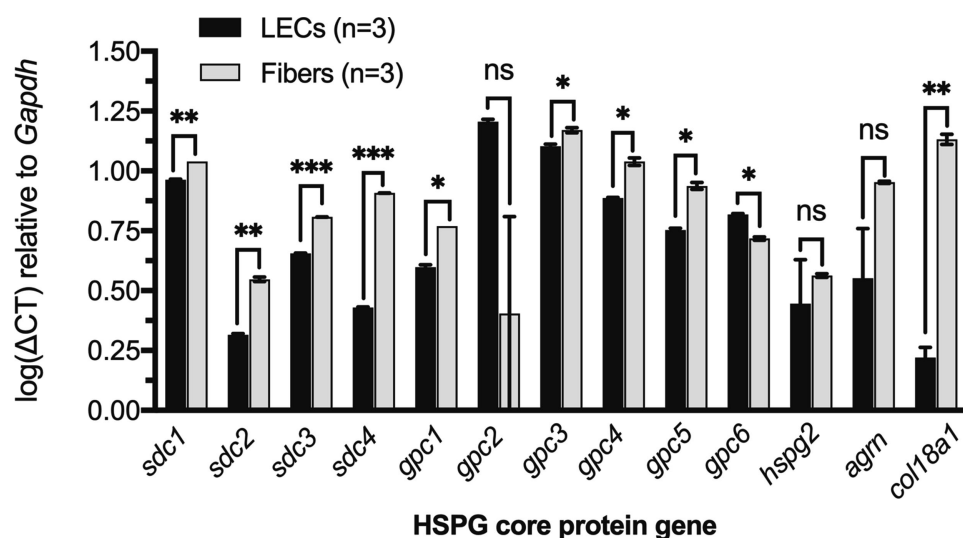
FIGURE 1. Relative expression of HSPG core protein transcripts in 10-day-old rat lens epithelial and fiber cells. Data are represented a *log(ΔCt) of* HSPG core protein transcript expression in lens epithelial cells (LECs; dark bar) or Fibers (grey bar), relative to *Gapdh* ± standard error of the mean (SEM), from at least three independent experiments ($n = 3$), applying unpaired t-tests with Welch's correction. Multiple comparisons were corrected for using the Holm-Šídák method. Significance threshold set at $P > 0.05$ ($P > 0.05$ = ns, not significant; $P < 0.05$ = *; $P < 0.01$ = **; $P < 0.001$ = ***).
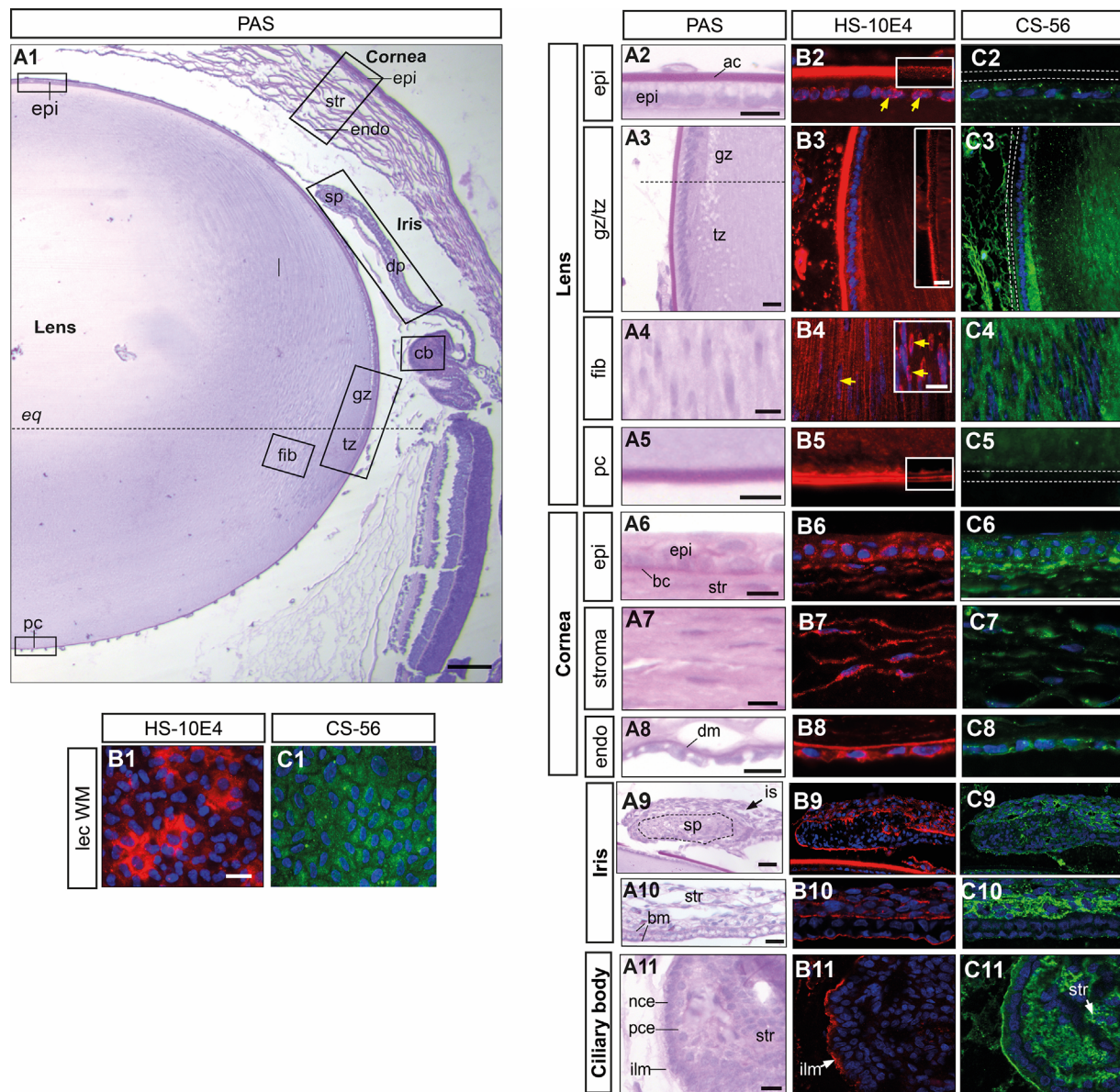
FIGURE 2. Distribution of sulfated glycosaminoglycans in the 10-day-old rat eye. Mid-sagittal eye sections stained with Periodic acid-Schiffs (PAS; **A**1–11) show morphology of regions of interest. Corresponding sections (**B**2–**B**11, **C**2–**C**11) and 10-day-old rat lens epithelial whole-mounts (lec WM; **B**1, **C**1) immunolabel the highly sulfated domains on heparan sulfate (HS; **B**1–11) or chondroitin sulfate (CS; **C**1–11) glycosaminoglycan chains. Yellow arrows (**B**2, **B**4) indicate nuclear localization. Dotted white lines demarcate outline of the lens capsule. Insets in **B**2, **B**3, and **B**5 represent images that were underexposed to show further detail of the HS-10E4 label in the anterior, equatorial, and posterior lens capsule, respectively. For immunofluorescence, nuclei were counterstained with Hoechst dye (blue). Abbreviations: lens; central anterior epithelium (epi) and capsule (ac), germinative zone (gz), transitional zone (tz), lens fiber cells (fib), posterior lens capsule (pc). Cornea; epithelium (epi), Bowman's capsule (bc), stroma (str), endothelium (endo), Descemet's membrane (dm). Iris; anterior iris/sphincter pupillae (sp), iridial stroma (str), corneal basement membrane (bm). Ciliary body; non-pigmented ciliary epithelium (nce), pigmented ciliary epithelium (pce), ciliary inner limiting membrane (ilm), ciliary stroma (str). Scale bars = 50 µm (**A**1), 25 µm (**A**2–11, **B**1–11, **C**1–11).

cells (see Figs. 2B1, 2B2, 2B3) and on the cell membranes of newly elongating lens fiber cells (see Figs. 2B3, B4). HS-GAG reactivity increased at the germinative and transitional zones (see Fig. 2B3), and adjacent lens capsule (see Fig. 2B3 inset). A subset of lens epithelial and fiber cell nuclei was positive for HS-GAG immunolabeling (see Figs. 2B2, B4 inset, arrows). Strong HS-GAG immunoreactivity was also observed in the corneal epithelium (see Fig. 2B6), stroma (see Fig. 2B7), and endothelium and associated Descemet's membrane (see Fig. 2B8), the basement membranes of the

iris (see Fig. 2B10), and the inner limiting membrane of the ciliary body (see Fig. 2B11). CS-GAG was absent from the lens capsule (see Figs. 2C2, C3, C5) with infrequent positive labeling for CS-GAG observed in the cytoplasm of lens epithelial cells (see Figs. 2C1, 2C2). Relative to the anterior lens epithelium, CS-GAG labeling was more prominent in the equatorial epithelium with a stronger label at the transitional zone (see Fig. 2C3). This label was further intensified in the cytoplasm of newly elongating lens fiber cells (see Fig. 2C3, C4). CS-GAG labeled strongly in the corneal
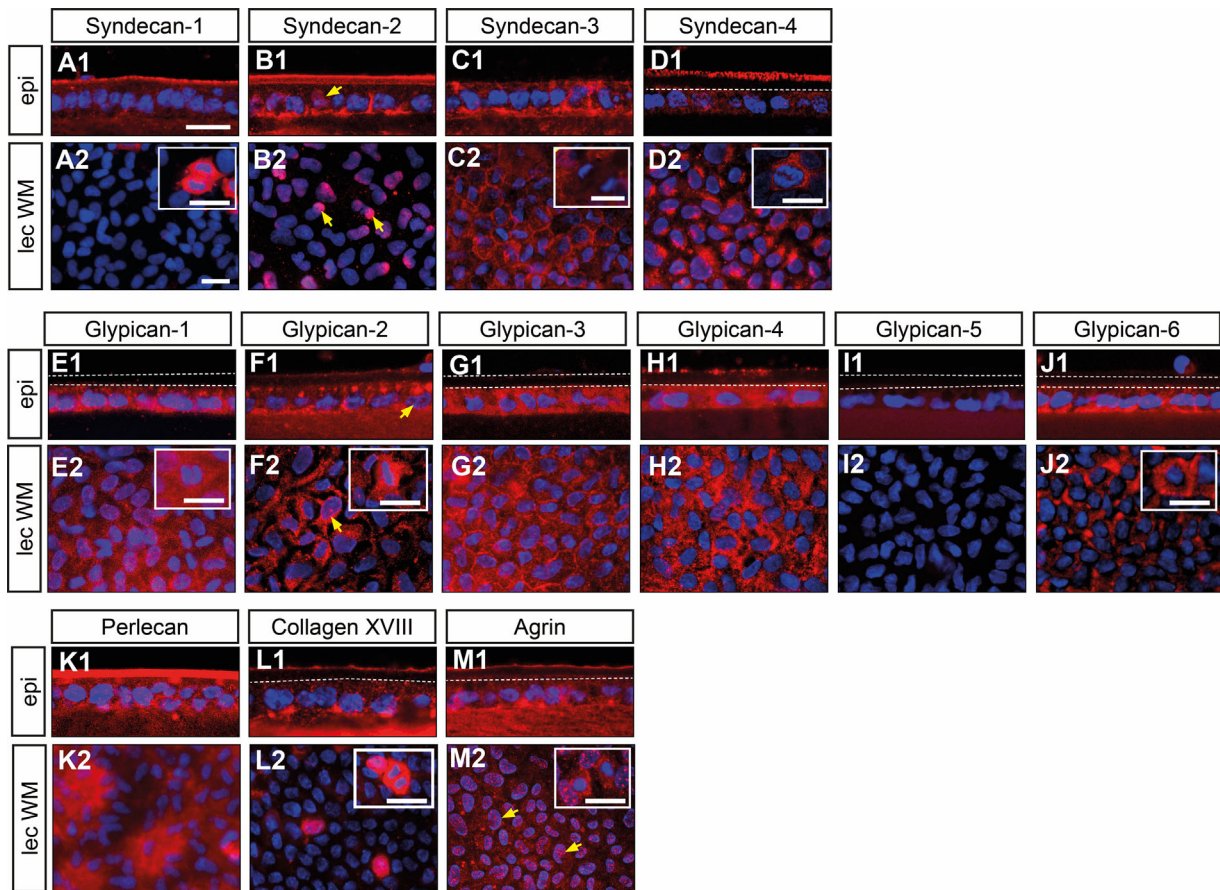
FIGURE 3. Anterior lens capsule and epithelia. Representative immunofluorescence (red) of HSPG core proteins for Syndecan-1 to −4 (**A–D**), Glypican-1 to -6 (**E–J**), Perlecan (**K**), Collagen XVIII/endostatin (**L**), and Agrin (**M**) in sections (1-series) or epithelial wholemounts (lec WM, 2-series) of 10-day-old rat lens. Yellow arrows indicate nuclear localization. Dotted white lines demarcate lens capsule. Cell nuclei counterstained with Hoechst (blue). Abbreviations: lens epithelium (epi). Scale bars = 25 μm.

epithelium, Bowman's capsule (see Fig. 2C6) and endothelium (see Fig. 2C8), with increased reactivity in the iridial stroma (see Fig. 2C10), and inner limiting membrane and stroma of the ciliary body (see Fig. 2C11). No CS-GAG was found in the iridial basement membranes (see Fig. 2C10).

HSPG Core Proteins are Differentially Localized Key Functional Regions in the Lens

All HSPG core proteins, except for glypican-5 (Fig. 3I, Fig. 4I), were readily detected in lens sections (see Figs. 3, 4). Expression patterns of individual core proteins were widely varied, both within families (e.g. syndecans) and between families (e.g. syndecans and glypicans). Despite this heterogeneity, most core proteins could be classified by their unifying expression patterns consistently observed in key "functional" regions of the lens (germinative and transitional zones).

Lens Capsule. The lens capsule labeled for three of the four transmembrane syndecans (syndecan-1, −2, and −4; see Figs. 3A1, 3B1, 3D1; Fig. 4A, 4B, 4D), two of the six glypicans (glypican-2 and −4; see Figs. 3F1, 3H1; Figs. 4F, H), and all three high-molecular weight HSPG core proteins (perlecan, collagen XVIII/endostatin and agrin; Figs. 3K1, 3L1, 3M1; Figs. 4K, 4L, 4M). Perlecan

and syndecan-2 were the most prominent species labeling the lens capsule. Syndecan-2 and perlecan labeling was observed throughout the lens capsule thickness (see Figs. 3B1, 3K1; Figs. 4B, 4K), whereas syndecan-4 (see Fig 3D1), glypican-2 (see Fig. 3F1), glypican-4 (see Fig. 3H1; Fig. 4H3), collagen XVIII/endostatin (Fig. 3L1), and agrin (see Fig. 3M1; Fig. 4M3) labeling was restricted to the outermost layer of the lens capsule. In contrast, syndecan-1 labeling (see Fig. 3A1; Fig. 4A3) appeared to be uniquely limited to the innermost capsule layer, opposing the cells. Capsular labeling of syndecan-1 (see Fig. 3A1; Figs. 4A1, 4A3), syndecan-2 (see Fig. 3B1; Figs. 4B1, 4B3), glypican-2 (see Fig. 3F1; Figs. 4F1, 4F3), glypican-4 (see Fig. 3H1; Figs. 4H1, 4H3), and to a lesser extent agrin (see Fig. 3M1; Figs. 4M1, 4M3), increased in an antero-posterior gradient, whereas labeling for syndecan-4 (see Fig. 3D1; Figs. 4D1, 4D3), and collagen XVIII/endostatin (see Fig. 3L1; Figs. 4L1, 4L3) was present in the anterior capsule but absent from the equatorial and posterior regions of the lens capsule. Syndecan-2, syndecan-4, glypican-4, perlecan, collagen XVIII/endostatin, and agrin were also localized throughout the corneal stroma (Supplementary Fig. S1), iridial stroma, ciliary body stroma, and iridial basement membranes (Supplementary Fig. S2). Glypican-2 did not appear in any ocular basement membranes (see Supplementary Figs. S1F, A2E) other than the lens capsule
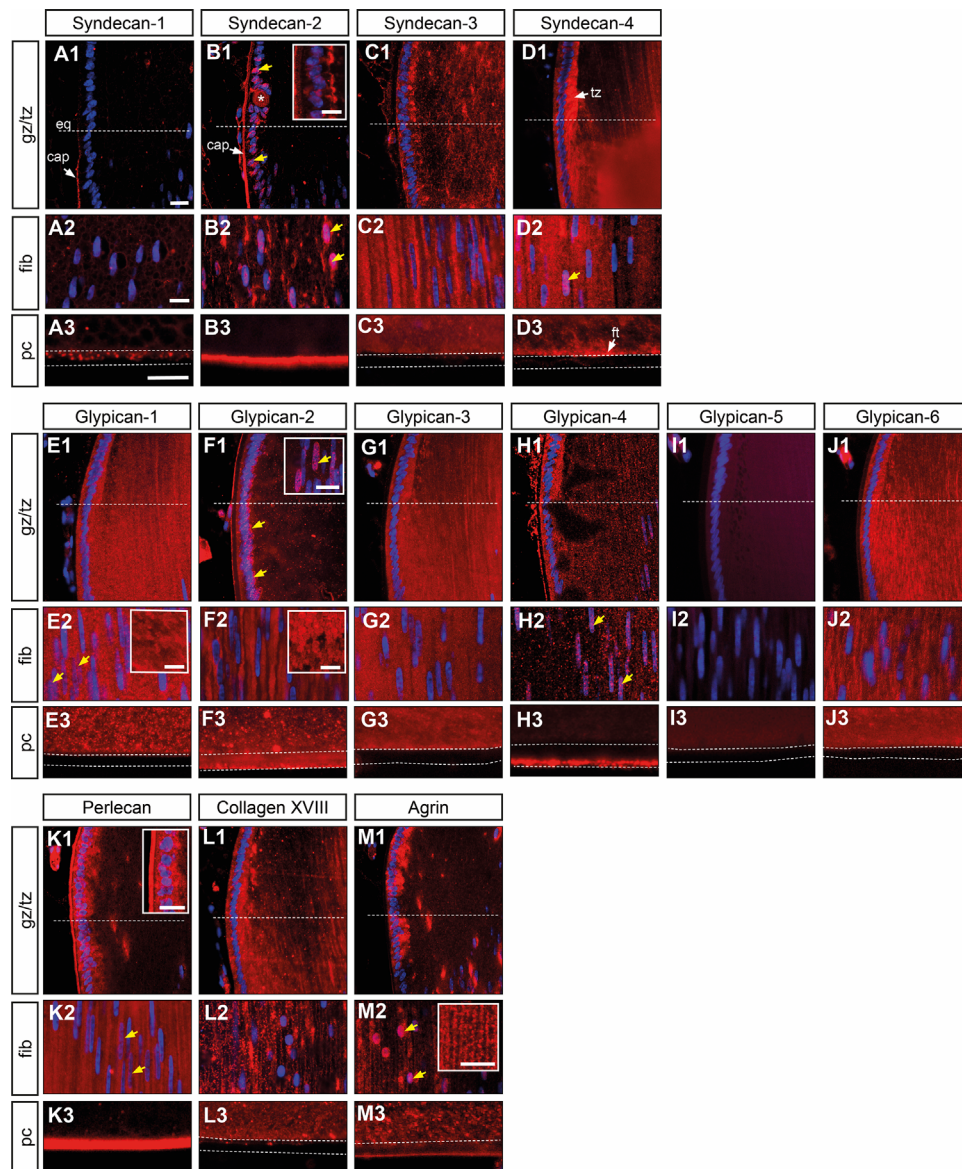
FIGURE 4. Equatorial lens, cortex and posterior capsule. Representative immunofluorescence (red) of HSPG core proteins for Syndecan-1 to -4 (**A–D**), Glypican-1 to -6 (**E–J**), Perlecan (**K**), Collagen XVIII (**L**), and Agrin (**M**) in sections of the equatorial germinative/transitional zone (gz/tz; 1-series), outer cortical fibers (fib; 2-series), and posterior capsule (pc; 3-series) of 10-day-old rat lens. Yellow arrows indicate nuclear localization. Dotted white lines demarcate lens capsule. Cell nuclei counterstained with Hoechst (blue). Scale bars = 25 μm.

(Fig. 3F1; Figs. 4F1, 4F3). Interestingly, syndecan-3 was absent from the lens capsule (see Fig. 3C1; Fig. 4C3), but strongly labeled in the basement membranes and stroma of the iris and ciliary body (see Supplementary Fig. S2C).

Lens Epithelium. Anterior LECs in tissue sections and wholemounts (see Fig. 3) labeled positively for all HSPG core proteins, apart from glypican-5 (see Figs. 3I1, 3I2); however, using dot blots, consistent with our transcript analysis, we were able to detect relatively low levels of glypican-5 protein in P10 rat lens epithelia (data not shown). There was diffuse labeling for glypican-1 (see Fig. 3E), glypican-3 (see Fig. 3G), and to a lesser extent syndecan-4 (see Fig. 3D), glypican-4 (see Fig. 3H), and perlecan (see Fig. 3K) in the cytosol of LECs. Positive labeling for syndecan-3 (see Fig. 3C) and glypican-2 (see Fig. 3F) was distributed in irregular patches within the cytoplasm of LECs. A similar level of

labeling was observed for syndecan-2 (see Fig. 3B), glypican-6 (see Fig. 3J), collagen XVIII/endostatin (see Fig. 3L), and agrin (see Fig. 3M), with reactivity concentrated at the basal pole of LECs. Syndecan-1 staining in LECs (see Fig. 3A) was weak relative to other syndecans, but levels were higher in what appeared to be mitotic figures, highlighting actively proliferating cells (see Fig. 3A2 inset, anaphase). Enriched reactivity of what appeared to be the mitotic spindle was observed for syndecan-4 (see Fig. 3D2 inset, anaphase), with similar stronger labeling for select epithelia for glypican-2 (see Fig. 3F2 inset, metaphase), glypican-6 (see Fig. 3J2 inset), collagen XVIII/endostatin (see Fig. 3L2 inset, telophase), and to a lesser extent glypican-1 (see Fig. 3E2 inset, telophase) and agrin (see Fig. 3M2 inset). These core proteins were also strongly expressed in the mitotically active basal

cells of the corneal epithelium, except for glypican-6 that was absent from the corneal epithelium (see Supplementary Fig. S1). Syndecan-3 (see Fig. 3C2) and glypican-3 (see Fig. 3G2) were localized to apical cell junctions in LEC wholemounts. Syndecan-2 (see Figs. 3B1–2, Fig. 4B1) and glypican-2 (see Fig. 3F1–2, Fig. 4F1) labeled positively in a subset of LEC nuclei. Positive labeling was enriched in LECs within the mitotically active germinative zone for all core proteins found in the anterior lens epithelium, with the highest label observed for syndecan-4 (see Fig. 4D1), glypican-4 (see Fig. 4H1), perlecan (see Fig. 4K1), collagen XVIII/endostatin (see Fig. 4L1), and agrin (see Fig. 4M1).

Lens Fiber Cells. Posterior to the lens equator, lens epithelial cells in the transitional zone undergoing early stages of fiber differentiation were labeled intensely for glypicans-1, -3, and -6 (see Figs. 4E1, 4G1, 4J1). To a lesser degree, cells in the transitional zone also labeled for syndecans-3 and -4 (see Figs. 4C1, 4D1), glypican-2 (see Fig. 4F1) and perlecan (see Fig. 4K1). Nuclear labeling for syndecan-2 (see Fig. 4B1–2, arrows) and glypican-2 (see Fig. 4F1, inset, arrows) was also observed in a subset of nuclei in newly differentiating lens fiber cells. HSPG core proteins were observed in four predominant expression patterns in equatorial fiber cells. Syndecan-4 (see Fig. 4D), and glypicans-1, -3, and -6 (see Figs. 4E, 4G, 4J) were uniformly found in the cytoplasm of fiber cells as diffuse punctae. Syndecan-3 (see Fig. 4C), glypican-2 (see Fig. 4F), and perlecan (see Fig. 4K) were also highly labeled in the cytoplasm of fiber cells, with striations of enriched labeling in new secondary fibers. Syndecan-2 (see Fig. 4B) and glypican-4 (see Fig. 4H) labels in fiber cells were sparse and irregular. Agrin (see Fig. 4M) and collagen XVIII/endostatin (see Fig. 4L) were concentrated at fiber cell membranes, with intensified labeling in the more mature fibers toward the nucleus of the lens. No label was observed for syndecan-1 (see Fig. 4A) or glypican-5 (see Fig. 4I) in lens fiber cells. As observed in lens epithelia, nuclear labeling for syndecan-2 (see Fig. 4B1–2, arrows) and glypican-2 (see Fig. 4H2, arrows) persisted in a subset of fiber cells. Some nuclear labeling for other core proteins, including syndecan-4 (see Fig. 4D2, arrows), glypican-1 (see Fig. 4E2, arrows), collagen XVIII/endostatin (see Fig. 4K2, arrows), and agrin (see Fig. 4M2, arrows) was observed in early differentiating secondary lens fiber cells. Syndecan-3 (see Fig. 3C1; Fig. 4C1), glypican-2 (see Fig. 3F1; Fig. 4F1), and agrin (see Fig. 3M1; Fig. 4M1) appeared to be enriched at the apical tips of fiber cells. On the other hand, labeling of syndecan-4 (see Fig. 4D3), glypican-3 (see Fig. 4G3), and glypican-6 (see Fig. 4J3) was stronger at the posterior basal fiber tips.

Non-Lenticular Regions of the Eye. In contrast to the lower expression levels for syndecan-1 observed in lens cells, strong labeling was found in the cornea (see Supplementary Fig. S1A), iridial stroma (see Supplementary Figs. S2 A1–2), ciliary non-pigmented epithelium (see Supplementary Fig. S2A3), and in the vascular endothelium of the choroid and remnant tunica vasculosa lentis (data not shown). Nuclear labeling of both syndecan-2, syndecan-3, and glypican-2 was not restricted to the lens, with both present in a subset of nuclei of corneal epithelial and stromal cells (see Supplementary Figs. S1B, S1C, S1F), iris, ciliary body, and retina (see Supplementary Figs. S2B, S2E, S2K). Glypican-5 and glypican-6 were absent from the corneal epithelium (see Supplementary Figs. S1I1, S1J1). Glypican-6 staining, however, was high in the corneal endothelium

(see Supplementary Fig. S1J3), with syndecan-3, syndecan-4, glypican-4, perlecan, collagen XVIII/endostatin (data not shown), and agrin labeling, delineating the limiting membranes of the iris (see Supplementary Fig. S2). Iridial stromal cells were strongly positive for syndecan-2, glypican-2, glypican-4, perlecan, and agrin (see Supplementary Figs. S2B1–2, S2E1–2, S2F1–2, S2H1–2, S2I1–2). Syndecan-4 alone appeared to be positively labeled in the myoepithelial cells of the sphincter and dilator pupillae of the iris (see Supplementary Fig. S2D1–2). Syndecans-1, -2, and -4 were selectively enriched in the non-pigmented epithelium of the ciliary body (see Supplementary Figs. S2A3, S2B3, S2D3). Conversely, syndecan-3, glypican-2, and agrin were more widely distributed in the ciliary epithelial bilayer, as well as in the stromal and endothelial cells of the ciliary processes (see Supplementary Figs. S2C3, S2E3, S2I3). Glypican-5 labeling was absent from all tissues investigated (see Supplementary Fig. S1I; Supplementary Fig. S2G).

As many of the HSPG core proteins in the lens were enriched in the germinative and transitional zones, where lens epithelial cells actively proliferate and undergo fiber differentiation, we investigated whether HS sulfation was required for these key cellular processes.

Surfen Blocks HS Sulfation and FGF-Induced ERK1/2 and Lens Epithelial Cell Proliferation. Surfen is a selective electrostatic inhibitor of sulfated HS chain-protein interactions.[57] Treatment with 5 µM Surfen for 1 hour decreased immunolabeling of heparan sulfate (HS-10E4; Fig. 5B2) relative to control explants (see Fig. 5B1), but not chondroitin sulfate (CS-56; see Figs. 5B3, 5B4). Explants treated with 1 or 5 µM Surfen alone for 24 hours showed no signs of decreased cell viability relative to controls ($P = 0.53$ and 0.91). A significant decrease in cell number relative to vehicle control was observed in explants treated with 10 µM ($P = 0.007$; see Fig. 5A). Phase micrographs of these showed pyknotic cell nuclei characteristic of cell death (data not shown). Cell proliferation was not significantly affected in lens epithelia treated with only 5 µM Surfen (see Fig. 5D). Proliferation of lens epithelial cells treated with a low proliferative dose of FGF-2 (5 ng/mL) was increased by 25.7% ($P < 0.001$) relative to control explants (see Fig. 5D). Cotreatment with Surfen partially blocked FGF-2-induced proliferation at 1 µM ($P = 0.02$; see Fig. 5D), and completely with 5 µM ($P < 0.001$; Figs. 5D, 5C4). A proliferative dose of FGF-2 induced phosphorylation of ERK1/2 at 1 hour ($P = 0.004$; see Fig. 5E) and after 4 hours (see Fig. 5C3). FGF-2-induced ERK1/2 phosphorylation was significantly reduced using either sodium chlorate ($P = 0.006$), and more specifically with Surfen ($P = 0.014$), comparable to vehicle controls with no FGF added (see Figs. 5E, 5F), consistent with immunofluorescent labeling for phosphorylated-ERK1/2 in explants treated with FGF-2, with and without Surfen at 4 hours (see Figs. 5C1–4).

Surfen Blocks FGF-Induced Lens Fiber Differentiation. After 5 days of culture with a fiber differentiating dose (150 ng/mL) of FGF-2,[58] cells in lens epithelial explants had an elongate morphology and labeled strongly for β-crystallin and aquaporin-0 (Figs. 6B2, 6C2), while vehicle control explants remained cuboidal and did not express lens fiber protein markers (see Figs. 6B1, 6C1). Explants treated with Surfen alone resembled control explants (data not shown), but when explants were cotreated with a differentiating dose of FGF-2 and Surfen, cell elongation, and the appearance of β-crystallin and AQP0 was significantly decreased in cells (see Figs. 6B3, 6C3), compared with cells
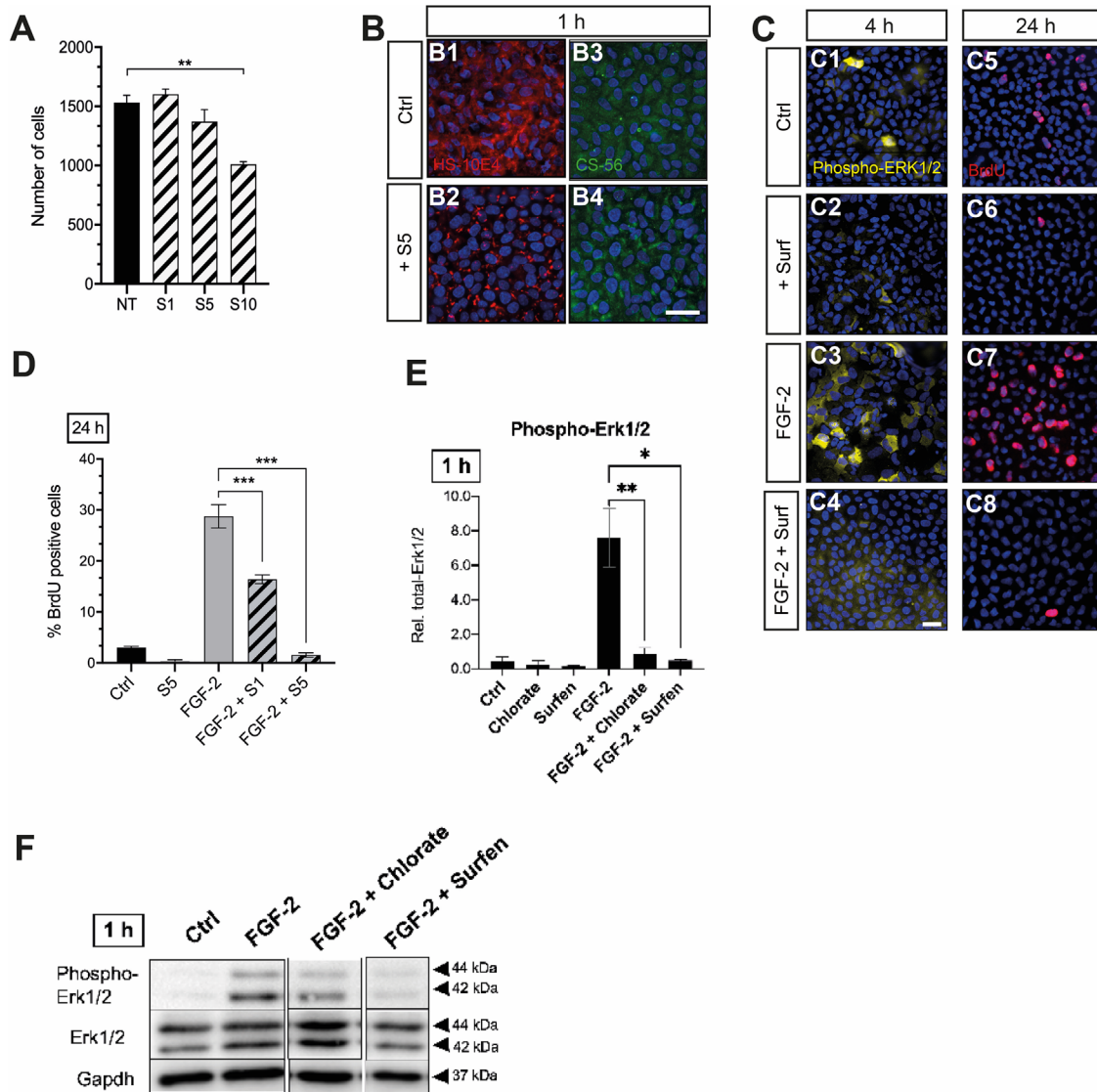
FIGURE 5. Surfen blocks FGF-2-induced lens epithelial cell proliferation and pERK1/2. The 10-day-old rat lens epithelial explants treated for 24 hours with increasing doses of Surfen (1 µM, S1; 5 µM, S5; or 10 µM, S10) had decreased cell viability (**A**; number of cells). (**B**) Treatment with 5 µM Surfen alone (**B**2, **B**4) for 1 hour decreased immunolabeling of heparan sulfate (HS-10E4; **B**2) relative to control explants (**B**1), but not chondroitin sulfate (CS-56; **B**3, **B**4). Surfen (up to 5 µM) also decreased FGF-2 (5 ng/mL)-induced lens epithelial cell proliferation (C5–8, **D**; % BrdU-labeling), as well as immunolabeling for phosphorylated-ERK1/2 (4 hours, **C**1–4). (**E**) Quantification of Western blots (**F**) for phosphorylated-ERK1/2, total-ERK1/2, and GAPDH in explants treated with FGF-2, with or without (Ctrl) sodium chlorate (30 mM) or Surfen (5 µM) at 1 hour. Data are the mean ± SEM from at least three independent experiments, applying unpaired two-tailed Students *t*-test analysis (*$P = 0.014$; **$P = 0.006$). Scale bar = 25 µm.

treated with FGF-2 alone. Surfen was also able to block phosphorylation of ERK1/2 after a 4-hour exposure to a high dose of FGF-2 (see Fig. 6D1–3).

As immunolabeling of HS-GAGs in intact lens showed differential patterns in lens epithelial cells (cytoplasmic; see Fig. 2B1–3) and differentiating lens fiber cells (cytoplasmic, cell membrane, and nuclear; see Fig. 2B4), we compared this to lens epithelial explants treated with either a low proliferative dose of FGF-2 for 24 hours (see Fig. 5), or a high differentiating dose of FGF-2 for 3 and 5 days and labeled with HS-GAG (see Fig. 6F). Although there was no change in HS-GAG labeling in explants treated with or without a low dose of FGF-2 (data not shown), when explants were treated with a differentiating dose of FGF-2, after 3 and 5 days of culture, HS-GAG was seen to translocate from the

lens capsule and cytosol of lens epithelia (see Fig. 6E1) to the cell nuclei of newly differentiating fiber cells (see Fig. 6E2–3), comparable to that seen in the intact lens.

DISCUSSION

Here, we present the most comprehensive analysis examining the spatial localization of all the different classes of HSPGs in the lens. Our identification of the differential cell compartment, cell type-, and functional regional-specific localization of key lens HSPGs highlights the lens as an ideal model in which to study the diverse roles of these different proteoglycans. The divergent localization and expression patterns of HSPG core proteins in the lens suggests unique and diverse roles for different HSPGs in the regulation of
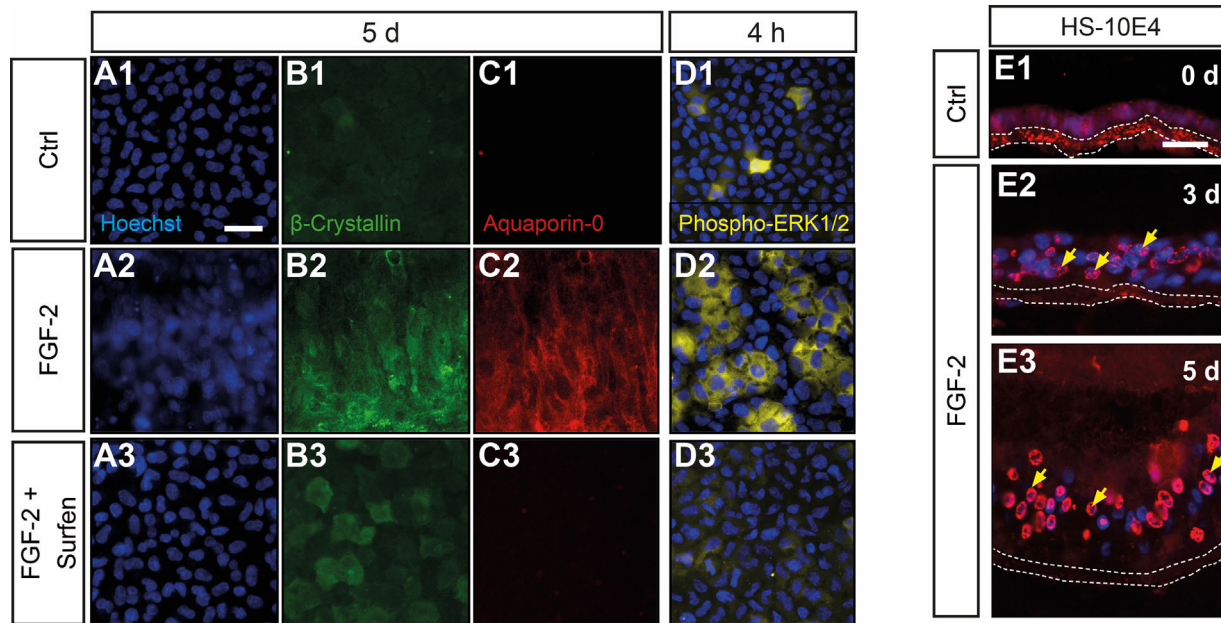
FIGURE 6. Sulfation of heparan sulfate is required for FGF-2-induced lens fiber differentiation. The 10-day-old rat lens epithelial explants were treated up to 5 days with a differentiating dose of FGF-2 (150 ng/mL), with or without Surfen (5 μM). Relative to controls (**A1–D1**), explants treated with FGF-2 alone for 5 days labeled for fiber protein markers, *β*-crystallin (B2) and aquaporin-0 (C2). FGF-2-treated explants showed increased phosphorylated-ERK1/2 labeling at 4 hours (D2) relative to controls (D1). Cotreatment with Surfen blocked FGF-induced fiber elongation, accumulation of fiber markers (B3, C3), and ERK1/2 phosphorylation (D3). Mid-sagittal sections through FGF-2-treated explants labeled for HS (E1–3, HS-10E4) up to 5 days show multilayering of elongating fiber cells (E2, E3) and translocation of HS from the lens capsule at day 0 (E1) to the nuclei of elongating cells at 3- and 5-days post-treatment (E2, E3). Yellow arrows indicate nuclear localization. Dotted lines demarcate lens capsule (E). Scale bars = 25 μm.

lens cell processes, including lens epithelial cell proliferation and fiber differentiation.

In this study, we found transcripts for all HSPGs (and for most, their corresponding protein) to be expressed in the lens, in agreement with other postnatal HSPG transcript expression profiles from published mouse lens datasets.[60–64] Our lack of protein labeling for glypican-5 may relate to its very low expression (lowest of all HSPGs), as reported in other lens transcriptome/microarray datasets (GEO series GSE69221),[60] (GEO series GSE100136),[64] with *Gpc5* mRNA also low or undetected in the developing lens (GEO series GSE69940).[63] We also detected *Gpc5* mRNA using a predesigned sensitive TaqMan Gene Expression Assay[65] against the *Gpc5* sequence; consistent with our detection of relatively low levels of glypican-5 protein using dots blots. Taken together, our findings suggest that all HSPG core protein transcripts and their respective proteins are expressed in the lens. Broad expression profiles of HSPG core proteins are not uncommon and have been reported for various other tissues and cell-types. For instance, despite us seeing some label for glypican-3 in human corneal stromal cells, other studies report the expression of transcripts for all HSPG core proteins in these cells, with the exception of *Gpc3*.[66]

HS is the predominant sulfated GAG in the postnatal murine lens, in accordance with previous studies.[67,68] The overlapping and divergent labeling patterns of HS and CS in the lens (e.g. in new differentiating secondary lens fibers) imply that these GAGs may both contribute to the regulation of some functional processes in the lens (e.g. fiber differentiation), whereas other processes are likely to be regulated predominantly by HS (e.g. gradient formation in the lens

capsule). Divergence in the functional roles of HS and CS has been described in other tissues, including the developing brain, where CS is important for the stabilization of existing synaptic connections, whereas the primary function of HS is to stimulate the formation of new synapses.[69] Given HS and CS each interact selectively with different proteins in a structure-dependent manner,[70] it is not surprising that they are often reported to be differentially localized,[69,71,72] consistent with their distinct functional roles.[73]

HSPG Expression Patterns in Lens Capsule and Functional Significance For ECM Interactions and Growth Factor Gradients

The lens capsule acts as a reservoir for growth and survival factors that can be sequestered by HSPGs or freed by catalytic enzymes, such as matrix metalloproteinases (MMPs). Both FGF[42,74,75] and HSPGs[37,42,76] have been localized to the lens capsule. FGF-1 and FGF-2 are colocalized with HSPGs in the capsule in an increasing antero-posterior gradient.[42,75] FGF-2 release from the lens capsule by MMPs, which can free HS-bound growth factors,[77,78] is essential for lens epithelial cell viability and survival.[79] Consistent with previous studies,[18,80,81] we found that perlecan is the predominant HSPG of the lens capsule. We also localized agrin and collagen XVIII/endostatin to the lens capsule, although to a lesser extent than perlecan, consistent with recent proteomic analyses of human lens capsules.[82] Moreover, the core proteins of these HSPGs are analogous in size to those of two unidentified HSPGs previously isolated from the calf lens capsule.[68] These three HSPGs (perlecan,

agrin, and collagen XVIII/endostatin) are commonly associated with basement membranes and extracellular matrix, where they play a key role in the formation of local gradients by binding to native factors and regulating their bioavailability to adjacent cells.[83,84] Previous studies have reported an increasing antero-posterior gradient of FGF-2, HS, and perlecan in the lens capsule,[20] suggesting that HSPGs native to the lens capsule may play a role in forming and/or regulating the distribution of growth factors available to lens cells. The lenses of transgenic mice expressing HS-deficient perlecan have structurally compromised lens capsules,[37] indicating that perlecan HS chains may also play an important structural role in the capsule. Our immunolocalization studies also indicate the presence of cell-associated HSPGs (syndecans-1/-2/-4 and glypicans-2/-4) in the lens capsule. With the exception of syndecan-4[85] this is the first report of these HSPGs in the lens capsule. Although most often localized to the cell surface, syndecan HSPGs also play well-established roles in basement membranes as regulators of ECM assembly, cell-matrix interactions, and focal adhesion. For instance, syndecans-1, -2, and -4 (no reports for syndecan-3) have binding domains for ECM proteins, such as fibronectin,[86–88] laminin[17,18,22] and collagen IV (Filla et al. 2004; Esko et al. 2017; Esko & Selleck 2002). Laminin and collagen IV are major components of the lens capsule, and promote the adhesion and migration of lens epithelial cells in vitro.[89,90] Syndecan-2 is codistributed with fibronectin, laminin, and collagen IV in human trabecular meshwork and Schlemm's canal cells,[91] and plays an important role in regulating the assembly of ECM components.[23,24] Syndecans-1 and -4 have been reported to promote cell adhesion through binding interactions with several ECM proteins native to the lens capsule,[82] including collagen IV[26,92] and laminin,[93,94] with syndecan-4 recently identified as essential for integrin signaling-dependent cell migration of lens epithelial cells.[85]

HSPG Expression Patterns in Lens Epithelium and Functional Significance For Epithelial Maintenance and Proliferation

All lens HSPGs were expressed in the lens epithelium; however, the patterns and intensity of labeling for the different HSPGs varied at the level of the subcellular compartment and functional regions of the lens epithelium (anterior versus equatorial). The subcellular localization of HSPGs in anterior lens epithelial cells can be categorized broadly as either diffusely cytoplasmic (syndecans-1/-4, glypicans-3/-4, perlecan), or apically polarized (syndecans-2/-3, glypicans-1/-2/-6, collagen XVIII/endostatin, and agrin). Further characterization in lens epithelial wholemounts revealed more detailed localization patterns at the apical surfaces of the epithelial cells including accumulation at cell-cell interfaces (syndecan-3 and glypican-3), within cell nuclei (syndecan-2), and to perinuclear regions (syndecan-4 and glypicans-2/-6). This differential localization of HSPGs within anterior lens epithelia suggests they may play distinct roles in the maintenance and polarity of the lens epithelium.

Although HSPGs are best known for their localization at the cell surface and within the pericellular matrix, HSPGs can also be found in more unexpected cellular compartments (reviewed in Couchman and Pataki[39] and Kolset et al.[95]). Beyond their predictable associations with organelles involved in their biosynthesis (endoplasmic reticulum) and glycosylation (Golgi apparatus), HSPGs have been reported to localize within the cytoplasm,[30,31,96,97] actin cytoskeleton,[98,99] vesicular trafficking networks,[29,95] and cell nuclei.[100,101] The localization of HSPGs to specific subcellular compartments is functionally relevant.[39,95] The strong diffuse cytoplasmic label we observed is most evident for syndecans-1/4 and glypicans-2/3. The cytoplasmic localization of these HSPGs has previously been reported in other cell types, including skin,[30] esophagus,[96] and cancer cells of the endometrium[102] and bladder.[97] Best characterized is the diffuse localization of glypican-3 to the cytosol of liver cancer cells,[103,104] where it drives the mitogenic activity of these cells by regulating the subcellular localization of β-catenin (nuclear versus cytoplasmic) to modulate the activation of canonical Wnt/β-catenin signaling.[105,106] Other members of the glypican family are also well-known regulators of canonical Wnt signaling.[34] In the lens, canonical Wnt signaling is involved in both the proliferation and maintenance of the lens epithelium and in the regulation of fiber cell differentiation.[107,108]

The mechanisms by which different HSPGs achieve specific polar distribution are diverse and have only been investigated in detail for a few proteins, with the intracellular localization resulting from HSPG-specific protein-binding interactions, secretion, signaling, or sorting. HSPG-protein binding interactions are a primary determinant of HSPG targeting, and these interactions may be unique to specific core proteins, families of HSPGs (e.g. core protein binding interactions) or ubiquitous among HSPGs (e.g. HS chain binding interactions). We observed Syndecans-2/-3 and glypicans-1/-2/-6 localized predominantly to the apical pole of anterior lens epithelial cells, with concentrated basal labeling for syndecan-3, glypican-2, and perlecan. Glypicans are usually targeted to the apical surface in polarized epithelial cells.[109]

The basolateral localization of syndecan-1 in epithelial cells is linked to its interaction with other proteins (such as ZO-1) via its cytoplasmic PDZ-binding domain.[110] PDZ proteins are thought to facilitate selective retention at the apical or basolateral surface of plasma membrane proteins that contain PDZ-binding motifs. All syndecan HSPGs have PDZ-binding motifs within their cytoplasmic tail, through which they can also bind integrins[105] and cytoskeletal components. This is a likely explanation for the basal labeling of syndecan-3. Another possible explanation for the localization of HSPGs to the apical pole of lens epithelial cells may imply a role in the regulation of events or interactions at the epithelial-fiber interface. For instance, members of the syndecan family are highly expressed at the epithelial-fiber interface and/or anterior and posterior poles of the lens fiber tips; similar in expression to F-actin[111] and $\beta1$ integrin,[38] that are known to interact directly with, and/or are regulated by syndecans.[39] On the other hand, HS-growth factor receptor complexing/activation and internalization may target several different HSPGs to different intracellular compartments for signaling, degradation, or recycling.[95]

Perlecan is primarily an ECM-localized core protein in the lens and becomes predominantly capsule-localized with increasing age (unpublished data, Wishart and Lovicu). Because this HSPG is synthesized by lens epithelial cells and deposited into the lens capsule as it is secreted, we suggest that the basal concentration of perlecan staining is likely related to its localized secretion.

HS-chain conjugation may also account for the polar nature of HSPG localization. Glypican HSPGs have been

reported as the major source of HS localized to the apical pole of epithelial cells.[36] The apical localization of glypican HSPGs in polarized epithelial cells is inversely related to the number of HS chains they carry.[37] In MDCK and CaCo2 cells, glypican HSPGs were transported predominantly to the basolateral membrane, whereas glypican lacking HS attachment sites were sorted to the apical surface.[109]

The intensity of sulfated HS labeling increased in an antero-posterior gradient from the central epithelium to the equatorial germinative zone. This increase in staining intensity from anterior-to-equatorial epithelium was also observed for a majority of HSPG core proteins. In rat lens epithelial wholemounts, some HSPGs labeled strongly in actively mitotic cells (syndecans-1/-4, glypicans-2, and collagen XVIII/endostatin). Taken together, these findings suggest a possible correlation between a change in the mitotic activity of lens epithelial cells in the germinative zone, and a change in the specific HSPG profile of these cells.

Localization of HSPGs in Fiber Cells

Several studies have demonstrated that organization of the actin cytoskeleton plays an important role in lens fiber differentiation,[112] as destabilizing the actomyosin cytoskeleton or disrupting RhoA/ROCK/Rac-signaling-activity in cultured lens cells with inhibitors leads to impaired fiber cell migration, elongation, and survival.[112–114] Syndecan-4 can signal to the cytoskeleton, primarily through Protein kinase C α (PKCα) that binds directly to the syndecan-4 core protein.[115,116] Phosphorylation of the syndecan-4 core protein is also sufficient for the assembly of focal adhesions and actin stress fibers.[117] Integrin signaling is key in several lens processes in morphogenesis and for lens maintenance throughout life.[118] In particular, $\beta 1$ integrin-signaling, that has been shown to be regulated by syndecans-2 and -4[87,119,120] and potentially other syndecans,[121] is essential for maintenance of the lens epithelium and for the structural maintenance and homeostasis of differentiating fiber cells.[122] Downregulation of syndecan-4 suppressed $\beta 1$ integrin-mediated activation and cell adhesion in human lens epithelial cells in vitro, and in syndecan-4-deficient murine lenses.[85] The association of syndecan-3 with fiber cells may relate to the association of this syndecan core protein with Src kinase and cortactin,[42] that regulate fiber differentiation in concert with Ephrin-signaling.[43–46] Moreover, in vitro the syndecan-3 core protein has been reported to be phosphorylated by Ephrin receptor activation.[123]

HSPGs in the Lens Cell Nuclei

In intact rat eyes, we observed nuclear localization of HS in newly differentiating secondary lens fibers. In agreement with this, in vitro we observed HS translocating to the nuclei of lens epithelial cells undergoing FGF-induced lens fiber differentiation. Nuclear HS was not observed in untreated controls, or cells treated with a lower proliferative dose of FGF. This is the first time this phenomenon has been described in the lens. Accumulating evidence suggests that HSPGs can localize to the nuclear compartment of various cell-types, although the functions of HS/HSPGs in the nucleus are yet to be completely understood.[124,125] HS and HSPGs transport HS-binding proteins, including growth factors and their high affinity receptors to the nucleus, where they can regulate gene expression to control cellular functions, such as proliferation and differentiation (reviewed in Stewart and Sanderson[124]). For instance, FGF-2 has been detected in the nuclei of many tissues and cell types, and induces the accumulation of FGFR-1 in the nuclear matrix in a concentration-dependent manner,[126] where it can influence both proliferation and differentiation in a cell type-specific manner.[127] FGFR-1 has been shown to be localized to the membrane and nuclei of epithelial lens cells and differentiating fiber cells,[24] suggesting possible internalization and trafficking of the receptor to the nucleus.

HSPGs also can colocalize with FGF in the nuclei of cells. Syndecan-1 and FGF-2, but not FGFR-1 colocalize in the nuclei of mesenchymal cancer cells.[128] CHO cells defective in the ability to properly synthesize heparan sulfate chains showed reduced nuclear FGF-2.[70] Syndecan-1 has also been reported to translocate together with FGF-2 to the nucleus in a highly regulated manner by a tubulin-mediated transport mechanism.[129] Nuclear HS/HSPGs have also been reported to negatively regulate cell proliferation by initiating cell-cycle arrest.[130–132] Syndecan-4 regulates FGF-2 signaling not only through PI3K/Akt and ERK1/2 activation, but also via internalization and nuclear localization.[133] Syndecan-1 nuclear localization of FGF-2 in corneal stromal fibroblasts is mediated by HSPGs through a protein kinase Cα-dependent-signaling mechanism.[134] During muscle cell differentiation, cell surface syndecan-4 is internalized and trafficked to cell nuclei, where it is suggested to play a regulatory role in the expression of $\beta 1$-integrin and actin.[135] Interestingly, syndecan-2, the HSPG we demonstrate most clearly to be associated with lens cell nuclei, is the only syndecan family member missing the juxta-membrane RMKKK nuclear localization sequence in its cytoplasmic domain; however, syndecan-2 has previously been reported in the nuclei of chondrosarcoma cells,[136] and co-localized with nuclear FGF-2 in neural cells.[137] Taken together, these observations suggest that the nuclear localization of syndecan-2 is likely mediated through its interaction with other proteins (e.g. FGF-2 and FGFR-1).

HS Sulfation is Essential for FGF-2-Induced Lens Epithelial Cell Proliferation and Fiber Differentiation

In this study, we showed that FGF-induced lens epithelial cell proliferation and early FGF-induced ERK1/2 phosphorylation in rat lens epithelial explants could be blocked by both chlorate (that blocks all sulfated GAGs, CS, and HS) and Surfen (that selectively blocks HS sulfation). This suggests that HS sulfation is required for FGF-induced lens epithelial cell proliferation, and that the functional roles of sulfated HSPGs in the lens are likely predominantly attributed to HS sulfation/HSPGs rather than any other sulfated GAG (e.g. CS). HS and CS often display opposite effects in cell function, with HS and CS structural motifs presenting unique binding sites for specific ligands. Relative to HS, CS-protein binding interactions are limited.[70] Although both HS and CS can bind to FGFs and regulate their activity, these binding interactions favor HS over CS. In agreement with this, even if an HSPG carries both HS and CS chains, it does not necessarily translate that they both contribute to FGF-binding and signaling. For instance, FGF-2-mediated chondrocyte proliferation is regulated by a form of perlecan carrying both HS and CS chains. Following enzymatic digestion of CS chains, the

HS-carrying perlecan augmented FGF2-FGFR binding and chondrocyte proliferation.[138]

Surfen was also shown to block FGF-induced lens fiber differentiation and its associated ERK activation, as indicated by impaired cell elongation and the accumulation of fiber-specific proteins, β-Crystallin, and AQP0/MIP. This suggests that HSPG sulfation is not only essential for FGF-2-induced lens epithelial cell proliferation, but also for fiber differentiation. FGF signaling is required for lens cell survival,[21,139,140] cell elongation,[141] and the accumulation of fiber-specific proteins β-crystallins[142,143] and AQP0/MIP[141] in the early stages of fiber differentiation. Although FGF-induced ERK-signaling is essential for fiber cell elongation[142,143] and expression of AQP0/MIP,[144] it is not required for the accumulation of β-crystallin, which is believed to be under the control of other signaling pathways, such as PI3K/Akt.[22] Inhibition of β-crystallin accumulation by Surfen suggests that the activation of PI3K/Akt signaling downstream of FGF-2, and alternative pathways that regulate crystallins, may also be dependent on HS sulfation, consistent with HSPGs acting upstream as a co-receptor for FGF/FGFR signaling in the lens. To date, FGF is the only growth factor known to be essential for the induction of lens fiber differentiation in mammals,[145] with our current studies now highlighting that FGF signaling is under the control of HSPGs, placing them as central regulators of lens cell fate.

Conclusions

The lens epithelium, fiber cells, and lens capsule display unique and distinct labeling profiles of the different HSPG core proteins. Specific cellular HSPGs (e.g. glypicans-1/2) and secreted HSPGs (e.g. perlecan) may play key roles in growth factor regulation in the lens (e.g. cell proliferation and differentiation). Cellular localization (lens epithelium and fiber cells) of the secreted HSPG agrin, is unique in the lens compared to other tissues where it is mostly associated with aquaporins. The differential spatial and temporal expression patterns of the different HSPG family members in lens, in combination with their interactions with key lens regulatory proteins, suggests that these HSPG core proteins may be differentially regulating different lens growth factors or specific cellular processes. The findings of this study highlight that different HSPGs may play distinct roles in lens function and growth processes. As HS-sulfation has been identified as critical for normal lens development, our ongoing studies aim to examine the spatiotemporal distribution of different GAGs and HSPGs during lens morphogenesis.

Due to the homogeneous nature of HSPGs, and given their activity is determined primarily by their sulfation, here, we initially targeted all HS sulfation given the likely functional redundancy among the different HSPGs. We validate the previously reported, well-established effects of sodium chlorate on lens and build on this by focusing only on the role of HS-sulfation using Surfen. Impaired HS blocks FGF-2-induced ERK1/2-phosphorylation and lens epithelial cell proliferation and fiber differentiation. Taken together with previous work from our laboratory, this data supports a requirement for HSPG/GAG sulfation in growth factor-induced signaling in the lens. Our future studies will seek to investigate the differential functions and protein interactions of specific HSPG members.

By further characterizing, and functionally modulating the different sulfated proteins, we hope to better understand how key lens HSPGs regulate different growth factor signaling associated with cellular processes. Moreover, gaining a better understanding of specific HSPG activity may allow us to develop novel ways of regulating cell behavior, that will not only be relevant to lens biology and disease, such as cataract, but may extend to other systemic growth factor-mediated pathologies.

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