

Heparan Sulfate Proteoglycans: Orchestrators of Lens Biology, Development and Pathology



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Time and the hour runs through the roughest day.

MacBeth I.III, William Shakespeare

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DECLARATION

I hereby declare that the content of this thesis is original and represents my own work. It has not been previously submitted for any academic degree or other purposes. Furthermore, I affirm that all sources and assistance received during the preparation of this thesis have been duly acknowledged and credited.

Tayler F.L. Wishart

September 30th 2023

ABSTRACT

Purpose: The arrangement of lens cells is regulated by ocular growth factors. Although the effects of these inductive molecules on lens cell behaviour (proliferation, survival, and fibre differentiation) are well-characterised, the precise mechanisms underlying the regulation of growth factor-mediated signalling in lens remains elusive. Increasing evidence highlights the importance of heparan sulfate proteoglycans (HSPGs) for the signalling regulation of growth factors; however, the identity of the different lens HSPGs and the specific roles they play in lens biology are still unknown. HSPGs can be considered as the “dark matter” of the biological universe: a major regulatory cellular component for which specific functions are only beginning to be elucidated. Ubiquitous to all animal cells, HSPGs are a major component of the cell surface and extracellular matrix, where they serve as the versatile interface of cells with their environment through regulation of protein-protein interactions associated with cellular events such as proliferation, differentiation and migration. Sulfated heparan sulfate glycosaminoglycan (HS-GAG) chains attached to HSPGs, are required for the extracellular distribution, gradient formation, and interactions of growth factors and their associated receptors. Accumulating evidence suggests that altered HSPG sulfation can profoundly influence growth factor-mediated processes, and drive pathology. Recent studies by our laboratory observed that impaired HSPG sulfation potently and reversibly blocked murine lens epithelial cell proliferation induced by growth factors, suggesting an important regulatory role for HSPGs in lens epithelial cell behaviour. The present study aims to determine which specific HSPGs are involved in regulating cell behaviour induced by ocular growth factors in the lens. To better understand the role of HSPGs in lens biology, here, we aimed to i) Identify the HSPG-associated GAGs and core proteins that are expressed in the postnatal and developing murine lens, ii) determine

whether HSPG sulfation is required for growth-factor-induced lens cellular processes (lens cell proliferation, fibre differentiation) *in vitro*, and iii) determine whether the expression of lens HSPGs is altered in lens pathogenesis (fibrotic cataract), and whether HSPG sulfation is required for TGF- β -mediated epithelial-to-mesenchymal transition (EMT) *in vitro*. We hypothesised that lens architecture is tightly controlled by different HSPGs that regulate lens development, ocular growth factor-induced lens cell proliferation and fibre differentiation, as well as aberrant TGF- β -mediated EMT and cataract development.

Methods: Semiquantitative real-time (RT)-PCR and immunolabeling were used to characterise the spatial distribution of all known HSPG core proteins and their associated GAGs in the postnatal and developing murine lens, and in a murine model of fibrotic cataract *in situ*. Lens epithelial explants, cultured in the presence of different ocular growth factors and different inhibitors of HSPG activity were used to investigate the requirement for HSPGs in lens cell proliferation, fibre differentiation, lens EMT and their associated downstream signalling pathways.

Results: The lens expresses all HSPGs, and HS-GAG is the predominant HSPG associated GAG in the postnatal and developing lens. HSPG core proteins were differentially expressed in 10-day old rat lens, and localised strongly to the germinative zone, and lens capsule, respectively. These HSPGs are differentially localised to distinct functional regions of the lens. In the developing lens, HSPG core proteins were differentially spatiotemporally regulated throughout lens morphogenesis. HS-GAG chains are strongly localised to fibrotic plaques in transgenic mice overexpressing TGF- β . Compared with normal lenses, the level of expression and spatial

localisation patterns of some HSPG core proteins were significantly modified in cataractous transgenic lenses. Pharmacological inhibition of HS-GAG activity in vitro blocked lens epithelial cell proliferation, fibre differentiation, and lens EMT highlighting that these cellular processes are dependent on HSPG activity.

Conclusions: The differential spatial and temporal expression of HSPGs in different lens cells and throughout lens morphogenesis suggests that lens cells acquire a distinctive repertoire of HSPGs as they differentiate, resulting in selective patterns of HS-protein interactions and activity. These data support differential roles for these HSPGs at key stages of lens induction and morphogenesis. These findings suggest that HS-GAG sulfation is essential for growth factor-induced cellular processes during embryogenesis, and the unique and divergent localisation of different lens HSPG core proteins indicates that different HSPGs likely play specialised roles during lens induction and morphogenesis. 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 signalling networks leading to specific biological events involved in lens growth and maintenance. These findings support a requirement for HSPGs in lens cell proliferation and fibre 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 signalling networks leading to specific biological events involved in lens growth and maintenance. The requirement of HS for lens cell EMT, combined with the differential expression of HS-GAG and specific HSPG core proteins in cataractous lenses suggests that changes in the selective patterns and interactions and activity of

specific HSPGs may point to candidate targets for the regulation of fibrotic changes underpinning the pathogenesis of fibrotic cataract. By further characterising and specifically modulating these sulfated proteins, we hope to develop novel ways of regulating cell behaviour that will not only be relevant to lens biology and disease, but may extend to other growth factor-mediated pathologies.

LIST OF ABBREVIATIONS AND SYMBOLS

α Alpha

β Beta

Δ Delta

κ Kappa

λ Lambda

μ Micro

α -crystallin Alpha-crystallin

α -SMA Alpha-smooth muscle actin

α -tubulin Alpha-tubulin

Ab Antibody

AB Alcian blue

ACO Anterior capsular opacification

AKT Protein kinase B

AP2 α Activator protein 2 alpha

AQ Aqueous humour

AQP0/MIP Aquaporin-0 / Membrane intrinsic protein

ASC Anterior subcapsular cataract

β -catenin Beta-catenin

β -crystallin Beta-crystallin

BM Basement membrane

BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CASK	Calcium/calmodulin-dependent serine protein kinase
CO ₂	Carbon dioxide
CS	Chondroitin sulfate
CSPG	Chondroitin sulfate proteoglycan
Ct	Cycle threshold
Dally	Division abnormally delayed
Dlp	Dally-like
DMSO	Dimethyl sulfoxide
E	Embryonic day
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
ERM	Ezrin/radixin/moesin
EXT	Exostosin
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor

GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GlcA	Glucuronic acid
Glce	d-glucuronyl C5-epimerase
GlcNAc	Glucuronic acid-N-Acetylglucosamine
GOF	Gain-of-function
Gpc	Glypican
GPI	Glycosylphosphatidylinositol
GRIFN	Galectin-Related Inter-Fiber Protein
Gt	Goat
GTPase	Guanosine-triphosphate binding proteins
H&E	Haematoxylin and Eosin
HB-EGF	Heparin-binding EGF
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
HS	Heparan sulfate
HSOST	Heparan sulfate O-sulfotransferase
HS2ST	Heparan sulfate 2-O-sulfotransferase
HS3ST	Heparan sulfate 3-O-sulfotransferase
HS6ST	Heparan sulfate 6-O-sulfotransferase
HSPG	Heparan sulfate proteoglycan
IGF	Insulin-like growth factor
IGF-BP	Insulin-like growth factor binding protein

IGFR	Insulin-like growth factor receptor
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IOL	Intraocular lens
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
LECs	Lens epithelial cells
M199	Medium 199
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MMP	Matrix metalloproteinase
Ms	Mouse
mTOR	Mammalian target of rapamycin
NaClO ₃ ⁻	Sodium chlorate
NBF	Neutral buffered formalin
Nd:YAG	Neodymium-doped yttrium aluminium garnet
NDST	N-deacetylase/N-sulfotransferase
NGS	Normal goat serum
P	Postnatal day
P38	Protein 38
pAb	Polyclonal antibody
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PAPSS	PAPS synthetase

PAS	Periodic acid Schiff's
PBS	Phosphate buffered saline
Pax6	Paired box protein 6
PCO	Posterior capsular opacification
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
Phospho-	Phosphorylated
PKC α	Protein kinase C alpha
PI3K	Phosphatidylinositol-3-OH kinase
Prox1	Prospero homeobox protein 1
PVDF	Polyvinylidene difluoride
Ras	Rat sarcoma virus
Raf	Rapidly accelerated fibrosarcoma
Rb	Rabbit
Rho	Ras Homolog Family Member
ROCK	Rho-associated protein kinase
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
RTK	Receptor tyrosine kinase
RT-qPCR	Reverse transcriptase-quantitative polymerase chain reaction
Sdc	Syndecan
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean

SMAD	Small mothers against decapentaplegic
SO ₄ ⁻	Sulfate
SPARC	Secreted protein acidic and rich in cysteine
Surf	Surfen
TBST	Tris-buffered saline with Tween-20
TGF-β	Transforming growth factor-beta
TMD	Transmembrane domain
tSmad	Total-smad
v/v	Volume / volume
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VIT	Vitreous humour
WM	Whole Mount
Wnt	Wingless/Integrated
WT	Wild-type
YAP	Yes-associated protein

PUBLICATIONS

INCLUDED AS PART OF THIS THESIS

The following published manuscripts emanate from this dissertation. Some parts of the manuscripts have been restructured for the purposes of coherence. Original manuscripts (See appendix 1). The work has arisen entirely during my PhD candidature under the guidance of my primary supervisor Professor Frank Lovicu who contributed to this manuscript by facilitating me with experimental design and manuscript writing.

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

Tayler F.L. Wishart

Sept 30th 2023

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Frank J. Lovicu (Primary supervisor)

Oct 4th 2023

- **TFL Wishart** and FJ Lovicu (2023). Spatiotemporal Localisation of Heparan Sulphate Proteoglycans throughout Mouse Lens Morphogenesis. Cells (DOI: 10.3390/cells12101364).
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- SJ Das, **TFL Wishart**, KAM Jandeleit-Dahm, FJ Lovicu (2020). Nox4-mediated ROS production is involved, but not essential for TGF- β -induced lens EMT leading to cataract. In Experimental Eye Research, vol.192 (DOI: 10.1016/j.exer.2020.107918).
- **TFL Wishart**, L Aw, K Byth, G Rangan, K Sud (2018). A Retrospective Sequential Comparison of Topical Application of Medicated Honey and Povidone Iodine for Preventing Peritoneal Dialysis Catheter-related Infections. In Peritoneal Dialysis International, vol.38 no.4 (DOI: 10.3747/pdi.2017.00224).

BOOK CHAPTERS

- Frank J Lovicu, **Taylor FL Wishart**, Brooke P Mao, Mary Flokis, Fatima Wazin and Daisy Y Shu (2019). ‘Chapter 1: Ocular Lens Epithelium: Modelling Growth Factor Signalling in Physiological and Pathological Conditions’ In Berhardt L.V. (vol. 146) Advances in Medicine and Biology. Nova Science Publishers, New York, pp.1-67. (ISSN: 2157-5398)

CONFERENCES AND PRESENTATIONS

ABSTRACTS

- **TFL Wishart**, FJ Lovicu. Heparan sulfate proteoglycans in fibrotic cataract. XXV Biennial Meeting of the International Society for Eye Research (ISER). Gold Coast, AUS Feb. 19-23rd, 2023
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- **TFL Wishart**, H Choucair, B Umashankar, K Bourget, T Rawling, M Murray. Protective effects of fatty acids against apoptosis in triple-negative breast cancer cells mediated by the ω -3 epoxy-lipid analogue CTU. The Experimental Pharmacologists and Toxicologists

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- **TFL Wishart**, L Aw, K Byth, G Rangan, K Sud. Topical Application of Medicated Honey for the Prevention of Peritoneal Dialysis Catheter-Related Infections. The 15th Asian Pacific Congress of Nephrology (APCN) and 52nd ANZSN ASM, Perth, WA, Sept 17–21 2016. In Nephrology (DOI: 10.1111/nep.12888).

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- **TFL Wishart**. “Heparan sulfate proteoglycans in fibrotic cataract”. XXV Biennial Meeting of the International Society for Eye Research (ISER). Gold Coast, AUS Feb 23rd, 2023
- **TFL Wishart**. “Seminar: An Atlas for Heparan Sulfate Proteoglycans in the Lens”. Combined Lens Laboratories Meeting. The University of Delaware, Newark, DE, USA. Aug 22nd, 2019.

AWARDS AND SCHOLARSHIPS

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Research Training Program Stipend Scholarship	Jan 2018 – Apr 2021
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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	2
DECLARATION	7
ABSTRACT	8
LIST OF ABBREVIATIONS AND SYMBOLS	12
PUBLICATIONS INCLUDED AS PART OF THIS THESIS	21
AWARDS AND SCHOLARSHIPS	22
TABLE OF CONTENTS	24
CHAPTER 1: GENERAL INTRODUCTION	32
1.1 The ocular lens	32
<i>Figure 1.1. Schematic drawing of the mammalian eye and lens</i>	33
1.1.1 Lens development	34
<i>Figure 1.2. Schematic representation of key stages of mouse lens development</i>	35
1.1.2 Normal lens physiology	35
<i>Figure 1.3. Schematic representation of functional regions of the lens</i>	36
1.2 Growth factor regulation of lens development and homeostasis	36
<i>Figure 1.4. Terminal differentiation of lens epithelial cells to lens fibre cells</i>	37
1.2.1 The ocular environment	37
1.2.2 Ocular growth factors	39
1.2.3 Growth factor signalling in the lens	40
1.3 Heparan sulfate proteoglycans	42
<i>Figure 1.5. Heparan sulfate proteoglycans (HSPGs).</i>	44
1.3.1 Expression and localisation of HSPGs	47
1.3.1 Functional roles of different HSPGs	47
<i>Figure 1.6. Common functional roles of HSPGs. .</i>	48
1.4 HSPG core proteins	49
1.4.1 The syndecans	49
	24

<i>Figure 1.7. HSPG core protein families</i>	53
1.4.2 The glypicans	54
<i>Table 1.1 HSPG mutant phenotypes</i>	57
1.4.3 Perlecan	60
1.4.4 Agrin	61
1.4.5 Collagen XVIII	62
1.5 HS-GAG chain structure and sulfation	63
1.5.1 HS-GAG Chain Polymerisation (EXTs)	65
<i>Figure 1.8. Schematic representation of heparan sulfate proteoglycan biosynthesis and sulfation</i>	67
1.5.2 PAPS/PAPS synthetases	68
1.5.3 HS N-Sulfation (NDSTs)	70
1.5.4 HS O-Sulfation (HS-OSTs)	71
1.5 Coordination of HSPGs in lens pathology	73
<i>Figure 1.9. Cataract surgery and intraocular lens placement</i>	73
<i>Figure 1.10. Aetiology of fibrotic cataract and lens TGF-β-induced epithelial-to-mesenchymal transition</i>	75
<i>Figure 1.11 TGF-β signalling pathways in TGF-β-induced EMT</i>	78
1.6 Aims and hypotheses	79
CHAPTER 2: GENERAL METHODS	86
2.1 Tissue	86
<i>Table 2.1. Source of ocular tissues</i>	87
2.2 Histological processing	88
2.3 Immunolabelling of HSPGs	89
<i>Table 2.2. Antibodies used for HSPG immunolocalization</i>	91
2.4 Preparation of Lens Epithelial Explants	93
<i>Figure 2.1. Schematic representation of lens explanting procedure</i>	94
2.5 Inhibiting HS-GAG sulfation	95
<i>Table 2.3. Inhibitors used to block HS-sulfation</i>	95
	25

<i>Figure 2.2. Structure of Surfen, a small molecule antagonist of heparan sulfate</i>	97
2.6 Immunofluorescent detection of proteins of interest in treated explants	97
<i>Table 2.4. Primary antibodies used for immunofluorescent labelling of explants</i>	98
<i>Table 2.5. Secondary antibodies used for immunofluorescent labelling of explants</i>	99
2.7 Lens epithelial proliferation and viability	99
<i>Table 2.6. Growth factors for lens epithelial cell proliferation</i>	100
2.8 Lens fibre cell differentiation	101
<i>Table 2.7. Growth factors for lens fibre cell differentiation</i>	102
2.9 TGF- β 2-induced epithelial-to-mesenchymal transition	103
2.10 Measuring changes in gene expression	103
2.11 Measuring changes in protein expression	104
2.12 Statistical analysis	105
CHAPTER 3: RESULTS I	107
AN ATLAS OF HEPARAN SULFATE PROTEOGLYCANS IN THE POSTNATAL RAT LENS	
3.1 Preface	107
3.2 Introduction	108
3.3 Results	112
3.3.1 Relative expression of HSPG core protein transcript in the lens.	112
<i>Figure 3.1. Relative expression of HSPG core protein transcripts in 10-day-old rat lens epithelial and fibre cells.</i>	113
3.3.2 localisation of Sulfated GAGs (HS and CS).	113
<i>Figure 3.2. Distribution of sulfated glycosaminoglycans in the 10-day-old rat eye.</i>	116
3.3.3 HSPG core proteins are differentially localised key functional regions in the lens.	117
3.3.4 Lens capsule	117
3.3.5 Lens epithelium	118
<i>Figure 3.3. Spatial localisation patterns of HSPG core proteins in the anterior lens capsule and epithelia.</i>	121
3.3.6 Lens fibre cells	122

<i>Figure 3.4. Spatial localisation patterns of HSPG core proteins in the equatorial lens, cortex and posterior capsule</i>	125
3.3.7 Non-lenticular regions of the eye	126
<i>Figure 3.5. Spatial localisation patterns of HSPG core proteins in the cornea</i>	128
<i>Figure 3.6. Spatial localisation patterns of HSPG core proteins in the iris and ciliary body</i>	130
<i>Figure 3.7. Heparan sulfate glycosaminoglycan immunolabelling in the lens across different vertebrate species.</i>	132
3.4 Discussion	133
3.4.1 The lens expresses all HSPG core proteins	133
3.4.2 HSPG expression patterns in lens capsule and functional significance for ECM interactions and growth factor gradients.	134
3.4.3 HSPG expression patterns in lens epithelium and functional significance for epithelial maintenance and proliferation	136
3.4.4 HSPGs in the lens cell nuclei	141
3.4.5 Conclusions	143
<i>Figure 3.8. Spatial localisation patterns of HSPG core proteins in lens cells</i>	145
<i>Table 3.1. Summary of postnatal HSPG immunolocalization.</i>	146
CHAPTER 4: RESULTS II	149
SPATIOTEMPORAL LOCALISATION OF HSPGS THROUGHOUT MURINE LENS MORPHOGENESIS	
4.1 Preface	149
4.2 Introduction	149
4.3 Results	152
4.3.1 HS-sulfation enzymes in the developing and postnatal lens.	152
<i>Figure 4.1. Immunolocalisation of PAPS Synthetase-2 (PAPSS2) in the murine eye</i>	154
<i>Figure 4.2. Heparan sulfate O-sulfotransferase expression in the murine and rat lens</i>	156
4.3.2 Heparan sulfate is the predominant sulfated glycosaminoglycan expressed throughout murine lens development.	157
	27

<i>Figure 4.3. Distribution of sulfated GAGs in mouse eye development</i>	160
4.3.3 Syndecan HSPGs are differentially spatially localised in the lens throughout development.	161
<i>Figure 4.4. Distribution of syndecan core proteins in the developing mouse eye.</i>	164
4.3.4 Glypican HSPGs differ in temporal expression at key stages of lens/eye development.	165
<i>Figure 4.5. Distribution of glypican core proteins in the developing mouse eye.</i>	167
4.3.5 Localisation of high molecular weight HSPGs extends beyond the lens capsule.	168
<i>Figure 4.6. Distribution of high molecular weight HSPG core proteins in the developing mouse eye.</i>	170
4.4 Discussion	171
4.4.1 HSPG sulfation enzymes in the lens.	171
4.4.2 HS-GAG is the predominant GAG associated with lens development.	173
4.4.3 HSPG core proteins are differentially spatiotemporally regulated throughout lens development.	174
4.4.3 Conclusions	182
<i>Table 4.1. Summary of HSPG core protein immunolocalization in mouse eye lens development</i>	183
CHAPTER 5: RESULTS III	187
HS-GAG SULFATION ORCHESTRATES LENS EPITHELIAL CELL PROLIFERATION AND FIBRE DIFFERENTIATION	
5.1 Preface	187
5.2 Introduction	187
5.3 Results	194
5.3.1 Surfen effectively blocks HSPG activity in lens cells in vitro	194
<i>Figure 5.1. Sodium chlorate effectively blocks lens cell proliferation induced by FGF-2 but not aqueous</i>	196
<i>Figure 5.2. Optimisation of Surfen hydrate, a selective inhibitor of HS-GAG activity</i>	199
5.3.3 Differential requirements for HS-GAG sulfation of aqueous humour and key ocular mitogens.	200
	28

<i>Figure 5.3. Surfen partially blocks aqueous-induced lens epithelial cell proliferation</i>	202
<i>Figure 5.4. Surfen blocks FGF-2-induced lens epithelial cell proliferation</i>	204
<i>Figure 5.5. Surfen blocks HB-EGF-induced lens epithelial cell proliferation.</i>	206
<i>Figure 5.6. Surfen partially blocks EGF-induced lens epithelial cell proliferation</i>	208
<i>Figure 5.7. Surfen partially blocks IGF-I-induced lens epithelial cell proliferation</i>	210
<i>Figure 5.8. Surfen does not block PDGF-AA-induced lens epithelial cell proliferation</i>	212
<i>Figure 5.9. Effects of sodium chlorate and surfen on downstream phosphorylation of ERK1/2 protein</i>	214
5.3.4 Surfen blocks vitreous and FGF-induced lens fibre differentiation	215
<i>Figure 5.10. HS-GAG sulfation is required for vitreous-induced lens fibre differentiation</i>	217
<i>Figure 5.11. HS-GAG sulfation is required for FGF-2-induced lens fibre differentiation</i>	220
5.4 Discussion	221
5.4.1 Surfen is an effective inhibitor of HS-GAG sulfation in vitro	221
5.4.2 Differential requirements of HSPG sulfation for lens cell proliferation induced by different ocular mitogens.	222
<i>Table 5.1. Summary of effects of surfen on lens mitogen-induced signalling activity and proliferation</i>	229
5.4.3 HSPG sulfation is required for lens fibre differentiation.	230
5.4.4 Conclusions	231
CHAPTER 6: RESULTS IV	233
HEPARAN SULFATE PROTEOGLYCANS IN FIBROTIC CATARACT	
6.1 Preface	233
6.2 Introduction	233
<i>Figure 6.1. Pathogenesis of fibrotic cataract and lens TGF-β-induced epithelial-to-mesenchymal transition</i>	235
6.3 Results	240
6.3.1 Heparan sulfate is the predominant HSPG-GAG associated with lens fibrotic plaques.	240
<i>Figure 6.2. Distribution of sulfated glycosaminoglycans in normal anterior lens vs fibrotic anterior subcapsular plaques of 21-day-old transgenic mice overexpressing TGF-β1</i>	243
	29

6.3.2 Immunolocalization of HSPGs is differentially altered in the pathogenesis of fibrotic cataract.	244
<i>Figure 6.3. Immunolocalisation of syndecan core proteins in the normal anterior lens vs fibrotic anterior subcapsular plaques of 21-day-old mice</i>	249
<i>Figure 6.5. Immunolocalization of collagen XVIII and agrin core proteins in the normal anterior lens vs fibrotic anterior subcapsular plaques of 21-day-old mice</i>	251
<i>Table 6.1. Summary of changes to HSPG core protein immunolocalisation in fibrotic cataracts</i>	252
6.3.3 The HS-GAG antagonist surfen blocks TGF- β 2- EMT in the lens.	253
<i>Figure 6.6. Surfen blocks TGF-β-induced epithelial-to-mesenchymal transition.</i>	255
<i>Figure 6.7. Effects of surfen on early TGF-β-induced signalling</i>	258
<i>Figure 6.8. Timing of Surfen treatment does not impact morphological changes associated with TGF-β-induced epithelial-to-mesenchymal transition</i>	261
6.4 Discussion	262
6.4.1 Distribution of sulfated glycosaminoglycans associated with lens fibrotic plaques.	262
6.4.2 Immunolocalisation of HSPGs is differentially altered in the pathogenesis of fibrotic cataract.	264
6.4.3 HSPGs are required for TGF- β 2-induced TGF- β 2 EMT in the lens.	271
6.4.4 HSPGs are differentially required for both the induction and progression of TGF- β 2-induced EMT.	274
<i>Figure 6.9. Schematic diagram of the aetiology and pathogenesis of fibrotic cataracts, and the role of HSPGs in the regulation of key cellular events</i>	277
6.4.5 Conclusions	278
CHAPTER 7: GENERAL DISCUSSION	279
7.1 Introduction	279
7.2 Heparan sulfate is the predominant HSPG-associated GAG in the lens	279
7.3 The lens has a unique profile of HSPG core proteins that are spatiotemporally regulated in lens development	281
7.4 HSPGs differentially regulate key lens growth factors and are required for lens epithelial cell proliferation and lens fibre differentiation	283
	30

7.5 HSPGs are differentially localised in fibrotic cataract, and HS-sulfation is essential for lens epithelial-to-mesenchymal transition	285
7.6 Conclusions	287
REFERENCES	291
APPENDIX 1	370

CHAPTER 1: GENERAL INTRODUCTION

1.1 The Ocular Lens

Located in the anterior aspect of the eye, the lens is a transparent, biconvex structure that, in conjunction with the cornea, plays a crucial role in facilitating vision by focusing incoming light onto the retina (Figure 1.1). Through the process of accommodation, the shape and curvature of the lens can be changed to refract light rays to allow for visual focus of objects at different distances. The lens is a relatively simple tissue, composed of two distinct cell types: a monolayer of cuboidal epithelial cells that overlies the anterior surface of elongate and precisely aligned fibre cells that make up the lens mass. The highly ordered spatial arrangement of the lens cells is established during embryogenesis and is maintained as the lens grows throughout life (Lovicu and McAvoy, 2005). The ocular lens is an elegant and well-established model for studying cell behaviour. In particular, the study of cellular processes involved in the development and maintenance of the lens in vertebrates has been a valuable and sophisticated framework for understanding the complex interplay of signalling and transcription events (Wormstone and Wride, 2011).

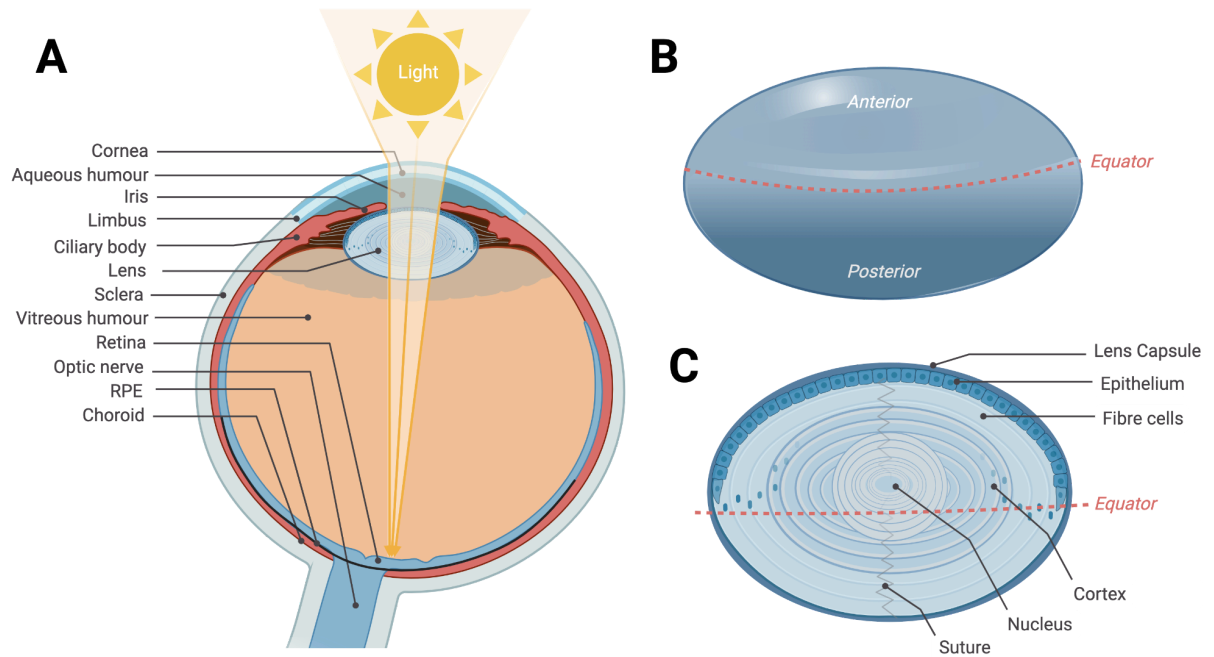


Figure 1.1. Schematic drawing of the mammalian eye (A) and lens. (B) gross structure, (C) mid-sagittal section.

1.1.1 Lens Development

During the early process of vertebrate development, the eye first takes shape when surface head ectodermal cells interact closely with the evaginating optic vesicle (McAvoy et al. 1999, Figure 1.2). This interaction leads to a thickening of the adjacent ectoderm, forming the lens placode. The placode cells then undergo apical constriction, leading to their inward folding, creating a surface depression known as the lens pit. Subsequently, the lens pit deepens and separates from the neighbouring head ectoderm, forming a hollow ball structure called the lens vesicle (Figure 1.2). Within the lens vesicle, the anterior cells differentiate into the lens epithelium, while the more posterior cells elongate to become the primary lens fibres (Lovicu and McAvoy 2005, see Figure 1.2). These primary fibre cells extend towards the overlying epithelium, resulting in the elimination of the vesicle cavity and the establishment of the lens's fundamental structure (Figure 1.2). From this stage onward, the lens continues to grow. Proliferating epithelial cells will exit the cell cycle and undergo pronounced elongation and differentiate, giving rise to secondary lens fibre cells (Figure 1.4). These new fibre cells displace the older fibres towards the centre of the lens. As the fibre cells mature, their nuclear material and organelles degrade, and the cell becomes primarily composed of water soluble crystallin proteins (Bassnett and Beebe 1992, Wride 2011, Figure 1.4). They also develop specialised membrane interdigitations, acquiring the transparency that is essential for the lens to fulfil its primary function: to transmit and focus light. Hence this distinctive pattern of growth throughout life maintains the normal architecture of the lens, and hence its transparency (Lovicu and McAvoy 2005).

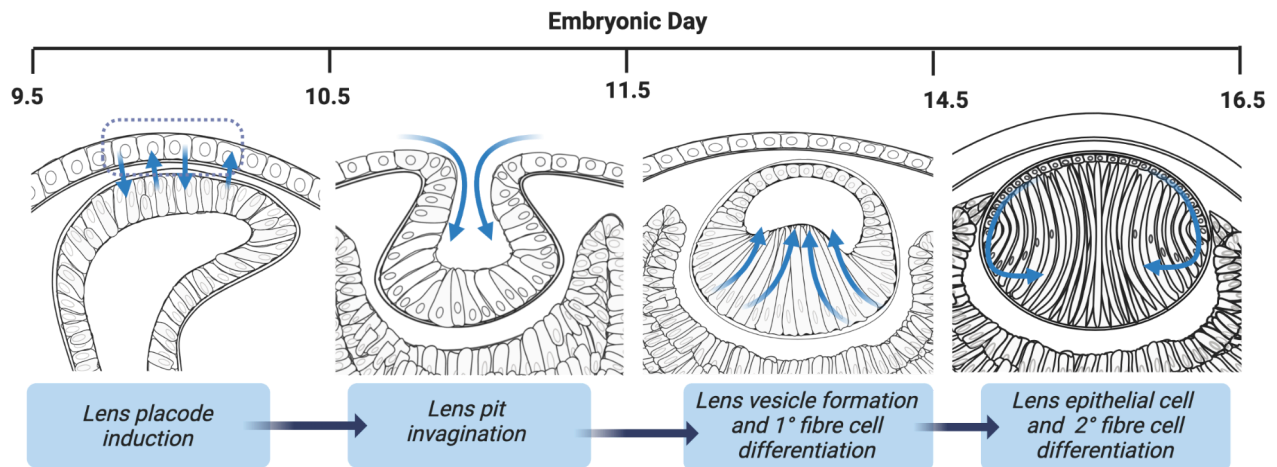


Figure 1.2. Schematic representation of key stages of mouse lens development. Embryonic day (9.5-16.5) relates to days of gestation.

1.1.2 Normal Lens Physiology

The unique architecture of the crystalline lens is characterised by the proliferation of the anterior lens epithelial cells and their differentiation into lens fibre cells (reviewed in Lovicu and McAvoy, 2005, Figure 1.4). As all differentiated lens cells (both epithelial and fibre cells) are derived from the lens epithelium, these cells can be considered as a ‘stem cell’ population, precursors responsible for all ongoing lens growth. A notable feature of the lens epithelium is that unlike most other epithelia in the human body, the lens cells continue to divide and differentiate throughout life (Bhat 2001). Lens growth is dependent on the proliferation of epithelial cells above the lens equator, in the germinative zone, and their subsequent differentiation into fibre cells at the transitional zone (Dawes et al. 2014, Mochizuki et al. 2014, Figure 1.3). This fibre differentiation is characterised by distinct molecular and morphologic changes, such as exit from the cell cycle, cell elongation, loss of cytoplasmic organelles

including nuclei, as well as the accumulation of fibre-specific crystallin proteins (Wride, 2011, Figure 1.4).

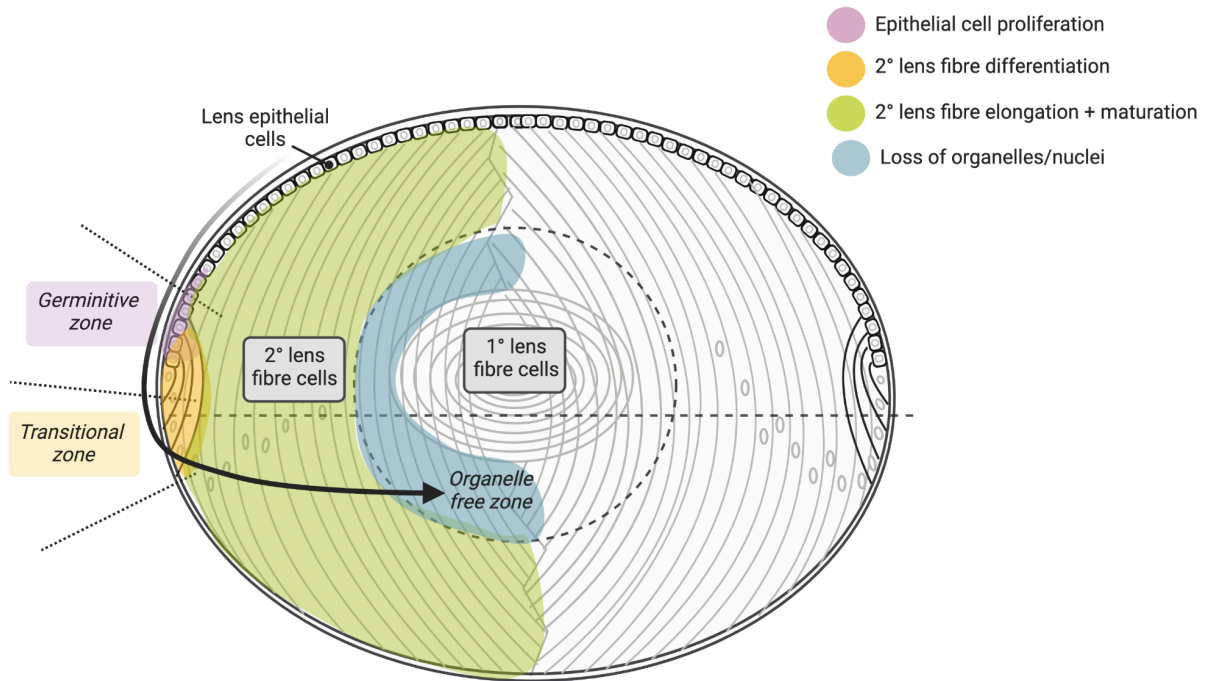


Figure 1.3. Schematic representation of functional regions of the lens.

1.2 Growth Factor Regulation of Lens Development and Homeostasis

The refined architecture and well characterised biology of the vertebrate lens makes it an excellent model to study how basic cellular processes are coordinated, through the specific activity and interplay of a complex array of signalling pathways regulated by native ocular growth factors (Lovicu and McAvoy, 2005, Lovicu et al. 2011). Growth factor activation of associated receptor tyrosine kinases (RTKs) that are found on the surface of the lens cells initiates signal transduction and activation of downstream effector pathways, subsequently leading to the induction of transcription factors responsible for the induction or inhibition of each

of the lens cellular responses. The interplay and tight control of these signalling pathways by both intrinsic and extrinsic mechanisms maintains the vertebrate lens throughout life (Lovicu et al. 2011).

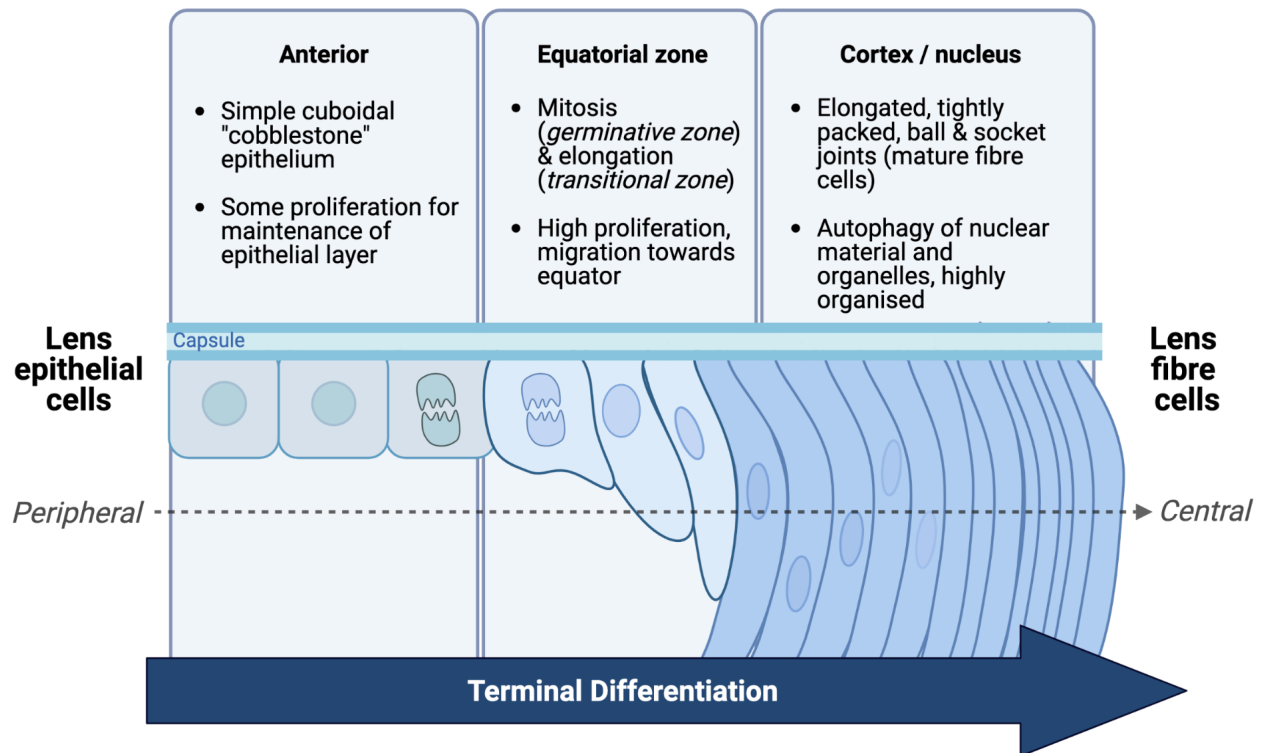


Figure 1.4. Terminal differentiation of lens epithelial cells to lens fibre cells.

1.2.1 The ocular environment

Pioneering research conducted in the 1960's initially illuminated the significant influence of the surrounding ocular milieu in establishing the distinctive polarity of the lens (Coulombre and Coulombre 1963). These investigations involved the surgical inversion of the lens in embryonic chicks, resulting in a repolarization phenomenon. The lens epithelium, now exposed to the posterior compartment of the eye and immersed in vitreous humour, underwent elongation as the

cells differentiated into fibre cells. Conversely, the anteriorly displaced primary fibre cells halted growth, and were subsequently enveloped by a nascent epithelial monolayer originating from the lens equator (Coulombre and Coulombre 1963). This led to the hypothesis that certain elements in the ocular environment were instrumental in triggering and sustaining the typical antero-posterior patterns of cellular proliferation, and fibre differentiation observed throughout the lifespan of the vertebrate lens.

Decades of research since then have identified the elements within the ocular environment responsible for controlling lens cell growth, and their individual and collective impact on lens cell behaviour (Lovicu et al. 2011). The aqueous and vitreous humours, bathing the anterior and posterior of the lens respectively, are a major source of mitogens and regulatory factors, including members of the fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), epidermal growth factor (EGF), bone morphogenetic protein (BMP), Wingless (Wnt), and transforming growth factor beta (TGF- β) families. Receptors for many of these growth factors are found on lens epithelial and fibre cells (Lovicu et al. 2005). The specific mitogens and morphogens comprising each ocular humour differ in makeup and concentration, and lead to different outcomes in exposed lens cells (McAvoy and Chamberlain, 1989, Ibaraki et al. 1995, Iyengar et al. 2009). The aqueous, acting primarily on the anterior lens epithelium, induces the survival and proliferation of this cell population (Iyengar et al. 2009). The vitreous, on the other hand, induces the terminal differentiation of lens fibre cells (Wang et al. 2010, Figure 1.4). Many of the growth factors present in both the aqueous and vitreous humours primarily transmit signals through the MAPK/ERK and PI3K/AKT pathways (Iyengar

et al. 2009). These signalling pathways are essential for both aqueous-induced lens epithelial cell proliferation and vitreous-induced lens fibre differentiation (Lovicu et al. 2001).

1.2.2 *Ocular Growth Factors*

Decades of research have identified key ocular molecules and characterised their differential and complementary roles in the coordination of specific morphogenetic events and cell behaviours. For instance, in vitro studies have shown that growth factors native to aqueous humour promote the mitogenic activity of the anterior lens epithelial cells (Iyengar et al. 2009), while posterior to the lens equator, factors in the vitreous humour induce the terminal differentiation of lens epithelial cells to secondary fibre cells (Lovicu and McAvoy, 2005, Wang et al. 2010). These diffusible native factors are secreted primarily from the retina and ciliary body, and include: FGF (Lovicu and McAvoy, 2005), IGF (Chamberlain et al. 1991, Shirke et al. 2001), EGF (Gospodarowicz et al. 1978, Majima, 1995), PDGF (Brewitt and Clarke, 1988, Kok et al. 2002, Li et al. 2019a), hepatocyte growth factor (HGF) (Choi et al. 2004, Tian, 2014), vascular endothelial growth factor (VEGF) (Saint-Geniez et al. 2009), ephrins (Cheng and Gong, 2011), as well as other non-RTK-binding morphogens, including BMPs (Boswell et al. 2008), TGF- β (de Iongh et al. 2001, Dawes et al. 2009) and Wnts (Lyu and Joo, 2004, Dawes et al. 2013). These factors traverse the ocular milieu to reach their target cells and act in concert to potentiate and regulate lens cell behaviour (i.e. differentiation, proliferation, migration and cell death), sometimes, in a concentration dependent manner, as reported for FGF (Schulz et al. 1993, Lovicu and McAvoy, 2001, Iyengar et al. 2006, 2007, Wang et al. 2009).

1.2.3 *Growth factor signalling in the lens*

Growth factors play a fundamental role in the development and maintenance of the lens. During embryogenesis, the tightly controlled spatial and temporal modulation of the expression of these growth factors regulates the induction, and antero-posterior patterning of the developing lens (Reneker and Overbeek, 1996, Faber et al. 2002). Overall, the role of growth factors in lens development is a complex and multi-faceted process. They are crucial for the normal development of the lens and any aberration in their signalling pathways can lead to lens abnormalities.

The importance of a better comprehension of the universally conserved signal transduction mechanisms that control cell behaviour for the understanding of normal cell biology and pathology is becoming increasingly evident. Crucial regulators of cell behaviour include the signalling pathways triggered by the binding and activation of receptor tyrosine kinases, such as mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK) and phosphoinositide-3-kinase–protein kinase B (PI3K/AKT)-signalling, which have been thoroughly studied (see Lovicu and McAvoy, 2001). The prevailing paradigm posits that the ligand binding to its high-affinity receptor induces trans- and/or autophosphorylation of the receptor's intracellular domains, thereby activating one or more distinct pathways. In the lens, growth factor-RTK signalling has been demonstrated to trigger various downstream pathways, including Ras/Raf/MEK/ERK (Lovicu and McAvoy 2001, Iyengar et al. 2009, Qin et al. 2017), PI3K/AKT (Lovicu and McAvoy 2001, Iyengar et al. 2009, Qin et al. 2017), Wnt/ β -catenin (Stump et al. 2003), and Hippo/YAP (Dawes et al. 2018), among others. The pathways activated can be influenced by several factors, such as growth factor concentration and receptor (including

co-receptor) availability (Qin et al. 2017). Lens-specific studies have verified that ocular growth factors activate these canonical pathways to induce lens cell proliferation and/or fibre differentiation (Lovicu and McAvoy 2001, Stump et al. 2003, Iyengar et al. 2009, Qin et al. 2017, Dawes et al. 2018).

Multiple growth factors are implicated in the initiation and regulation of lens cell proliferation and differentiation. For example, *in vitro* studies suggest that there is no one specific mitogen in the aqueous humour that regulates lens cell proliferation (Iyengar et al. 2009), although FGF is believed to play a pivotal role (McAvoy et al. 1991, Robinson, 2006). Moreover, several factors, including FGF, IGF, EGF, PDGF, and BMPs, interact to strengthen the downstream signalling pathways necessary for lens fibre differentiation (Richardson et al. 1993, Faber et al. 2002, Iyengar et al. 2009, Wang et al. 2010, Li et al. 2019).

It appears that lens epithelial cell behaviour is coordinated by the synergistic interactions of numerous ocular growth factors (Lovicu et al. 2005, Wang et al. 2010, Li et al. 2019). Although FGFs essential role in regulating lens epithelial cell proliferation is clear, some redundancy with other mitogens in aqueous-induced lens epithelial cell proliferation has been reported (Iyengar et al. 2009). This redundancy, often observed in other systems (Salmerón-Sánchez and Dalby 2016), likely provides regulatory flexibility and complexity by ensuring optimal activation of key downstream signalling pathways, offering an alternative activation mechanism. Given this complexity, identifying the factors that coordinate these signalling events is critical to our understanding of normal lens development and the lens pathology that arises when these signalling pathways are disrupted.

Although the many distinct and overlapping effects of growth factors on lens cell behaviour are well-characterised, the mechanisms underlying how these proteins travel through the extracellular space, arrive precisely at target cells, and transmit their signalling within cells at the appropriate level, remain elusive. Studies in mice and other model animals have revealed the importance of heparan sulfate proteoglycans (HSPGs) for the signalling and extracellular distribution of many of these different growth factors.

1.3 Heparan sulfate proteoglycans

HSPGs are complex glycoconjugates composed of a core protein “trunk” decorated with covalently attached linear anionic polysaccharide heparan sulfate (HS) glycosaminoglycan (GAG) chains (“branches”) (Figure 1.5). HSPGs are a component of all known cells, and can be localised to the cell surface, intracellularly in the cytoplasm and nucleus, or secreted by cells into the extracellular space where they form a major component of basement membranes and the extracellular matrix (ECM) (Sarrazin et al. 2011). Until relatively recently, our understanding of HSPGs was limited to their role as essential matrix components, lending structural stability to basement membranes. As our understanding of proteoglycans has expanded, the exciting discovery was made that HSPGs can bind to and regulate a variety of proteins, including growth factors, chemokines, matrix proteins and intracellular components (e.g. FGFs, integrins, laminin, fibronectin, collagens) (Bishop et al. 2007, Vallet et al. 2021). The negatively charged sulfate (SO_4^-) groups of HS-GAG chains electrostatically interact with positively charged lysine/arginine-rich regions of many proteins, influencing their structure and activity (Billings and Pacifici, 2015). Importantly, the interactions of growth factors with native HSPGs influences

their receptor binding and signalling activation (Park et al. 2000, Forsten-Williams et al. 2008, Yu et al. 2018), gradient formation (Yan and Lin, 2009, Matsuo and Kimura-Yoshida, 2014), potentiates their signalling activity (Dreyfuss et al. 2009) and protects them from degradation (Sun et al. 2019) (See Figure 1.6).

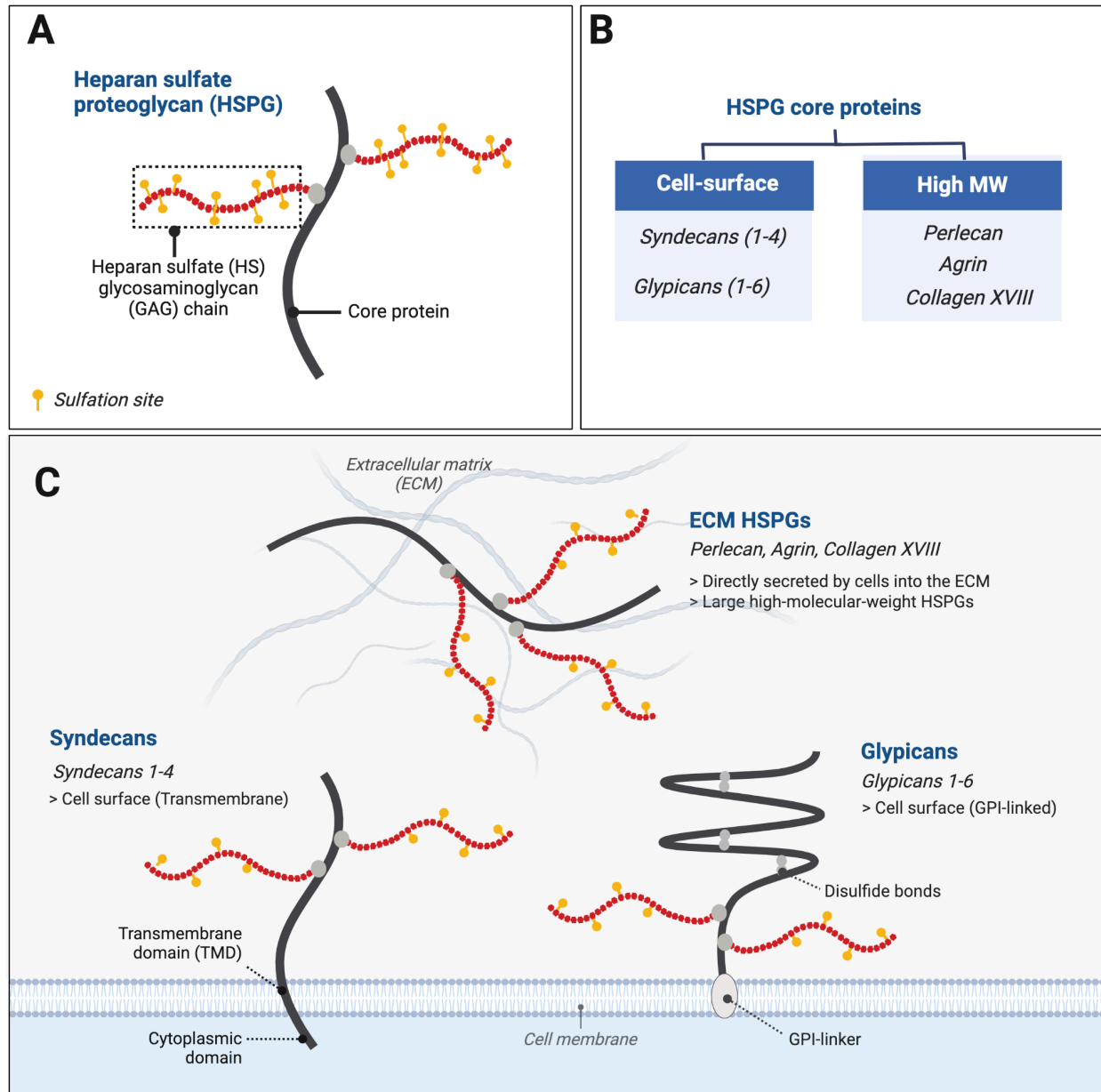


Figure 1.5. Heparan sulfate proteoglycans (HSPGs). (A) General structure of a heparan sulfate proteoglycan (HSPG). (B) HSPG core protein families. (C) Schematic representation and general localisation of HSPG families.

glomerular basement membrane (Rossi, 2003), and overexpression of agrin in transgenic mice causes a dysgenic phenotype that is specific to the eye (Fuerst et al. 2007).

The localisation of HSPG core proteins can provide valuable information about their functional roles in a specific tissue. Immunolabelling 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 neural precursor cells, identified these HSPGs as key regulators of mitogens in early brain development (Ford-Perriss et al. 2003). The specificity of these HSPG-protein interactions not only depends on the core protein type (e.g. syndecan, glypican), and their localisation (e.g. the cell surface or ECM), but also on the fine structure (i.e. sulfation) of the HS chains that associate with HSPGs.

At the cell surface, HSPGs bind growth factors and their cognate receptors to enhance formation of receptor-signalling complexes, as well as immobilise and regulate the turnover of ligands that act at the cell surface (Bishop et al. 2007, Figure 1.6). The pleiotropic modulation of growth factor receptors by HSPGs can control different outcomes from the same growth factor-signalling interaction, by altering ligand density, period of activation to say the least. HSPGs can also directly and indirectly influence intracellular signalling cascades, leading to the activation of key effector genes, changes in cell behaviour as well as cell phenotype (Bishop et al. 2007, Figure 1.6). Within the ECM, HSPGs can bind to factors to regulate their bioavailability at the cell surface and stabilise dynamic mitogen and morphogen gradients locally at the tissue level that is critical for organogenesis and the maintenance of tissue homeostasis (Figure 1.6). HSPGs also regulate the binding and activation of several proteins required for

cell-matrix interactions (e.g. integrins, laminin, collagens), thus regulating cell attachment, migration, transdifferentiation and polarity (Iozzo, 2005). Through modulation of the binding and activation of these key proteins, HSPGs are positioned as master regulators of the physiology, development and pathophysiology of many tissues (Bishop et al. 2007), not least in the eye (Puri et al. 2020).

1.3.1 Expression and localisation of HSPGs

HS-GAGs are present in every ocular tissue in cell-, tissue- and developmental stage-specific patterns (Sarrazin et al. 2011). Pioneering sulfate-radiolabelling studies revealed that ocular cells incorporate highly anionic-GAG species in prominent ocular basement membranes including the lens capsule (Young and Ocumpaugh, 1966), Bruch's membrane (Hewitt et al. 1989) and Descemet's membrane (Davies et al. 1999), later expanding to many other ocular cell types.

1.3.1 Functional roles of different HSPGs

Not surprisingly given the extent of their expression, diverse roles for HSPGs have been described in the regulation of multiple key lens signalling molecules and developmental processes. By mediating the binding of many extracellular ligands to their receptors, HSPGs trigger a cascade of reactions that subsequently regulate various cell processes including proliferation, differentiation, adhesion, migration, cell survival, and matrix assembly and organisation (Bishop et al. 2007). HSPGs can bind to and regulate the activity of PDGF, for example, that is essential for lens growth and transparency (Brewitt and Clark, 1988) through the regulation of lens epithelial cell proliferation (Iyengar et al. 2009) and fibre differentiation (Li et al. 2019). With the diverse structure and overlapping temporal and spatial distribution of the

different HSPGs in many tissues, it does not easily allow us to delineate their precise roles and activity. While mouse models with deficiencies in different HSPGs provide some insights, without compound knockouts to account for any redundancy, it is difficult to ascertain the precise role of any one specific HSPG core protein type. Here, we have introduced different HSPG members in the context of what is currently known regarding their functional roles in the lens and its surrounding tissues.

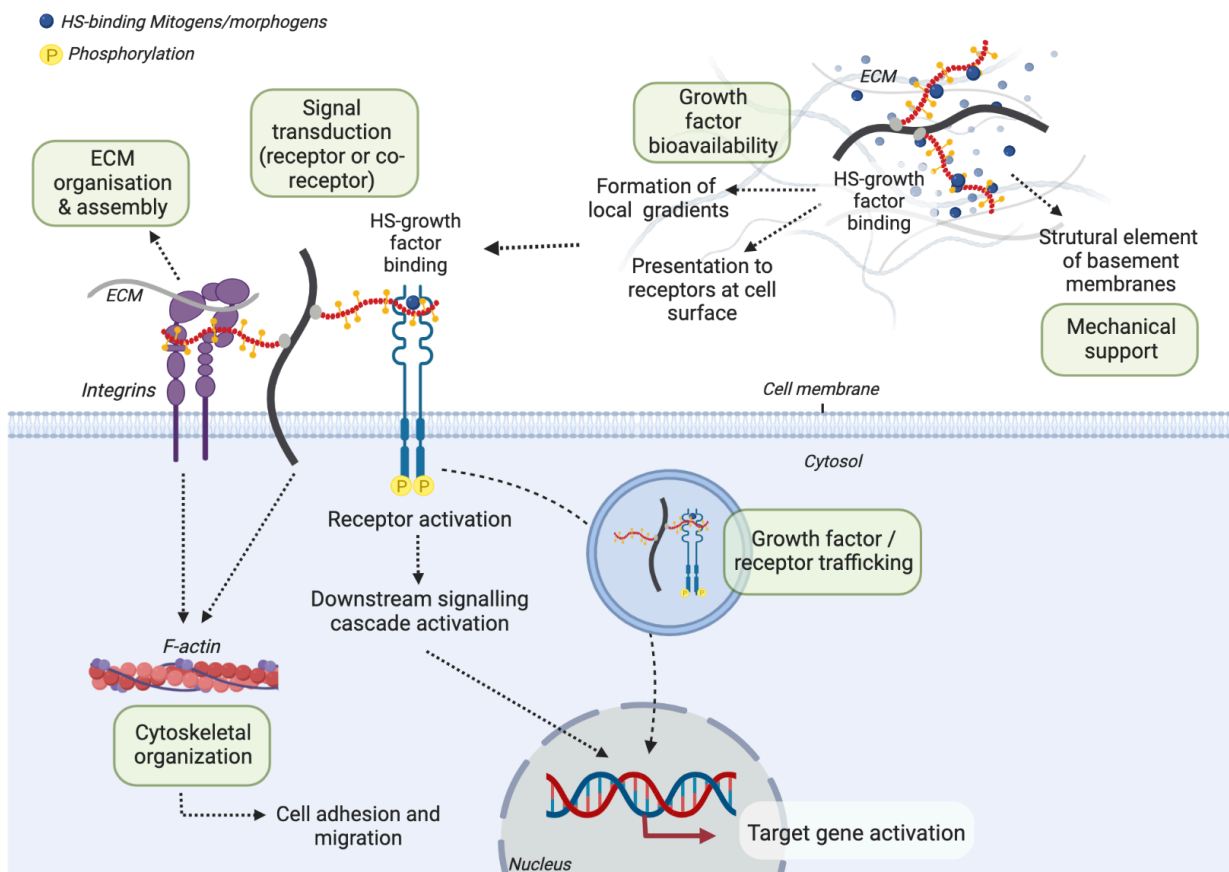


Figure 1.6. Common functional roles of HSPGs. Abbreviations: extracellular matrix (ECM).

1.4 HSPG core proteins

1.4.1 The Syndecans

The syndecan family of HSPGs (Figure 1.7) are a unique and highly versatile family of four, syndecan-1 (syndecan, CD138), syndecan-2 (fibroglycan, CD362), syndecan-3 (N-syndecan, neuroglycan), and syndecan-4 (amphiglycan, ryudocan), primarily localised to the cell surface, mediating numerous cellular processes through signalling pathways that control cell proliferation, migration, mechano-transduction and endocytosis (Bernfield, 1992, Couchman et al. 2015, Afratis et al. 2017). Common to all syndecans is a highly conserved type-I transmembrane domain (TMD) and extracellular domains, that function to target the syndecans to sites at the cell surface and pericellular matrix where they collaborate with other proteins to regulate growth factor signalling and cytoskeletal organisation (Rapraeger, 2001). The intracellular domain of the syndecan core protein comprises two “conserved” domains that flank a short “variable” region that binds cytosolic regulatory factors and uniquely defines the intracellular signalling interactions of each syndecan family member (Schaefer et al. 2010). While all syndecans have HS-GAG chains that mediate their interactions with extracellular signalling molecules, syndecans-1 and -3 may also carry CS-GAGs (Rapraeger et al. 1985). While the functions of each syndecan family member are distinct, there is higher structural and functional similarity between syndecans-1 and -3 (Afratis et al. 2017), and similarly between syndecans-2 and -4 (Oh, 2004).

All syndecans have been identified as essential regulators of the signalling of several key ocular growth factors and morphogens, including FGFs (Shia et al. 2003, Zong et al. 2009, Jang et al.

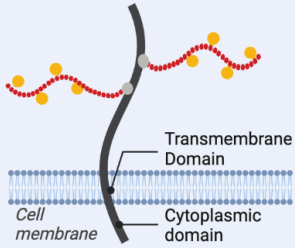
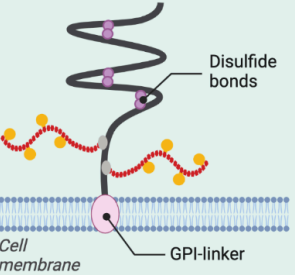
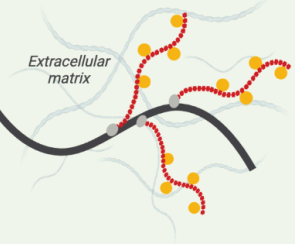
2012, Qin et al. 2017), IGFs (Ruiz et al. 2012), EGFs (Dobra et al. 2003, Ibrahim et al. 2017), PDGFs (Das et al. 2016), HGF (Seidel et al. 2000), BMPs (Fisher et al. 2006), Wnts (Pataki, 2015) and Ephrins (Ethell et al. 2001). Moreover, syndecans can influence the downstream signalling pathways of these molecules, including the activation of MAPK/ERK (Bellin et al. 2009, Qin et al. 2017) and PI3K/AKT/ mammalian target of rapamycin (mTOR) (Partovian et al. 2008, Qin et al. 2017) signalling. In addition to their role as receptors and co-receptors, many other studies have suggested that signalling through the core protein of syndecans can regulate cytoskeletal organisation through their clustering, association with cytoskeletal structures, binding to cytoplasmic binding proteins, and intracellular phosphorylation (Yoneda and Couchman, 2003). For instance, the cytoplasmic domains of their core proteins can interact directly with cytoskeletal proteins (e.g. α -tubulin, cortactin, α -actinin), GTP-ases (RhoA, Rac1), kinases (protein kinase C- α , PKC α , Src kinase, calcium/calmodulin-dependent serine protein kinase, CASK, focal-adhesion kinase, FAK), cytoplasmic ERM proteins ezrin/radixin/moesin) and other cell-surface receptors (integrins) to directly influence cell architecture, adhesion and motility (Afratis et al. 2017).

Mutations in syndecans (Table 1.1) have revealed critical roles in development in lower vertebrates (Kuriyama and Mayor, 2009, Muñoz et al. 2006, Olivares et al. 2009), with similar roles described in mammals, however, mutant mice with compromised syndecans-1, and -4, do not show any gross developmental abnormalities (Echtermeyer et al. 2001, Gougoula et al. 2019). Syndecan-3 null mice have been described to have some abnormalities in their memory (Kaksonen et al. 2002), locomotion (Cornelison et al. 2004) and metabolism (Dickinson et al. 2016). Following these reports, it was suggested that the syndecans may be functionally

redundant in their regulatory roles, however, recent closer characterisation of these syndecan-deficient animals has revealed abnormalities at the molecular level, suggesting distinct and separable roles for different syndecan family members. For instance, syndecan-3 deficiency impairs neural migration in the brain (Hienola et al. 2006), and explanted satellite cells from *Sdc3* null mice display aberrant differentiation, with a general increase in overall tyrosine phosphorylation (Cornelison et al. 2004). A role for syndecan-3 in lens fibre cells may relate to its association with SRC kinases and cortactin (Filla et al. 2004, Kinnunen et al. 1998) that regulate fibre differentiation in concert with Ephrin signalling (Cheng et al. 2013) which is required for the phosphorylation of the syndecan-3 core protein (Asundi and Carey, 1997). Mice deficient in *Sdc4* have delayed wound healing, angiogenesis and FGF-2/integrin signalling (Echtermeyer et al. 2001, Morgan et al. 2007, Qin et al. 2017). In the lens, syndecan-4 is an essential regulator of FGF-2 and integrin signalling (Qin et al. 2017), acting as a FGFR co-receptor, strengthening the duration and intensity of downstream FGF-induced MAPK/ERK signalling, that has been shown to be required for lens fibre differentiation (Lovicu and McAvoy, 2001, Iyengar et al. 2007, Wang et al. 2010).

Integrin signalling is key in several lens processes in morphogenesis and for lens maintenance throughout life (Walker and Menko, 2009). In particular, $\beta 1$ -integrin-signalling that has been shown to be regulated by syndecans-2 and -4 (Couchman and Woods, 1999, Filla et al. 2006, Kusano et al. 2000) and potentially other syndecans (Morgan et al. 2007), is essential for maintenance of the lens epithelium and for the structural maintenance and homeostasis of differentiating fibre cells (Simirskii et al. 2013). Activation of the canonical fibronectin receptor $\alpha 5 \beta 1$ -integrin requires a HSPG co-receptor such as syndecan and involves the activation of

protein kinase C α (PKC α). In syndecan-4-deficient murine lenses or with downregulation of syndecan-4 in human lens epithelial cells *in vitro*, this suppresses β 1 integrin-mediated activation and cell adhesion (Qin et al. 2017).

HSPG Family		Core Protein
Syndecans Type-I transmembrane proteins		Syndecan-1*
		Syndecan-2
		Syndecan-3*
		Syndecan-4
Glypicans GPI-anchored		Glypican-1
		Glypican-2
		Glypican-3
		Glypican-4
		Glypican-5
		Glypican-6
High Molecular Weight HSPGs Large high molecular weight HSPGs		Perlecan (HSPG2)*
		Agrin*
		Collagen XVIII / endostatin

*may also carry chondroitin sulfate

Figure 1.7. HSPG core protein families.

Other novel syndecan-associated molecules include equarin, that was first identified as a secreted molecule using a signal sequence trap screen in chick and shown to be specific to the lens (Mu et al. 2003), in both chick and mice (Song et al. 2012). Consistent with its co-expression with lens fibre-specific β -crystallin (Mu et al. 2003), it is thought to complex with syndecan-3 HS chains to regulate FGF-induced lens fibre differentiation and cell-adhesion, with its loss abrogating lens fibre cell markers (Song et al. 2013).

1.4.2 The Glypicans

The glypican family (Figure 1.7) is composed of six HSPGs (glypicans-1 to -6) that form two subgroups, with glypicans-3/5 and glypicans-4/6 respectively sharing high homology (De Cat, 2001). Glypicans only carry HS-GAG chains, and each is linked to the cell surface via a GPI anchor (Sarrazin et al. 2011). With the exception of glypican-3, that has been extensively characterised in its role in the pathogenesis of hepatocellular carcinoma and other cancers (Zhou, 2018), the glypicans are arguably the least-studied of all the HSPGs, and their precise roles remain elusive.

In *Drosophila*, glypican homologues, division abnormally delayed (Dally) and dally-like (Dlp), have been implicated as key orchestrators of cell polarity, morphogenesis, patterning, differentiation and growth during development, through their regulation of growth factor, cytokine, and morphogen signalling (Lin and Perrimon, 1999, Khare and Baumgartner, 2000, Williams et al. 2010, Zhang et al. 2013). Dally has been reported to be required for ordered cell division in the eye (Nakato et al. 1995), with dally mutants showing defects in progression through G2-M for specific sets of dividing cells. A number of studies have demonstrated that

Dally serves as a regulator of growth factor signalling during development, affecting the activity of Wingless (Wg, Wnt homologue, Lin and Perrimon, 1999, Khare and Baumgartner, 2000, Waghmare et al. 2020), Decapentaplegic (Dpp, TGF- β / BMP homologue, Jackson et al. 1997, Akiyama et al. 2008) and Hedgehog (Williams et al. 2010, Ayers et al. 2012) in a tissue-specific manner, consistent with Dally serving as a molecule that promotes the assembly of discrete signalling complexes on the cell surface, as we see for HSPGs. The interplay between secreted morphogens Wingless (Wg), Dpp and Hh regulates important events in *Drosophila* eye development, controlling tissue specification, growth, and patterning (Ma and Moses, 1995, Chang et al. 2001, Won et al. 2015). Similarly, in the vertebrate eye, their homologues Wnt (Fujimura, 2016), TGF- β /BMP (Furuta and Hogan, 1998, Wawersik et al. 1999, Flügel-Koch et al. 2002,) and Hh (Kerr et al. 2012) are major players in eye development.

Studies using transgenic animals are beginning to clarify the key roles of glypicans as cell signalling regulators (See Table 1.1). Glypicans can negatively regulate Wnt signalling by competitively binding to secreted Wnt proteins, controlling their bioavailability to cell surface Frizzled receptors as a co-receptor (Sakane et al. 2012, Wang et al. 2016, Li et al. 2019b, Shi et al. 2020). Glypican-1 and -3 can act as both positive and negative regulators of canonical Wnt signalling (Stigliano et al. 2009, Shiau et al. 2010, Capurro et al. 2014). Expression of *Gpc4* enhanced the Wnt3a-dependent β -catenin pathway and the Wnt5a-dependent β -catenin-independent pathway, while knockdown of *Gpc4* suppressed both pathways (Sakane et al. 2012). *Gpc3* expression is also correlated with nuclear/cytoplasmic localisation of β -catenin (Akutsu et al. 2010), however, its HS chains were reported not to be required for its stimulatory activity on Wnt5a signalling and for the formation of glypican-3-Wnt5a complexes in mice

(Song et al. 2005), highlighting HS-chain independent regulation. Glypican-3 knockout mice exhibited alterations in Wnt signalling associated with the regulation of cell proliferation, the loss of glypican-3 inhibited non-canonical Wnt/JNK signalling, with concomitant activation of canonical Wnt/beta-catenin signalling and increased mitotic activity (Song et al. 2005). Contrary to these studies, glypican-3 can directly bind to Frizzled through its HS-GAG chains, consistent with Wnt binding at the cell membrane triggering the endocytosis of a complex that includes Wnt, Frizzled and glypican-3 (Capurro et al. 2014).

Glypicans also play an important role in orchestrating growth factor signalling to modulate proliferation and cell growth (Filmus and Capurro, 2008, Qiao et al. 2008, Cano-Gauci et al. 1999). For example, glypican-1 deficiency leads to reduced brain size associated with loss of developmental FGF signalling (Jen et al. 2009). Modulation of glypican-1 levels by expression of full-length *Gpc1*, or a truncated form that is thought to act in a dominant-negative fashion, prevents trigeminal placodal differentiation or proper ganglion formation, respectively, with particularly strong effects on the ophthalmic lobe (Shiau et al. 2010). *Gpc1* overexpression phenocopies Wnt inhibition in causing loss of ophthalmic placodal neurons, and increased Wnt activity can rescue the effects of *Gpc1* overexpression (Shiau et al. 2010).

Table 1.1 *HSPG mutant phenotypes.*

Core protein/ gene	Phenotype
Syndecan-1 <i>Sdc1</i>	<i>Sdc1</i> -deficiency causes defects in cell proliferation, migration, and regulation of integrin expression in corneal epithelial cells (Stepp et al., 2002), inflammation-mediated corneal angiogenesis (Gotte et al., 2002), and resistance to corneal infection (Hayashida et al., 2011, 2015, Jinno et al., 2020).
Syndecan-2 <i>Sdc2</i>	<i>Sdc2</i> -deficiency causes (endothelial-cell specific) delayed retinal vascular development (inhibition of VEGFA ₁₆₅ -signalling and angiogenesis) (Corti et al., 2019). <i>Sdc2</i> ^{-/-} (<i>Sdc2</i> ^{tm1b(KOMP)Wtsi}) mice show abnormal lens morphology/cataract (Dickinson et al., 2016).
Syndecan-3 <i>Sdc3</i>	<i>Sdc3</i> -deficiency causes impaired locomotion, fibrosis, and hyperplasia, aberrant differentiation and increase in overall tyrosine phosphorylation in muscle cells (Cornelison et al., 2004), impaired hippocampus-dependent memory (Kaksonen et al., 2002).

Syndecan-4 <i>Sdc4</i>	<i>Sdc4</i>-deficiency causes increased ocular pathological angiogenesis and decreased retinal neovascularization. <i>Sdc4</i>^{-/-} mice (<i>Sdc4</i>^{tm2b(KOMP)Wtsi}/<i>Sdc4</i>⁺) show abnormal retinal blood vessel morphology, mydriasis (Dickinson et al., 2016), and tissues do not respond to VEGF-A with abnormal VE-Cadherin trafficking (De Rossi et al., 2021). <i>Sdc4</i>^{-/-} mice have delayed wound healing associated with decreased cell proliferation/migration (Echtermeyer et al., 2001, Corti et al., 2013). <i>Sdc4</i>^{-/-} mice show reduced capsular opacification after injury (Qin et al., 2017). Downregulation blocks FGF-signalling and integrin-mediated cell adhesion/migration (Qin et al., 2017).
Glypican-1 <i>Gpc1</i>	<i>Gpc1</i>^{-/-} mice (<i>Gpc1</i>^{em1(IMPC)Mbp}/<i>Gpc1</i>^{em1(IMPC)Mbp}) have an abnormal eye phenotype (Dickinson et al., 2016). Overexpression of <i>Gpc1</i> in the chick trigeminal placodal ectoderm blocks proliferation and differentiation and can lead to the loss of the ophthalmic trigeminal ganglion (Jen et al., 2009).
Glypican-2 <i>Gpc2</i>	No reported eye phenotype for <i>Gpc2</i> deficiency (Dickinson et al., 2016)
Glypican-3 <i>Gpc3</i>	Despite Wnt and JNK signalling being altered in other <i>Gpc3</i>^{-/-} tissues (Song et al., 2005), no eye phenotype has been reported (Cano-Gauci et al., 1999, Chiao et al., 2002)
Glypican-4 <i>Gpc4</i>	No reported eye phenotype associated with <i>Gpc4</i>-deficiency (Amor et al., 2019, Dowling and Allen 2018). GPC4 variants associated with Keipert Syndrome (Amor et al., 2019).

Glypican-5 <i>Gpc5</i>	There is no reported eye phenotype associated with a <i>Gpc5</i>-deficiency (Joslyn et al., 2011, Okamoto et al., 2015).
Glypican-6 <i>Gpc6</i>	<i>Gpc6</i>-deficiency causes neonatal lethality, with no reported eye phenotype (Campos-Xavier et al., 2009, Capurro et al., 2017), however, reduced <i>GPC6</i> may be linked to sporadic retinoblastoma (Lau et al., 2010).
Perlecan <i>Hspg2</i>	<i>Hspg2</i>-deficiency is embryonic lethal and causes basement membrane malformations (Arikawa-Hirasawa et al., 1999), microphthalmia and thin corneal epithelium associated with decreased differentiation (Inomata et al., 2012). Pathological variants of <i>Hspg2</i> are linked to Schwartz-Jampel Syndrome (where Type 1 is associated with cataract) (Stum et al., 2006). HS-deficient perlecan (<i>Hspg2</i>Δ3/Δ3) results in an eye-specific phenotype with lens degeneration, corneal abnormalities, impaired corneal angiogenesis, delayed wound healing and microphthalmia (Rossi, 2003, Zhou et al., 2004).
Agrin <i>Agrn</i>	<i>Agrn</i> deficiency in zebrafish causes microphthalmia, retinal mis-differentiation, abnormal retinal lamination, optic nerve hypoplasia, delayed regression of retinal blood vessels, lack of or abnormal outgrowth of retinal vessels, reduced size of the optic fibre layer, and disruption of retinal ganglion cell axon growth to the optic tectum (Kim et al., 2003, Kim et al., 2007, Liu et al., 2008). <i>Agrn</i> overexpression causes anophthalmia, persistence of vitreal vessels, retinal

	mis-differentiation and fusion of anterior chamber structures (Fuerst et al., 2007).
Collagen XVIII <i>Coll8a1</i>	<i>Coll8a1</i> deficiency causes widespread anterior and posterior eye defects including lens subluxation, macular degeneration, ciliary body atrophy, iris fragility, delayed regression of retinal blood vessels, abnormal retinal vasculature (Fukai et al., 2002), attenuated visual function associated with impaired retinal pigmented epithelium function that worsens with age (Marneros and Olsen 2003, Marneros et al., 2004). Mutations of <i>Coll8a1</i> are associated with Knobloch syndrome (severe myopia, vitreoretinal degeneration, anterior eye defects, occipital encephalocele) (Passos-Bueno et al., 2006, Khan et al., 2012). Overexpression of <i>Coll8a1</i> (endostatin domain) can cause cataract (Elamaa et al., 2005, Aikio et al., 2013).

1.4.3 *Perlecan*

Perlecan is a large multidomain high-molecular weight HSPG that is a ubiquitous component of cellular basement membranes and extracellular matrices (Iozzo 2005, Whitelock et al. 2008), dynamically regulated throughout vertebrate development (Handler et al. 1997). Perlecan plays an important role in controlling the bioavailability of growth factors at the cell surface, and in the formation and orchestration of mitogenic and morphogenetic gradients (Gubbiotti et al. 2017). It can bind to and influence the signalling activity of other mitogens and morphogens including FGFs (Knox et al. 2002), BMPs (Decarlo et al. 2012), Hh (Palma et al. 2011) and EGF (Zoeller

et al. 2009). Perlecan and matrix-bound FGF-2 are co-expressed in the lens capsule in a complimentary antero-posterior gradient, with the levels of both, highest at the lens equator correlating with proliferating lens epithelial cells and phospho-ERK1/2 labelling (Wu et al. 2014).

In zebrafish, perlecan helps form a VEGF gradient, and the regulation of VEGF-VEGFR plays an important role in developmental angiogenesis (Zoeller et al. 2009), potentially explaining why perlecan (*Hspg2*)-deficient mice are embryonic lethal. In mice lacking exon 3 of perlecan (*Hspg2*^{Δ3/Δ3}), that results in loss of HS side chain attachment to its core protein (Rossi, 2003), mice are viable but present with microphthalmia. In these *Hspg2*^{Δ3/Δ3} mutant mice, the lens capsule was compromised by cell remodelling that led to its rupture at birth, and subsequent lens degeneration by weaning age. As mentioned earlier, other perlecan-rich basement membranes in these mice, such as the GBM were intact with normal kidney function (Rossi, 2003). The lens phenotype in *Hspg2*^{Δ3/Δ3} mice was exacerbated with the added loss of collagen XVIII, with lens degeneration proceeding faster. This suggested that the HS chains of perlecan have a structural function in the lens and can readily regulate incoming signals protecting the lens from its environment (Rossi, 2003).

1.4.4 Agrin

Agrin is a large high-molecular weight HSPG that can exist as either a membrane-bound or secreted HSPG (Tsen et al. 1995, Iozzo 2005). It is associated with the cell bodies and processes of retinal neurons and is concentrated at retinal synapses (Fuerst et al. 2007), as it is at neuromuscular junctions where it was first identified as essential for clustering acetylcholine

receptors (Bezakova and Lømo, 2001). The functions of agrin also encompass other important roles in the central nervous system such as regulation of brain development, plasticity, and maintenance of the blood-brain barrier (reviewed in Kröger and Pfister, 2009 and Daniels, 2012). Not surprisingly then, systemic knockout of agrin is embryonic lethal (Gautam et al. 1996), however, its overexpression led to a phenotype specific to the eye (Fuerst et al. 2007). Ocular dysgenesis was correlated with the amount of agrin expressed, with microphthalmia and anophthalmia resulting from fusion of anterior chamber structures, and persistent hyperplastic primary vitreous associated with a failure of foetal fissure closure, coloboma and aberrant formation of the optic stalk (Fuerst et al. 2007).

1.4.5 Collagen XVIII

Collagen XVIII is a high-molecular weight HSPG, predominantly found in the ECM and basement membranes (Iozzo 2005). Variations of the N-terminal domain of Collagen XVIII produce three distinct isoforms (short, medium/intermediate, and long) with distinct activity and tissue distribution (Muragaki et al. 1995, Rehn and Pihlajaniemi, 1995, Saarela et al. 1998). In the eye, the short isoform is localised to the lens capsule and inner limiting membrane of the retina, while the medium/long isoforms are found in the ciliary and iris epithelia, and Bruch's membrane. All isoforms are also present in the *tunica vasculosa lentis* (Ohlmann et al. 2012, Aikio et al. 2013). The C-terminus of all Collagen XVIII proteins contains an anti-angiogenic endostatin domain, and all isoforms have conserved HS-GAG attachment sites (Dong et al. 2003).

Given its varied ocular expression, Collagen XVIII plays a key role in eye formation and homeostasis (Heljasvaara et al. 2017). Mutations of *Coll8a1*, (the gene encoding the collagen XVIII core protein), are linked to Knobloch syndrome, characterised by severe and diverse ophthalmic defects in both anterior and posterior segments (Passos-Bueno et al. 2006, Khan et al. 2012). Similarly, *Coll8a1*^{-/-} mice display widespread ocular dysgenesis and malformation of several eye tissues (Fukai et al. 2002, Ylikärppä et al. 2003, Marneros et al. 2004). Moreover, overexpression of the endostatin domain of Collagen XVIII leads to cataract and ultrastructural abnormalities in basement membranes (Elamaa et al. 2005, Aikio et al. 2013).

Collagen XVIII binds to and regulates the activity of many proteins directly through interactions with its core protein (Rychkova et al. 2005), and via its associated HS-GAG chains (Dong et al. 2003), as reported in the regulation of integrin and Wnt signalling. Collagen XVIII also binds to and interacts with several ECM proteins (e.g. laminin, collagens, nidogen), and plays an important role in maintaining the structural integrity of ocular basement membranes. Collagen XVIII likely plays a distinct role from other major basement membrane HSPGs in ocular basement membrane maintenance, as mentioned earlier lens degeneration due to impaired HS activity of perlecan is exacerbated when Collagen XVIII is also compromised (Rossi, 2003).

1.5 *HS-GAG Chain Structure and Sulfation*

HSPGs have been shown to directly or indirectly regulate most lens mitogens and morphogens, primarily through binding to sulfated domains of their associated HS-GAG chains (Dreyfuss et al. 2009). Therefore, it is not surprising that HS sulfation is required for normal lens development and molecular functions (Pan et al. 2006). To elucidate the biological significance

of HS-GAG chains, animal models together with chemical approaches have been employed to alter or inhibit their biosynthesis or function. As ocular morphogenesis is difficult to evaluate in constitutive mutant animals deficient for HS-GAGs or HS-GAG sulfation, due to defective gastrulation or death at an early stage of embryonic development (Bullock et al. 1998, Lin et al. 1999) or at birth (Fan et al. 2000, Ringvall and Kjellén, 2010), the generation of structurally-defined HS oligosaccharides (Jemth et al. 2003) and phenotypic analyses of animal models with conditional deletions of genes encoding different HS-sulfotransferases (HSTs) (Forsberg and Kjellén, 2001) have proven invaluable in the elucidation of relationships between HS structure and molecular phenotype.

Once specific core proteins are synthesised, GAG chains, including HS are synthesised from cytoplasmic nucleotide sugars and assembled onto core proteins by HS-polymerases, glycosyltransferases, epimerases, and sulfotransferases in the Golgi apparatus (Li and Kusche-Gullberg, 2016) ([Figure 1.8](#)). There is significant heterogeneity in HS chain-link, structure, modification sites, extent of sulfation, electrostatic charge distribution, and epimerization. The “sugar code” hypothesis proposes that the biological activity of HS is encoded in its sulfation patterns (Poulain and Yost, 2015). The heterogeneity of HS chains is due to a series of sulfation modifications of its chains that occur after the initial synthesis of the HS chain precursor, through a non-template driven process that does not necessarily go to completion (Li and Kusche-Gullberg, 2016). The patterning and degree of HS chain sulfation is not predetermined but a result of the isoforms of HS sulfation enzymes expressed by a given cell. This results in modifications of variable size along the chain resulting in highly heterogeneous products with variable sulfation. HS sulfotransferase modifications occur with high specificity

(Zhang et al. 2016b) in a cell-type and developmental stage specific manner (David et al. 1992, Maccarana et al. 1996, Poulain and Yost, 2015) resulting in highly heterogeneous HS chains interspersed with un-sulfated and highly sulfated regions, with differential levels of 2-O-, 6-O- and 3-O-sulfation (Figure 1.8). Furthermore, the specific HS sulfation pattern requirements for ligand interactions vary with the ligand. This is important as variations in the sulfation patterns of HS chains can confer functionally important differences in HS-ligand binding interactions and therefore differential regulation of biological functions (Kato et al. 1994, Pratt et al. 2006). Phenotypes resulting from the conditional and selective depletion of specific HS biosynthesis and sulfation enzymes, or combinations of HS biosynthesis and sulfation enzymes from the developing eye tissues, exemplifies their importance and provides clues that point to the distinct and complementary roles of different HS sulfation and fine structure in the regulation of developmental and biological events.

1.5.1 HS-GAG Chain Polymerisation (EXTs)

HS chains are composed of repeating units of glucuronic acid (GlcA)-N-Acetylglucosamine (GlcNAc) synthesised by HS-polymerases Exostosin-1 and -2 (EXT1/EXT2) (Busse et al. 2007). This step is referred to as “chain extension” and determines the length of the GAG chain (Li and Kusche-Gullberg, 2016) (Figure 1.8). The number of disaccharide repeats, and therefore the length of the chain, are variable, and clearly important for growth factor signalling. For example, HS-deficient Ext2 null mutant mice embryos fail to respond to FGF (Shimokawa et al. 2011).

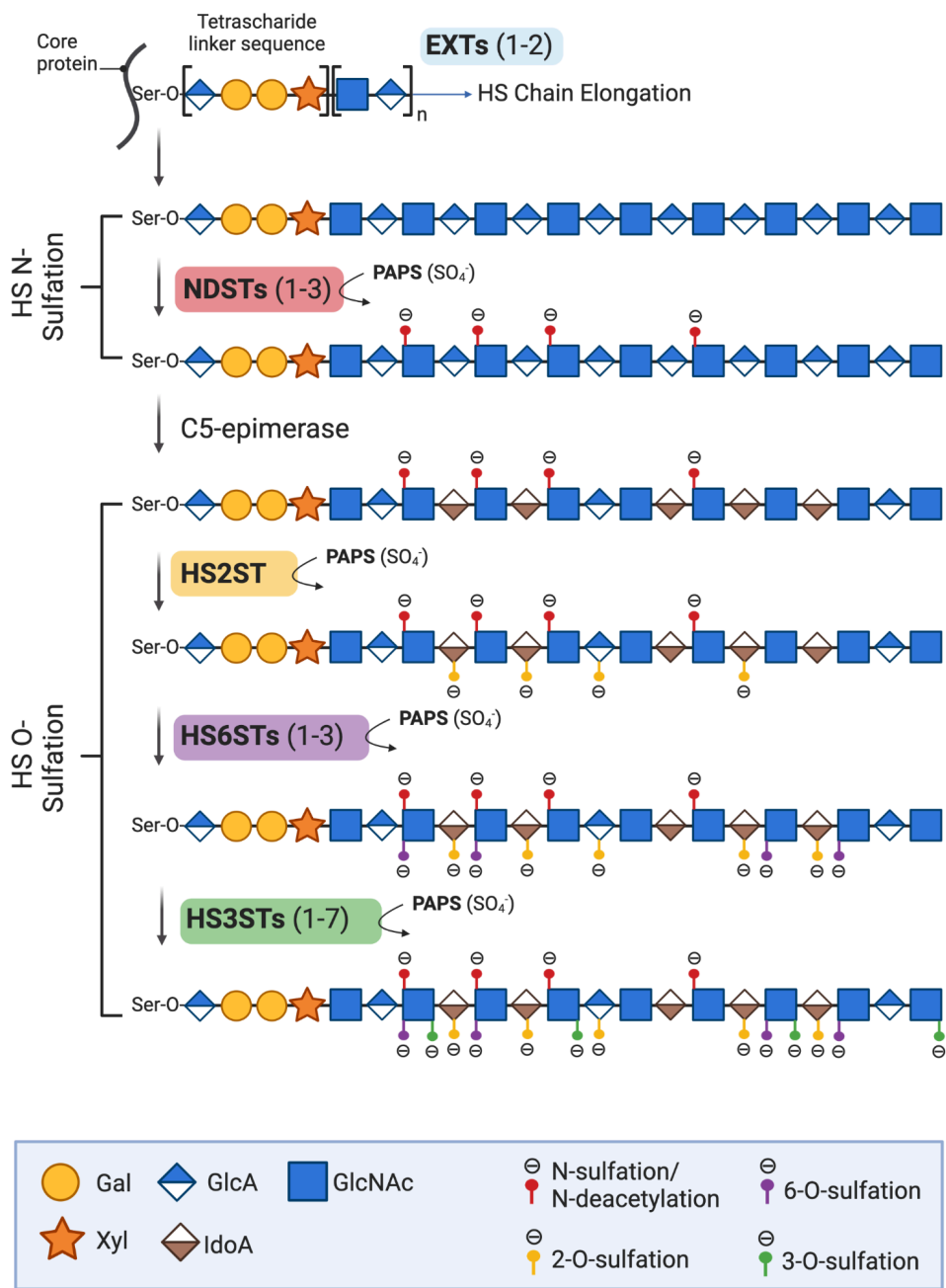


Figure 1.8. Schematic representation of heparan sulfate proteoglycan biosynthesis and sulfation. Heparan sulfate (HS) glycosaminoglycan chain synthesis begins in the Golgi apparatus when Exostosin (EXT1/2) polymerises a series of repeating disaccharide units of glucuronic acid (GlcA)-N-Acetylglucosamine (GlcNAc) to the HS core-protein-associated tetrasaccharide linker sequence. Following HS-chain elongation, HS sulfotransferases transfer sulfate from the sulfate-donor molecule, 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to specific positions on the HS chain. The N-sulfated domains of the HS-chains are followed by 2-O-sulfation of selected iduronic acid (IdoA) residues by HS 2-O-sulfotransferase (HS2ST). Next, HS 6-O-sulfotransferases (HS6STs 1-3) transfer sulfate to the sixth position of N-Acetylglucosamine (GlcNAc) or N-sulfated GlcNAc (GlcNAS) residues. Finally, HS 3-O-sulfotransferases (HS3STs 1-6) transfer sulfate to the GlcNAS residues.

Ext1 depletion (lacking all HS chains) in the periocular mesenchyme during embryogenesis leads to microphthalmia with reduced axial lengths, lens diameters, and vitreous volume (Iwao et al. 2010). Similarly, *Ext1* deletion in neural crest cells of the developing eye causes microphthalmos, dysgenesis in the primitive iris and ciliary body, the failure of lens-cornea separation and optic nerve head agenesis (Iwao et al. 2006). Given our understanding of the wide array of protein interactions orchestrated by HSPGs, it is perhaps not surprising that complete loss of HS-GAG would have detrimental effects on development. More complex and elusive are the divergent functional roles of the different HS sulfotransferases.

1.5.2 PAPS/PAPS Synthetases

Following HS-chain elongation by EXT enzymes, HS sulfotransferases transfer sulfate from the sulfate-donor molecule, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), synthesised by cellular PAPS synthetases (PAPSS1/2) to specific positions on the HS chain (Figure 1.8). The two isoforms of PAPS synthase, PAPSS1 and PAPSS2 are localised primarily to the nucleus and cytoplasm of cells, respectively (Schröder et al. 2012). Transcriptome data identifies both PAPS synthetase isoforms in the lens (Kakrana et al. 2018).

Of all the chemical inhibitors of HS activity, sodium chlorate (NaClO_3) is the best characterised. Sodium chlorate inhibits the formation of PAPS, the sulfate donor, by competitively binding to PAPS synthases thereby blocking the production of PAPS, the essential rate-limiting sulfate (SO_4^-) donor required for all cellular sulfation events in the Golgi, including HS-GAG sulfation. Sodium chlorate treatment inhibits different types of HS-GAG sulfation in a concentration-dependent manner (Safaiyan et al. 1999) without compromising HS-GAG chain

length or structural properties (Safaiyan et al. 1999, Conrad, 2001). This effectively reduces the sulfation of all sulfated GAGs (i.e. both CS- and HS-GAG), with the production of under-sulfated proteoglycans whose secretion is partially prevented by the quality control mechanisms of the cell (Fannon and Nugent, 1996). As a result, sodium chlorate-supplemented media has been widely used in various cellular systems to define the biological functions of sulfated GAG chains/HSPGs, however, a relatively high concentration of sodium chlorate (up to 30 mM) is required to completely block all HS-GAG sulfation (Safaiyan et al. 1999, Conrad, 2001). Such high concentrations are not suitable for *in vivo* studies in model organisms (Humphries and Silbert, 1988). High concentrations of sodium chlorate (20-50 mM) have been shown to block the signalling activity of aqueous humour (Iyengar and Lovicu, 2017) on lens epithelial cells, as well as the activity of several ocular mitogens and morphogens including FGF-2 (Iyengar and Lovicu, 2017) and other heparin-binding growth factors such as PDGF-A (Iyengar and Lovicu, 2017), and VEGF (Ashikari-Hada et al. 2005). FGF- and aqueous-induced ERK1/2 signalling in lens epithelia was completely disrupted in the presence of sodium chlorate while PDGF-induced ERK and aqueous-induced AKT phosphorylation were not impacted (Iyengar and Lovicu, 2017) (Iyengar and Lovicu, 2017). The effects of sodium chlorate can be replicated by digestion of cell-surface HS with heparitinase and rescued by simultaneous addition of sodium sulfate (restores HS sulfation, Iyengar and Lovicu, 2017) or porcine heparin (exogenous sulfated GAGs), indicating that the observed effects of sodium chlorate treatment in these studies are likely primarily attributable to the de-sulfation and transient loss of HS-GAG activity.

These experimental blocking studies *in vitro* focus attention on the different modalities of HSPG function, as co-receptors for matrix molecules and their ability to serve as independent receptors for heparin-binding growth factors. HSPGs also indirectly influence the intracellular signalling (e.g. MAPK/ERK, PI3K/AKT/mTOR, and the Rho GTPases of these molecules) by regulating receptor binding interactions, activation and trafficking (Bishop et al. 2007).

1.5.3 HS N-Sulfation (NDSTs)

HS sulfation proceeds in a stepwise manner, beginning with the N-sulfation of specific glucosamine (GlcNAc) residues of the HS chain by N-deacetylase/N-sulfotransferase (Ndsts 1-3) enzymes (Poulain and Yost, 2015) (Figure 1.8). These enzymes play important roles in eye development with mutations in *Ndst1/2* displaying highly variable dysgenic eye defects, ranging from bilateral anophthalmia, microphthalmia, and/or aphakia, and to a lesser degree unilateral aphakia in embryos (Qu et al. 2011b) (see Table 1.1). N-sulfation of HS by *Ndst1* has been reported to be required for lens induction and development, with *Ndst1*^{-/-} mice displaying reduced lens cell proliferation and delayed or failed induction and vesicle formation of the lens (Pan et al. 2006). This was accompanied by severely disrupted expression of α A-crystallin and key lens proteins and transcription factors, including AP2 α , Pax6 and Prox1 (Pan et al. 2006). Interestingly, depletion of *Ndst1* caused the dysregulation of FGF-signalling in early lens development, but not other important pathways such as BMP- or Wnt-signalling (Pan et al. 2006). Conditional ablation of *Ndst1* and *Ndst2* (lacking HS N-sulfation) after the lens induction stage caused reduced lens cell proliferation, increased cell death and defective lens fibre differentiation in later lens development (Qu et al. 2011b). Thus, it is clear that HS sulfation is important in lens morphogenesis and postnatal growth, and that there is specificity in the binding

interactions of growth factors and specific sulfated residues (e.g. N-sulfated) of HS. HS sulfation is also critical for normal growth factor signalling and development of other ocular tissues, such as the lacrimal gland (Qu et al. 2012) with N-sulfation of HS required for FGF-induced induction events in lacrimal gland development (Pan et al. 2008), as seen in lens (Pan et al. 2006).

1.5.4 HS O-Sulfation (HS-OSTs)

The N-sulfated domains of the HS-chains are followed by 2-O-sulfation of selected iduronic acid (IdoA) residues by HS 2-O-sulfotransferase (Hs2st). Next, HS 6-O-sulfotransferases (Hs6st1-3) transfer sulfate to the sixth position of GlcNAc or N-sulfated GlcNAc (GlcNAS) residues.

Finally, HS 3-O-sulfotransferases (Hs3st1-6) transfer sulfate to the GlcNAS residues (Li and Kusche-Gullberg, 2016) (Figure 1.8). HSPGs carrying differentially O-sulfated HS have distinct and complementary roles in their regulation of developmental and biological events (Tillo et al. 2016). The interactions of HS with FGF-2 and its cognate receptors (FGFRs) have been characterised in detail. HS chains strongly facilitate the formation of ternary complexes with FGF and FGFR (Escobar Galvis et al. 2007), serving to lower the activation energy required for the conformational change of the FGFR and initiation of intracellular signalling, prolonging the duration of FGFR activation (Forsten-Williams et al. 2008). Distinct sulfation patterns can modulate different FGF-10-mediated gland developmental processes, such as proliferation, duct elongation, bud expansion and differentiation (Pan et al. 2008, Patel et al. 2008).

Mice deficient in *Hs2st* (lacking HS 2-O-sulfation) display eye defects, including the formation of cataracts, abnormal pigmented retinal epithelium and bilateral coloboma of the iris (Bullock et al. 1998). The disruption of the mouse gene encoding d-glucuronyl C5-epimerase (Glee, the

precursor for HS 2-O-sulfation) also causes frequent association with iris coloboma (Li et al. 2003). Despite binding of HS to HGF and PDGF-A being dependent on 6-O-sulfation, mice with systemic inactivation of *Hs6st1* (lacking HS 6-O-sulfation) develop without any major gross abnormalities, with the exception of errors in axonal guidance at the optic chiasm (Pratt et al. 2006). In early adulthood they develop growth retardation, body weight loss and lung defects leading to late postnatal mortality (Izvolosky et al. 2008). Few studies have investigated the interactions of HS-3-O-sulfation due to its rarity (De Agostini et al. 1990). Further investigations revealed unique and differential roles for 2-O- and 6-O- sulfation of HS in the regulation of later stages of lacrimal gland development. *Hs6st*^{-/-} mutants exhibited lacrimal gland hypoplasia and disrupted FGF signalling, while *Hs2st*^{-/-} mutants had a less severe phenotype. In *Hs2st*^{-/-}/*Hs6st*^{-/-} double mutants, lacrimal gland development and associated FGF-induced downstream ERK-signalling activation were completely abolished (Qu et al. 2011a). While not directly reported in this study, the developing lens of these mutants was also much smaller (Xin Zhang, personal communication, FJ Lovicu).

Compared with 2-O- and 6-O-sulfation, 3-O-sulfation of HS is relatively rare, and typically comprises less than 1% of overall HS sulfation (De Agostini et al. 1990). HS3STs are also expressed by fewer cell types (De Agostini et al. 1990). Out of the seven HS 3-O-sulfotransferase genes present in the mouse, transcripts for *Hs3st1* represent 66% of all the HS 3-O-sulfotransferase transcripts in the newborn lens (Hoang et al. 2014). The *Hs3st1*^{-/-} genotype is early postnatal lethal on a C57BL/6 inbred background where homozygous embryos exhibit intrauterine growth retardation (HajMohammadi et al. 2003), however, F1-hybrids are

relatively normal, but have been shown to have an enhanced sensitivity to pro-inflammatory molecules, such as tumour necrosis factor (TNF) (Smits et al. 2017).

1.5 Coordination of HSPGs in lens pathology

Cataract is one of the leading causes of blindness worldwide (Foster and Resnikoff, 2005, Lam et al. 2015, Pesudovs et al. 2021). Currently, the only effective cure for cataracts is cataract surgery, which remains the most frequently performed surgical procedure worldwide (Chen et al. 2021). The surgery involves removing a portion of the anterior lens capsule and the lens cells, and inserting an intraocular lens (IOL) into the denuded capsular bag to regain vision (Allen and Vasavada 2006, Lam et al. 2015) (Figure 1.9).

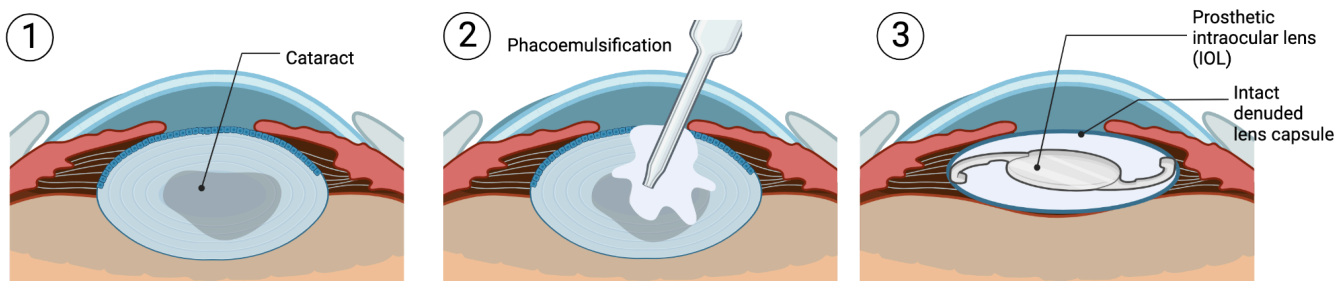


Figure 1.9. Cataract surgery and intraocular lens placement.

However, despite the high success rate of cataract surgery, it is prone to develop postoperative complication known as posterior capsular opacification (PCO), involving the formation of a fibrotic lesion on the newly inserted IOL (Gu et al. 2022). This results in the development of a secondary cataract that impairs vision to different extents (Konopińska et al. 2021). PCO affects

30-50% of adults and all children within five years of undergoing cataract surgery (Konopińska et al. 2021). The only existing treatment for PCO is the Neodymium-doped yttrium aluminium garnet (Nd:YAG) laser capsulotomy (Karahan et al. 2014). However, this procedure is not accessible in most underdeveloped countries and imposes a significant financial strain on the healthcare systems of developed countries (Ursell et al. 2018, Raulinajtys-Grzybek et al. 2020). Furthermore, Nd:YAG laser capsulotomies can lead to complications like ocular inflammation, retinal detachment, and retinal tear (Javitt et al. 1992, Gu et al. 2022). Despite these complications occurring at fairly low rates, they limit the availability of Nd:YAG laser capsulotomy to severe PCO cases (Gu et al. 2022, Wesolosky et al. 2017). However, any level of vision impairment due to PCO significantly diminishes patient satisfaction and degrades their quality of life (Gu et al. 2022). Hence, there is an urgent need to devise new treatments for PCO, particularly for those cases with minor vision impairment where Nd:YAG laser capsulotomy is not recommended.

Other forms of cataract, such as fibrotic subcapsular cataracts (anterior or posterior) result from aberrant lens epithelial trans-differentiation processes (See Figure 1.10). Following surgery, residual lens epithelial cells that remain attached to the lens capsule undergo EMT, migrating posteriorly to form plaques of myofibroblastic cells that cause lens capsule wrinkling, distorting the passage of light, a condition known as posterior capsular opacification (PCO). ASC also involves disruption of the lens epithelium and has many similarities to PCO, including EMT (Figure 1.9). Fibrotic forms of cataract, such as subcapsular cataracts (anterior or posterior) result from aberrant lens epithelial differentiation processes. In anterior subcapsular cataract or capsular opacification (ASC/ACO), LECs transdifferentiate into myofibroblasts under the

influence of TGF- β 2, secreting abundant ECM containing fibronectin and collagen I, as they adhere to the overlying lens capsule to create a fibrotic plaque (de Jongh et al. 2005).

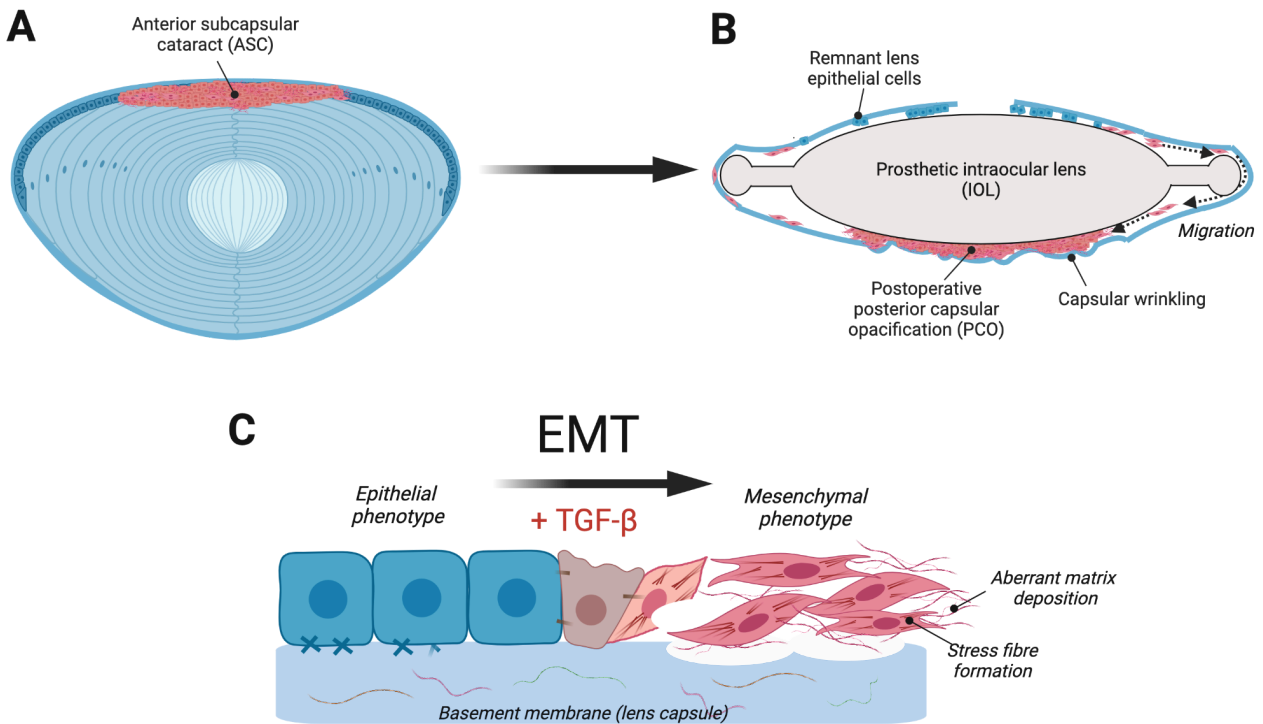


Figure 1.10. Aetiologies of fibrotic cataract (A, B) and lens TGF- β -induced epithelial-to-mesenchymal transition (C).

TGF- β -induced EMT (Figure 1.10) is a complex stepwise phenomenon that plays a crucial role in fibrogenic disease, that is defined by a common set of intertwined cellular events, which are each differentially regulated by signalling molecules and pathways. First, induction of an injury response to lens epithelial cells causes the up-regulation of TGF- β production by lens epithelial cells, and its increased release from reservoirs in the lens capsule, along with other growth

factors including FGF-2, EGFs and PDGFs by matrix metalloproteinases (MMPs) (Tholozan et al. 2007, Zelenka and Arpitha 2008). TGF- β binding to receptors on lens epithelial cells leads to the initiation of canonical TGF- β SMAD signalling, where SMAD2/3 forms a complex with SMAD4 and translocates to the nucleus, where it activates EMT-response genes and transcription factors (Figure 1.11). The result is the transdifferentiation of cuboidal lens epithelial cells to myofibroblastic cells (Wormstone et al. 2009). The TGF- β -induced transdifferentiation of lens epithelial cells to myofibroblastic cells (see Figure 1.10) is characterised by i) cytoskeletal reorganisation, including the formation of actin stress fibres and increased expression of pro-contractile molecules (e.g. α -SMA, vimentin, tropomyosins), that manifest in spindle-shaped cell morphology, increased cell stiffness, reoriented microtubule polarity, and elevated cell motility (Korol 2007, Kubo et al. 2013), and ii) focal adhesion turnover and destabilisation of adherens junctions, indicated by the loss of epithelial markers (i.e. loss of junctional E-cadherin localisation) and the gain of mesenchymal markers (i.e. N-Cadherin, *Sail*) (Taiyab et al. 2022). Alterations to the actin cytoskeleton, focal adhesions, and stability of adherens junctions in TGF- β -induced EMT are regulated via a RhoA/ROCK-dependent pathway (Bhowmick et al. 2001). Having completed EMT, the myofibroblastic cells secrete abundant modified ECM, rich in fibrillar collagen I and fibronectin (Johar et al. 2007, Saika et al. 1998, Sappino et al. 1990). The motile myofibroblastic cells adhere to this modified matrix through interactions between matrix proteins (i.e. fibronectin) which induce focal adhesion formation at the cell–ECM interface through focal adhesion kinase (FAK)/integrin signalling-dependent mechanism. This alteration in both the epithelial cells and ECM results in a corresponding change to the lens capsule, which due to the increased adhesion of the contractile myofibroblastic cells, becomes wrinkled. At the same time, other factors in the ocular milieu, notably FGF-2, induce increased

proliferation and migration of lens epithelial and myofibroblastic cells. The migratory and proliferative effects of FGF-2 have distinct pathways (Boilly et al. 2000). While FGF-2-induced proliferation is primarily mediated via ERK1/2 (Boilly et al. 2000, Lovicu and McAvoy 2001) and PI3K/AKT signalling (Boilly et al. 2000), FGF-2-induced migration rely on the signalling activity of Src kinase, p38 MAPK (Boilly et al. 2000) and integrins. Alongside FGF-2, other factors present in ocular milieu (i.e. PDGF, HB-EGF, IGFs) synergize with TGF- β to augment fibrosis through crosstalk, transactivation and feed-forward mechanisms that include regulation of TGF- β levels, potentiation of pro-fibrotic pathways and suppression of TGF- β regulatory signalling (Flokis and Lovicu, 2023).

To date, the main PCO-preventative strategy has been to eliminate all epithelial cells from the lens, however, this is problematic, with attempted interventions inducing other ocular problems. Strategies to prevent EMT associated with cataract to maintain the normal epithelia are an alternative.

HSPGs have been reported to play a key role in the damage and repair processes of diseased ocular tissues. These processes are often linked to significant changes in specific HSPGs (Heimann et al. 2001, Ibrahim et al. 2017) or HS sulfation enzymes (Saravanan et al. 2019) that can either support these pathogenic processes, as seen in cataract (the opacification of the lens) (Qin et al. 2017), or can promote healing (reviewed in Park and Shukla, 2013). These changes can affect both specific core proteins (i.e. cleavage of specific cell surface HSPGs, up-/down-regulation of specific core proteins, changes in tissue expression patterns) and/or HS

chain structure (i.e. cleavage of HS chains, up-/down-regulation of specific HS biosynthesis/sulfation enzymes).

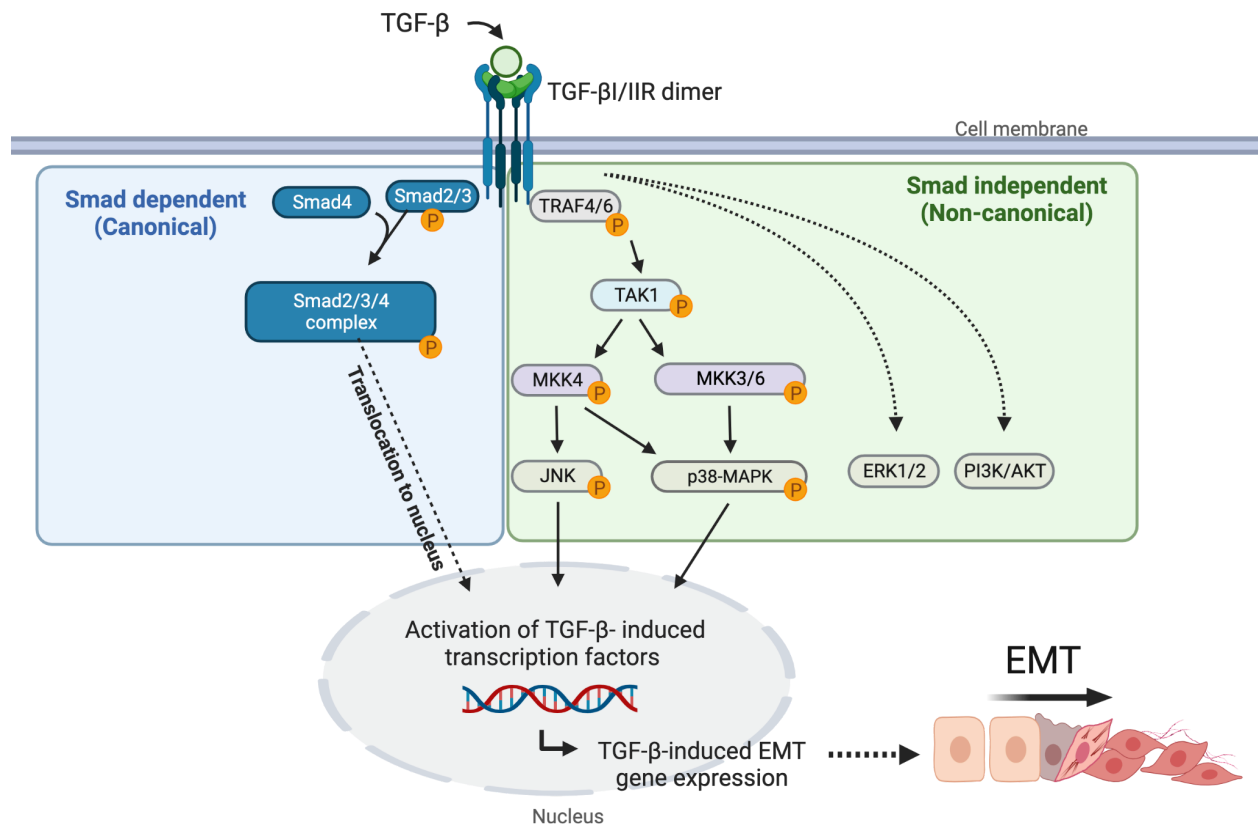


Figure 1.11 TGF- β signalling pathways in TGF- β -induced EMT.

1.6 Aims and hypotheses

Here, we hypothesise that lens architecture is tightly controlled by different HSPGs that regulate lens development, ocular growth factor-induced lens cell proliferation and fibre differentiation, as well as aberrant TGF- β -mediated EMT and cataract development.

Aim 1: Identify the HSPG-associated GAGs and core proteins that are expressed in the postnatal rat lens.

Aim 2: Identify the HSPG-associated GAGs and core proteins that are expressed in the developing lens.

Aim 3: Determine whether HSPG sulfation is required for growth-factor-induced lens cellular processes (lens cell proliferation, fibre differentiation) *in vitro*.

Aim 4: Determine whether the expression of lens HSPGs is altered in lens pathogenesis (fibrotic cataract), and whether HSPG sulfation is required for TGF- β -mediated EMT *in vitro*.

1.6.1 Aim 1: Identify the HSPG-associated GAGs and core proteins that are expressed in the postnatal rat lens.

Addressed: Chapter 3 Results Part I

Rationale: The organisation of lens cells is regulated by the ocular environment, namely, the aqueous and vitreous humours that bathe the lens. *In vitro* studies have shown that growth factors native to the aqueous humour promote the mitogenic activity of the lens epithelial cells (Iyengar et al. 2009), while factors in the vitreous humour induce the differentiation of secondary lens fibre cells (Wang et al. 2010). The interactions of growth factors with native HSPGs can

influence their receptor binding and signalling activation (Park et al. 2000, ForstenWilliams et al. 2008), gradient formation (Matsuo et al. 2014), modulation of their signalling activity (Shworak et al. 2011), as well as protecting cells from degradation (Sun et al. 2019). Through modulation of the bioavailability, receptor binding and signalling activation of mitogens and morphogens, HSPGs are positioned as master regulators of growth factor signalling in cell biology and development (Bishop et al. 2007, Lin et al. 2004). To determine which specific HSPG core proteins could potentially be involved in the regulation of signalling events important for lens cell biology, here we comprehensively characterised the presence, and comparative spatial localisation of all known HSPG core proteins in the postnatal rat lens.

1.6.2 Aim 2: Identify the HSPG-associated GAGs and core proteins that are expressed in the developing lens

Addressed: Chapter 4 Results Part II

Rationale: The development and maintenance of the vertebrate lens throughout life is orchestrated by the interplay of a complex network of signalling molecules within the native ocular environment (Lovicu et al. 2005). Decades of research have identified these key molecules and characterised their differential and complementary roles in the coordination of specific morphogenetic events and cell behaviours (Cvekl et al. 2014, Cvekl et al. 2017). For the defined stages of lens morphogenesis, these factors need to be tightly controlled in a spatial and temporal manner to regulate the induction, and anteroposterior, left–right and dorsoventral patterning of the lens (Cvekl et al. 2014, Cvekl et al. 2017). Although the many distinct and

overlapping effects of these factors on lens cell behaviour are well-characterised, the mechanisms underlying how these growth factors traverse the extracellular space, arrive precisely at, and transmit signalling within target cells at the appropriate level, remain elusive. Recent studies in mice and other model animals have revealed the importance of HSPGs, that are localised to the cell membrane and in the ECM of tissues, to promote the signalling and extracellular distribution of growth factors, respectively (Sarrazin et al. 2011). While we know much of vertebrate lens morphogenesis, less is understood about the regulation of the growth factors driving this development. Given the important role of HSPGs in regulating diverse growth factors, and with recent studies implicating HSPGs as essential for the induction and development of the lens (Rossi et al. 2003go, Pan et al. 2006) and accessory glands (Qu et al. 2011), to identify the specific roles of the diverse HSPG core proteins, here, we characterise the most comprehensive spatiotemporal localisation of key HSPGs throughout lens development, the first known study to map the HSPG expression and localisation profile of a developing tissue over the course of organogenesis.

1.6.3 Aim 3: Determine whether HSPG sulfation is required for growth-factor-induced lens cellular processes (lens cell proliferation, fibre differentiation) in vitro.

Addressed: Chapter 5 Results Part III

Rationale: The refined architecture and well characterised biology of the vertebrate lens makes it an excellent model to study how these basic cellular processes are coordinated. The transitional cellular processes of proliferation, migration and fibre cell differentiation are distinct, and each is orchestrated by the interplay of a complex array of signalling pathways regulated by growth

factors native to the surrounding ocular media, the aqueous and vitreous humours (Lovicu and McAvoy, 2005). Mitogens present in the aqueous humour, bathing the posterior aspect of the lens, promote the proliferation, survival, and maintenance of the anterior epithelial monolayer. Posterior to the lens equator, factors found in the vitreous humour drive the differentiation of lens epithelial cells into elongated lens fibre cells (Lovicu and McAcoy 2005). Numerous studies have been conducted to investigate and characterise the various factors present in the ocular environment and their individual and collective effects on lens cell behaviour. Despite the well-characterised effects of various growth factors on lens cell behaviour, the precise mechanisms by which these factors navigate the extracellular space, reach their intended target cells, and initiate signalling at the appropriate intensity remain largely unknown. The interplay of lens growth factors and their interactions with ECM proteins influence the precise timing and location of cellular events such as proliferation, migration, and differentiation along the anterior-to-posterior axis of the lens. Given the complexity of these processes, identifying factors that coordinate these signalling events is crucial for understanding normal lens physiology and pathologies arising from the disruption of these pathways. Research conducted in mice and other model organisms points to a key regulatory role for HSPGs in the signalling and extracellular distribution of growth factors in the lens. HSPGs can bind to and influence the signalling activity and distribution of a wide range of growth factors. HSPGs can serve as receptors or co-receptors for various signalling molecules and their downstream signalling pathways. Within the ECM, HSPGs serve as molecular scaffolds capable of binding and sequestering growth factors, stabilising dynamic gradients of mitogens and morphogens at the tissue level, and orchestrating the bioavailability of these factors at the cell surface. Investigating the intricate interplay between growth factors and HSPGs holds promise for uncovering the elusive mechanisms that govern

their precise actions within the lens and other tissues. Although previous findings lend strong evidence to suggest an important role for HSPGs in the regulation of mitogen-induced epithelial proliferation (Iyengar et al. 2017) and pathogenesis in the lens (Qin et al. 2017), the localisation and interactions of HSPGs with different growth factors and their signalling complexes, remain largely uncharacterised in the lens (see chapters 3 and 4). Evidence compiled from ours, and other preliminary studies in other tissues, suggest that growth factor-mediated processes in the epithelium can be regulated by modulating HS-GAG sulfation (Wang et al. 2017, Iyengar and Lovicu, 2017). Here, we investigate the differential requirements for HSPG/HS-GAG sulfation in essential lens cellular processes – lens epithelial cell proliferation and fibre cell differentiation – induced by ocular humour and some of the major growth factors known to drive these signalling processes in the lens. By specifically modulating HS-GAG sulfation using surfen and further characterising which growth factors are modulated by HSPGs, we hope to better understand how key lens HSPGs regulate different growth factor signalling cellular processes.

1.6.4 Aim 4: Determine whether the expression of lens HSPGs is altered in lens pathogenesis (fibrotic cataract), and whether HSPG sulfation is required for TGF- β -mediated EMT in vitro

Addressed: Chapter 6 Results Part IV

Rationale: The lens is an excellent model for the study of fibrotic disease (Eldred et al. 2011), and a range of *in vitro* and *in vivo* experimental models have been established to study the events underpinning fibrotic cataractogenesis (Wormstone and Eldred 2016). A growing body of work over the past decades in the lens field has made significant advances in our understanding of the

underpinnings of fibrotic cataract, but there are still many unanswered questions. What is clear is that understanding the molecular basis of PCO is essential to elucidate potential strategies for prevention and treatment. The specific molecules and mechanisms that regulate PCO and its associated molecular mafia, remain largely unidentified and unexplored. Similarly, though the pathogenesis of fibrotic cataract has been extensively characterised both *in vitro* and *in vivo*, the field has yet to fully elucidate the role of external factors influencing the activation and progression of TGF- β -mediated EMT in the lens. Identifying the molecules that regulate cell behaviour and maintain the integrity of the lens is fundamental to discerning the underlying mechanisms involved in cataract formation. One such class of potential regulatory candidates are the HSPGs, which are known to impart a multifactorial influence over several key players and pathways involved in fibrosis.

Progressive advances in the field of glycobiology have revealed a critical role for proteoglycans in the activation and regulation of growth factor signalling and processes associated with mitogen-driven pathogenesis. As different HSPGs appear to influence overlapping signalling pathways with diverse and cell type-specific outcomes (Rossi 2003), characterising different HSPG-growth factor interactions to develop a more comprehensive understanding of the functional significance of HSPG-signalling changes with the onset of pathology, is an important area of study. Studies from many tissues lend strong evidence to suggest an important role for HSPGs in the regulation of fibrotic disease (Solic et al. 2005, Westergren-Thorsson et al. 2017, Lord et al. 2018), however, the functional roles of HSPGs in fibrotic cataract remain largely uncharacterised. A better understanding of these underlying regulatory mechanisms may serve as a basis for the identification of novel therapeutic targets and molecular strategies that could be implemented to slow or prevent the development and progression of diseases characterised by

abnormal growth factor-mediated epithelial cell proliferation and HSPG sulfation profiles, such as fibrotic cataract. Here, we investigate the spatiotemporal immunolabelling of HSPGs in the pathogenesis of fibrotic cataract and investigate the functional requirement for HSPGs in driving TGF- β -induced EMT in the lens *in vitro*.

CHAPTER 2: GENERAL METHODS

2.1 Tissue

Animals were bred at the Medical Foundation Building Animal House (K25), The University of Sydney, Australia, with an alternating 12-hour light-to-dark cycle. 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 (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research. Animals were sacrificed by asphyxiation with carbon dioxide followed by cervical dislocation. Eyes were collected for lens explanting or histological processing (see below, Table 2.1).

Post-mortem human foetal eyes (17-weeks gestation) were obtained from the Lions NSW Eye Bank, with informed consent, and approval from the University of Sydney Human Research Ethics Committee. All methods applied in the study were performed in accordance with the approved Human Research Ethics guidelines.

Table 2.1. *Source of ocular tissues.*

Tissue collection	Phenotype assessment	Animals used
<i>Embryonic day 11.5 (E11.5)</i>	Lens induction/invagination, vesicle formation/primary lens fibre differentiation	Wild-type mice
<i>Embryonic day 14.5 (E14.5)</i>	Lens growth, lens epithelium maintenance, secondary lens fibre cell differentiation, denucleation/organelle loss	Wild-type mice
<i>Embryonic day 16.5 (E16.5)</i>	Lens growth, lens epithelium maintenance, secondary lens fibre cell differentiation, denucleation/organelle loss	Wild-type mice
<i>Postnatal day 0 (P0)</i>	Lens growth, lens epithelium maintenance, secondary lens fibre cell differentiation, denucleation/organelle loss	Wild-type mice
<i>Postnatal day 10 (P10)</i>	Lens growth, lens epithelium maintenance, secondary lens fibre cell differentiation, denucleation/organelle loss.	Wild-type mice Wistar rats
<i>Postnatal day 21 (P21)</i>	Lens growth, lens epithelium maintenance, secondary lens fibre cell differentiation, denucleation/organelle loss	Wild-type mice 918 TGF GOF mice Wistar rats

Conception was recorded as midnight on the day of mating confirmed by the presence of a vaginal plug. Embryos were routinely collected post conception at embryonic (E) day 9.5 (E9.5), E10.5, E11.5, E14.5 and E16.5, representing key stages of lens development (See Figure 1.1, Table 2.1). Pregnant FvB/N mice were sacrificed by asphyxiation with carbon dioxide followed by cervical dislocation, and embryos were extracted and sacrificed by decapitation. Embryonic heads were collected for histological processing.

2.2 Histological processing

Intact eyes or embryonic heads were collected and fixed for 24 h, in 10% neutral buffered formalin (NBF, Sigma, Castle Hill, NSW, Australia) before processing for routine paraffin embedding. Lens epithelial whole mounts were fixed for 5 min in 10% (v/v) NBF and pre-embedded in 1% noble agar (Sigma) prior to paraffin embedding. Paraffin-embedded tissues were serially sectioned at 5 μ m. Mid-sagittal sections and lens epithelial whole mounts from at least three independent animals were used for staining and immunolabelling.

Prior to staining and immunolabelling, slides were deparaffinized and rehydrated: three 5 min Xylene washes, two 3 min 100% ethanol washes, 95% ethanol for 2 min, and 70% ethanol for 2 min and finally slides were washed with distilled water. Haematoxylin and Eosin (H&E) or periodic acid–Schiff (PAS) stains were used to stain sections and whole mounts to show cell morphology. Alcian Blue (AB) staining was also performed to show regions rich in highly sulfated GAGs (Chute et al. 2019).

2.3 *Immunolabelling of HSPGs*

Intact eyes or uncultured lens epithelial whole mounts were collected and fixed for 24 h or 5 min, respectively, in 10% neutral buffered formalin (NBF, Sigma, Castle Hill, NSW, Australia) before processing for routine paraffin embedding (lens epithelial whole mounts were pre-embedded in 1% noble agar, Sigma). Paraffin-embedded tissues were serially sectioned at 5 μ m. Lens epithelial whole mounts were also isolated and immediately fixed for 1 min in cold methanol (Sigma) in preparation for histochemical staining or immunolabelling procedures. Mid-sagittal sections and lens epithelial whole mounts from at least three animals were used for staining and immunolabelling. Representative sections were also stained with PAS and H&E to highlight lens morphology, or AB to show sulfated GAG localisation.

Whole eye sections and lens epithelial whole mounts were first labelled for HS using a monoclonal antibody (HS F58-10E4, 370255, Amsbio LLC, Abingdon, UK). As some HSPGs (i.e. Syndecans -1 (Kokenyesi et al. 1994) and -3 (Gould et al. 1995), agrin (Winzen et al. 2003) and collagen XVIII (Dong: et al. 003)) may carry CS-GAG chains, in addition to the predominant HS chains, we also labelled 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 (Avnure et al. 1984, David et al. 1992, VanDenBorn et al. 2005). 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 behaviours, and their differential expression is reported to be spatiotemporally regulated in a cell type- and tissue-specific manner (Ford-Perriss et al. 2003, Couchman et al. 2012), we characterised the spatial localisation of the different HSPGs in the postnatal lens. localisation of HS and HSPG core proteins were investigated by immunolabelling 10-day-old rat lens mid-sagittal sections and lens epithelial whole mounts using commercially available antibodies (see Suppl. Table 1). For all immunofluorescent labelling studies, cell nuclei were counter-labelled with bisbenzimidazole (Hoechst 33258, Sigma). We included the following controls for the immunofluorescence studies, i) unlabelled 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 v4.8.0 (Leica Microsystems), a ZEISS LSM-800 microscope with an AxioCam 506 camera using ZEN Blue Edition v2.3 software (Carl Zeiss, Jena, Germany), or a ZEISS AxioScan.Z1 microscope (Carl Zeiss) with a Hamamatsu ORCA-flash 4.0 v2 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 v1.5 (Leica Microsystems). Images were saved as TIFF (Tagged Information Field Format) without compression at 8-bit (phase-contrast images) or 16-bit depth. Photoshop v21.1.1 (Adobe, Inc., San Jose, CA, USA) and ImageJ (FIJI) v2.0 (Wayne Rasband, NIH, 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.

Table 2.2. *Antibodies used for HSPG immunolocalisation.*

			Antibody
HSPG-associated GAGs	HS-GAG	Sulfated HSPG-associated GAG chain (major species)	mAb (F58-10E4) Ms IgMκ Amsbio, Cat#: 370255-1
	CS-GAG	Sulfated HSPG-associated GAG chain (minor species)	mAb (CS-56), Ms IgM Sigma, Cat#: C8035
Core proteins	Syndecan-1	Syndecan family (transmembrane)	pAb, Ms IgG1κ Santa cruz, Cat#: sc-12765
	Syndecan-2	Syndecan family (transmembrane)	pAb, Ms IgG1κ Santa cruz, Cat#: sc-376160
	Syndecan-3	Syndecan family (transmembrane)	pAb, Ms IgMκ Santa cruz, Cat#: sc-398194

	Syndecan-4	Syndecan family (transmembrane)	pAb, Rb IgG Abcam, Cat#: ab24511
	Glypican-1	Glypican family (GPI-anchored)	mAb (A-10), Ms IgG1k Santa cruz, Cat#: sc-365000
	Glypican-2	Glypican family (GPI-anchored)	Ms mAb (F-5), IgG1 λ Santa cruz, Cat#: sc-393824
	Glypican-3	Glypican family (GPI-anchored)	Ms mAb IgG1 (9C2) Abcam, Cat#: ab129381
	Glypican-4	Glypican family (GPI-anchored)	Rb pAb, Invitrogen Cat#: PA5-88766
	Glypican-5	Glypican family (GPI-anchored)	Ms IgG1 κ (F-3) Santa cruz, Cat#: sc-390838
	Glypican-6	Glypican family (GPI-anchored)	Rb pAb, Abcam Cat#: ab251716
	Perlecan	High-molecular weight	Rb pAb, gift from Prof. Djajek Save Sight Institute
	Aggrin	High-molecular weight	Ms IgM κ (D2)

			Santa cruz, Cat#: sc-374117
	Collagen XVIII	High-molecular weight	Ms IgG2b (1837-46) Santa cruz, Cat#: sc-32720
HS-GAG Sulfation enzymes (See Figure 1.8)	PAPSS2	PAPS synthase	Rb PAb Abcam, Cat#: ab155588
	HS2ST	HS-2-O-sulfotransferase	Ms mAb IgG (G-10) Santa cruz, Cat#: sc-376530
	HS3ST1	HS-3-O-sulfotransferase	Rb pAb Abcam Cat#: ab91065
	HS6ST1	HS-6-O-sulfotransferase	Ms mAb IgG (C-9) Santa cruz, Cat#: sc-398231

2.4 Preparation of Lens Epithelial Explants

The *ex vivo* rat lens epithelial explant system (Lovicu et al. 2001, West-Mays et al. 2010) is a well-characterised method used to study the effects of different treatment conditions on the behaviour of lens epithelial cells attached to their native basement membrane, the lens capsule.

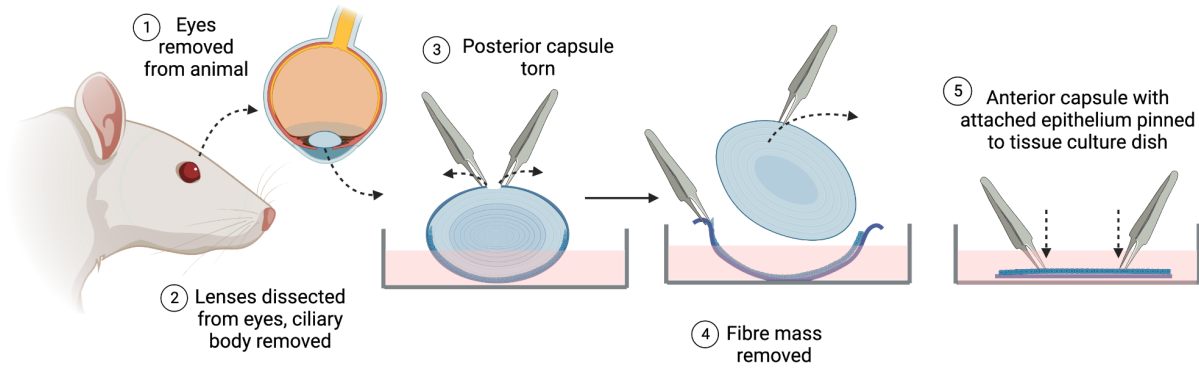


Figure 2.1. Schematic representation of lens explanting procedure.

All tissue culture procedures were performed in a laminar flow cabinet. Lenses were dissected from rat eyes and prepared as previously described (See figure 2.1) (West-Mays et al. 2010). Immediately following sacrifice of the animals, eyeballs were removed and placed into dishes containing fresh 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, NSW, Australia) equilibrated to 37°C with 5% CO₂. Using an inverted stereo microscope, forceps were used to make an incision in the posterior aspect of the eye and the lens was removed. Following removal of the ciliary body, 2-3 isolated lenses were transferred to dishes with fresh media. Lenses were positioned with the anterior surface and epithelium facing down. The posterior capsule was torn, and the fibre mass was carefully removed and discarded, leaving the anterior capsule and epithelium behind. Anterior capsule was carefully pinned to the tissue culture dish. Explants were cultured at 37°C in 5% CO₂. Explants were allowed to equilibrate overnight, and the following day, media was changed to fresh media followed by any treatments (See tables 2.3, 2.4, 2.5).

2.5 *Inhibiting HS-GAG sulfation*

To block HS-GAG sulfation, explants were co-treated with either 30 mM sodium chlorate (NaClO_3 , in M199, Sigma) (Iengar et al. 2017) or up to 10 μM Surfen (bis-2-methyl-4-amino-quinolyl- 6-carbamide, in dimethyl sulfoxide (DMSO, Sigma)(Schuksz et al. 2008) (Table 2.3). Control explants were untreated or treated with an equal volume of DMSO vehicle (in experiments with Surfen). Explants were pre-treated overnight (for sodium chlorate) or 1h (for surfen) prior to the addition of growth factors.

Table 2.3. *Inhibitors used to block HS-sulfation.*

Growth factor	Treatment	Source
Sodium chlorate (NaClO_3)	30 mM in M199 (overnight pre-treatment) as in (Iyengar et al. 2017)	Sigma, Catalog #403016
Surfen (bis-2-methyl-4-amino-quinolyl-6-carbamide, $\text{C}_{21}\text{H}_{20}\text{N}_6\text{O}$)	up to 10 μM (1 h pre-treatment unless otherwise specified)	Sigma, Catalog #S6951 in Dimethyl sulfoxide (DMSO) vehicle (Sigma Catalog #D2438)

Sodium chlorate is a chemical compound that has been found to inhibit the biosynthesis of sulfated glycosaminoglycans, such as HS-GAG. Sodium chlorate specifically inhibits the activity of PAPS synthetases, which produce PAPS, the rate limiting sulfate donor which is crucial for the sulfation of HS-GAG. This results in the production of under-sulfated HS-GAG.

Under-Sulfated HS-GAG can have altered biological activity, as the degree of sulfation can influence the ability of HS-GAG to interact with various proteins, such as growth factors, chemokines, and adhesion molecules. Thus, sodium chlorate can potentially affect various biological processes by altering the sulfation and, therefore, the function of HS-GAG.

Surfen, on the other hand, is a small molecule that acts as a HS-GAG antagonist (Schuksz et al. 2008). Surfen is an electrostatic inhibitor, which is to say the positively charged regions of surfen bind strongly to the negatively charged highly sulfated regions of HS-GAG, which are also the sites where HS-GAG interacts with various proteins, including growth factors (Figure 2.2). By blocking these interactions, Surfen can effectively inhibit the biological activities of heparan sulfate (Schuksz et al. 2008).

Surfen

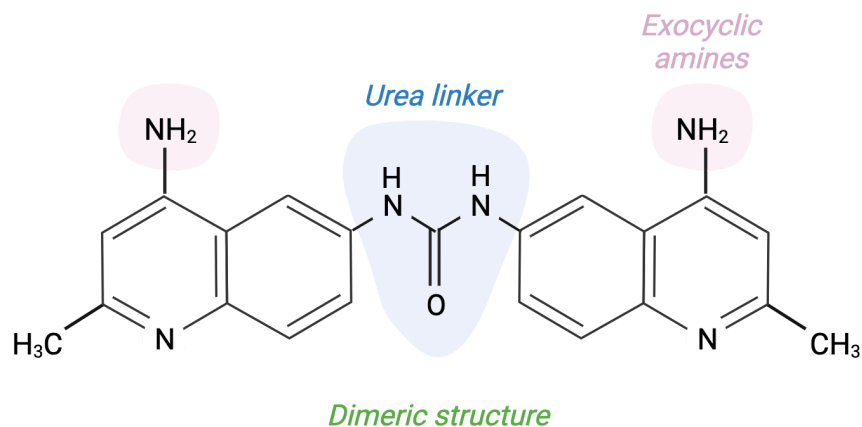


Figure 2.2. Structure of Surfen, a small molecule antagonist of heparan sulfate. Regions required for the biological activity of surfen (Schuksz et al. 2008) have been highlighted.

As Surfen binds strongly to plastic, Surfen was stored in amber glass vessels, and plastic tissue culture dishes were pre-saturated with a solution containing Surfen. 5 mM stock solutions were prepared in DMSO and stored in glass at -80°C . Fresh aqueous solutions were prepared as needed. Experiments involving Surfen included a DMSO control treated with an equal volume of DMSO.

2.6 Immunofluorescent detection of proteins of interest in treated explants

At the endpoint of a treatment period, explants were fixed for either 1 min in methanol AnalaR (for α -SMA, anti-phospho-ERK1/2 and HS-10E4 labelling) or with 10% NBF for 5 min (all other labelling). Following fixation, explants were washed 3 x 5 min washes in 70% ethanol, and

3 x 5 min washes in PBS/BSA (phosphate-buffered saline, 0.1% w/v bovine serum albumin) at room temperature. Explants were blocked in 3% normal goat serum (NGS) in PBS/BSA for 1 h before applying the primary antibody (diluted at 1:100 in 3% NGS) (See Table 2.4) and incubating in a humidified chamber at 4°C overnight. The next day, explants were equilibrated to room temperature and washed in PBS/BSA (3 x 5 min). The appropriate secondary antibodies (See Table 2.5) (diluted 1:1000 in PBS/BSA) were then applied for 2 h in a dark humidified chamber at room temperature.

Table 2.4. *Primary antibodies used for immunofluorescent labelling of explants.*

Target	Antibody source
Anti-BrdU	Ms mAb,
phospho-p44/42 MAPK (Thr202/Tyr204)	Rb mAb, Cell signalling Technology, Cat# 9106
anti- β -crystallin	Rb mAb, Lens Research Laboratory
anti-Aquaporin-0/MIP	Ms mAb, Santa Cruz Biotech, Cat#:
α -smooth muscle actin (α -SMA)	Ms mAb, Sigma, Cat#:A2547
β -catenin	Rb mAb, Clone E274, Abcam, Cat#: ab32572

Total SMAD2/3	Rb mAb, Cell Signalling Tech, Cat#: 8685, 1:100
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Table 2.5. *Secondary antibodies used for immunofluorescent labelling of explants.*

Antibody	Source
Gt anti-Rb Alexa-Fluor 488 IgG	Abcam, Cat#: ab150077
Gt anti-Rb Alexa-Fluor 594 IgG	Abcam, Cat#: ab150080
Gt anti-Ms Alexa-Fluor 488 IgG	Abcam, Cat#: ab150113
Gt anti-Ms Alexa-Fluor 594 IgG	Abcam, Cat#: ab150116
Gt anti-Ms Alexa-Fluor 568 IgM	Abcam, Cat#: ab175702

2.7 *Lens Epithelial Proliferation and Viability*

To induce lens epithelial cell proliferation, explants were treated for up to 24 h with aqueous humour (AQ) or exogenous growth factors found in the ocular media previously described to induce proliferation.

Table 2.6. *Growth factors for lens epithelial cell proliferation*

Growth factor	[Treatment]	Source
Aqueous humour (AQ)	10% v/v in M199	Freshly extracted from bovine eyeballs and filtered (Sourced from Hawkesbury valley meat Processors pty. Ltd.)
FGF-2 (low-dose)	1 ng/ml	R&D Systems, Minneapolis, MN, USA, Cat# 3718-FB
EGF	5 ng/ml	R&D Systems, Cat# 236-EG
HB-EGF	50 ng/ml	R&D Systems, Cat# 259-HE
IGF-I	50 ng/ml	R&D Systems, Cat# 291-G1
PDGF-AA	10 ng/ml	R&D Systems, Cat# 221-AA

As in McAvoy et al. 1989, Liu et al. 1996, Majima et al. 1998, Kok et al. 2002, Iyengar et al. 2006, 2009.

Cell counts (nuclei) were performed on representative images of explants (at least two per treatment group) cultured with increasing doses of Surfen (1, 5 or 10 μ M) for 24 h to assess cell viability. Explants treated with Surfen (5 μ M) for 1 h were immunolabelled for HS and CS GAGs. To identify cells in S-phase of the cell cycle, 5'-2'-bromo-deoxyuridine (BrdU, 150 μ g/ml) was added to explant cultures 18 h post-treatment and cultured for an additional 6 h prior to fixation in 10% neutral buffered formalin (10 min). BrdU-immunolabelling was performed as previously described (Iyengar:2006cs) and explants were counterstained with Hoechst 33258

(Sigma). Representative images were taken of each explant from at least three explants per treatment group. Cell counts (total cells and BrdU-positive) were performed using an optimized batch processing macro using ImageJ (FIJI) software (v2.0, Wayne Rasband, NIH, USA) from images captured using an epifluorescent microscope (Leica Microsystems, Wetzlar, Germany), and averaged for each treatment group. BrdU-proliferation assays were performed on representative images of explants (at least two per treatment group) cultured with a proliferating dose of FGF-2 (1 ng/ml), together with increasing doses of Surfen (1, 5 or 10 μ M) for 24 h. To visualise early changes in downstream MAPK/ERK signalling activation, explants treated with aqueous humour or different growth factors (see Table 2.6) with or without Surfen (5 μ M), were fixed with methanol 4 h after treatment and immunolabelled for mouse anti-phospho-ERK1/2 (p-ERK1/2, 9106, Cell signalling Tech., 1:100).

2.8 *Lens fibre cell differentiation*

To induce lens epithelial cell proliferation, explants were treated for up to 5 days with vitreous humour (VIT) or exogenous high-dose FGF-2 (See Table 2.7). Fibre differentiation was monitored over a 5-day period, and phase contrast images were taken each day to document changes to morphology.

Table 2.7. *Growth factors for lens fibre cell differentiation*

Growth factor	[Treatment]	Source
Vitreous humour (VIT)	10% v/v in M199	Freshly extracted from bovine eyeballs and filtered (Sourced from Hawkesbury valley meat Processors pty. Ltd.)
FGF-2 (high-dose)	150 ng/mL	R&D Systems, Minneapolis, MN, USA, Catalog # 3718-FB

as in McAvoy et al. 1989, Wang et al. 2009, 2010.

To visualise early changes in downstream MAPK/ERK signalling activation, explants treated with vitreous humour or a differentiating dose of FGF-2 (See Table 2.7), with or without Surfen (5 μ M), were fixed with methanol 4 h after treatment and immunolabelled for mouse anti-phospho-ERK1/2 (p-ERK1/2, 9106, Cell Signalling Tech., 1:100). Explants treated with vitreous humour or a differentiating dose of FGF-2 (See Table 2.7), with or without Surfen (5 μ M), were fixed (10% NBF for 5 min) 5 days after treatment and immunolabelled for markers of fibre differentiation: rabbit anti- β -crystallin (Lovicu et al. 2001) and mouse anti-Aquaporin-0/MIP (Lo et al. 2014) (Santa Cruz Biotechnology Inc., Dallas, TX, USA, 1:100).

2.9 *TGF- β 2-induced epithelial-to-mesenchymal transition*

To induce epithelial-to-mesenchymal transition (EMT), lens cells were treated with 300 pg/ml recombinant human TGF- β 2 (302-BP, R&D Systems, MN, USA) for up to 5 days (Wojciechowski et al. 2017). EMT was monitored over a 5-day period, and phase contrast images were taken each day to document changes to morphology.

To investigate the nuclear translocation of total SMAD2/3, lens epithelial explants were treated with TGF- β 2 as described above for 2 h and subsequently processed for immunolabelling with total SMAD2/3. Nuclei were stained with Hoechst, and at least 3 images were taken of each explant. Relative nuclear translocation was quantified using a nuclear localisation score: at least 3 images from each of 3 explants, from 3 independent experiments, were scored as either 0 (no nuclear tSMAD2/3 labelling), 0.5 (some nuclear tSMAD2/3 labelling) or 1 (predominantly nuclear tSMAD2/3 labelling). Total scores were calculated for each explant (mean of images of explant) and each treatment group (mean of explants within the treatment group). Differences in nuclear localisation score were calculated between treatment groups for all experiments using Unpaired t-tests with Welch's correction (mean $\pm$ SEM, with $P < 0.05$ considered statistically significant).

2.10 *Measuring changes in gene expression*

Total RNA was extracted from dissected 10-day-old rat lens epithelial cells and lens fibre cells (n = pooled tissue from 5 animals) using the ISOLATE II RNA Micro Kit (Bioline, Eveleigh, NSW, AU) according to 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 semi-quantitative reverse transcription polymerase chain reaction (RT-qPCR). RT-qPCR was performed as biological triplicates ($n = 3$), according to the manufacturer's instructions with the QuantStudio 12K Flex Real-Time PCR System (ThermoFisher Scientific, Waltham, MAS, 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 fibre cells respectively. Data were represented a $\log(\Delta Ct)$ relative to *Gapdh* $\pm$ standard error of the mean (SEM), from at least three independent experiments ($n = 3$).

2.11 Measuring changes in protein expression

Lens epithelial explants (at least five 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, NSW, 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 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, Cell signalling Tech., 1:1000), rabbit anti-ERK1/2 (ERK1/2, 9102, Cell signalling Tech., 1:1000) and mouse anti-Gapdh (G8795, Sigma, 1:5000). Following primary antibody incubation, membranes were rinsed in TBST and incubated with secondary antibodies (1:5000 in TBST) for 2 h at room temperature. Secondary antibodies used were a horse anti-mouse IgG HRP-linked antibody (7076, Cell Signalling Tech.) and a goat anti-rabbit IgG HRP-linked antibody (7074, Cell Signalling Tech.). 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 to Gapdh internal control.

2.12 Statistical Analysis

Data were presented as the mean or change in mean $\pm$ standard error of the 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 was performed using grouped samples one-way ANOVA

with Šidák corrections for multiple comparisons. Effects were considered statistically significant if $P < 0.05$.

CHAPTER 3: RESULTS I

AN ATLAS OF HEPARAN SULFATE PROTEOGLYCANS IN THE POSTNATAL RAT LENS

3.1 Preface

The following manuscripts comprise the majority of the following results chapter. Some sections of the following manuscripts have been reordered and/or edited for the purposes of elaboration or continuity. Original unedited manuscripts are attached in [appendix 1](#).

- 1) **TFL Wishart** and Frank J. Lovicu (2021). An Atlas of Heparan Sulfate Proteoglycans in the Postnatal Rat Lens. Investigative Ophthalmology & Visual Science, (DOI: 10.1167/iovs.62.14.5)
- 2) **TFL Wishart** and FJ Lovicu (2023). Spatiotemporal Localisation of Heparan Sulphate Proteoglycans throughout Mouse Lens Morphogenesis. Cells (DOI: 10.3390/cells12101364).

These manuscripts were published in Investigative Ophthalmology & Visual Science in 2022 (1) and Cells in 2023 (2). The work comprising these manuscripts was completed entirely during my PhD candidature under the guidance of my primary supervisor Professor Frank Lovicu who contributed to this manuscript by facilitating experimental design and manuscript editing.

Note that figures including images of glypican-5 have been updated from original figures in manuscript (1) to updated images later published in manuscript (2), which repeats the same immunolocalisation studies undertaken in manuscript (1) with an updated commercial antibody

which successfully showed glypican-5 localisation in the 10-day-old rat eye. For the original published figure see supplementary figure 1 (Wishart and Lovicu 2023) in [appendix 1](#).

3.2 Introduction

The ocular lens is a well-established model for studying growth factor regulation of cell behaviour (Wormstone et al. 2011). 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 fibre 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 fibre cells at the transitional zone (Mochizuki et al. 2014, Lovicu et al. 2014). fibre differentiation is characterised 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 fibre-specific crystallin proteins (Wride et al. 2011). The normal architecture, and hence transparency of the lens, depends on the maintenance of this distinctive pattern of growth.

The organisation of lens cells is regulated by the ocular environment, namely, the aqueous and vitreous humours that bathe the lens. *In vitro* studies have shown that growth factors native to the aqueous humour promote the mitogenic activity of the lens epithelial cells (Iyengar et al. 2009), while factors in the vitreous humour induce the differentiation of secondary lens fibre cells (Wang et al. 2010). These native factors, IGF-I (Shirke et al. 2001), EGF (Majima et al. 1995), PDGF-AA (Kok et al. 2002, Li et al. 2019), hepatocyte growth factor (HGF) (Choi et al. 2004), ephrins (Cheng et al. 2011, Zhou et al. 2018), and bone morphogenetic proteins (BMPs) (BeleckyAdams et al. 2002) act in concert with FGFs (Zhao et al. 2008, Iyengar et al. 2017) to

potentiate and regulate lens epithelial cell proliferation (Iyengar et al. 2006, Iyengar et al. 2009) and fibre differentiation (Wang et al. 2009).

FGFs have been reported to be particularly important in the regulation of lens cell survival, proliferation, and differentiation (Robinson et al. 2006, Zhao et al. 2008, Iyengar et al. 2017). They activate their high affinity RTKs) (e.g. FGFRs) in lens in a concentration-dependent manner (Chamberlain et al. 1997), stimulating a proliferative and/or fibre differentiation response (Chamberlain et al. 1989) via downstream signalling including, the MAPK/ERK and PI3K/Akt pathways (Lovicu et al. 2001, Iyengar et al. 2006, Wang et al. 2009). Differential distribution of FGF bioavailability in the eye can regulate these lens growth processes (Chamberlain et al. 1989), with the proliferating lens epithelial cells exposed to lower levels of FGF activity in the aqueous, and the differentiating fibre cells exposed to higher levels of FGF activity from the vitreous (deLongh et al. 1996, Lovicu et al. 2001). Although the many distinct and overlapping effects of growth factors on lens cell behaviour are well-characterised, the mechanisms underlying how they traverse the extracellular space, arrive precisely at target cells, and transmit signalling within cells at the appropriate level, is still unclear. Recent studies in mice and other animal models have revealed the importance of cellular and high molecular weight HSPGs for the signalling and extracellular distribution of growth factors (Bishop et al. 2007).

HSPGs are complex glycoconjugates composed of a core protein backbone decorated with covalently linked linear HS and/or CS 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, collagens, (Esko et al. 2017). The interactions of growth factors with native HSPGs can influence their receptor binding and signalling activation (Park et al. 2000, ForstenWilliams et al. 2008), gradient formation (Matsuo et al. 2014), modulation of their signalling activity (Shworak et al. 2011), as well as protecting cells from degradation (Sun et al. 2019). Through modulation of the bioavailability, receptor binding and signalling activation of mitogens and morphogens, HSPGs are positioned as master regulators of growth factor signalling in cell biology and development (Bishop et al. 2007, Lin et al. 2004).

The HSPG superfamily is divided into three major groups based on their core-protein structure. The transmembrane syndecans (1-4) (Afratis et al. 2017), and GPI-anchored glypicans (1-6) (Fico et al. 2011), are localised to the cell surface, while the large secreted HSPGs, including perlecan, agrin and collagen XVIII are primarily secreted by cells and deposited to the ECM and basement membranes (Schaefer et al. 2014). Different HSPG core proteins play unique roles in tissue homeostasis, and these roles are often tissue and cell-type specific (Bishop et al. 2007). Moreover, the number, localisation and HS/CS sulfation patterns of HSPGs can modify their protein interactions, and therefore their function (Poulain et al. 2015). 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 (Rossi 2003), while overexpression of agrin in the lens causes a dysgenic phenotype that is specific to the eye (Fuerst et al. 2007). The localisation of HSPG core proteins can hence provide valuable information pertaining to their functional roles in specific tissues (Couchman et al. 2012).

Immunolabelling 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 (Ford-Perriss et al. 2003). 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 (Hubka et al. 2019). 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 signalling activity (Chamberlain et al. 1997, Wu et al. 2014). Other studies have also provided support for a direct role of cell-surface HSPGs, rather than basement membrane localised HSPGs, in FGF signalling (Dealy et al. 1997, Galli et al. 2003, Zhang et al. 2003, Shimokawa et al. 2011).

To determine which specific HSPG core proteins could potentially be involved in the regulation of signalling events important for lens cell biology, here we comprehensively characterised the presence, and comparative spatial localisation of all known HSPG core proteins in the postnatal rodent lens. We found that the lens has a unique profile of HSPG core proteins, that are differentially localised 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, fibre 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 fibre differentiation.

3.3 Results

3.3.1 Relative expression of HSPG core protein transcript in the lens.

Transcripts for all thirteen HSPG core proteins were detected in the lens (Figure 3.1). *Gpc2* mRNA was the most highly expressed in lens epithelial cells, while all other HSPG core protein transcripts were the highest for fibre cells (Figure 3.1). Moreover, mRNA encoding *Sdc2*, *Sdc4* and *Coll8a1* were the lowest of any HSPG core protein transcript expressed in lens epithelial cells, while for fibre cells the lowest expression levels were observed for *Sdc2*, *Gpc2* and *Hspg2* (Figure 3.1).

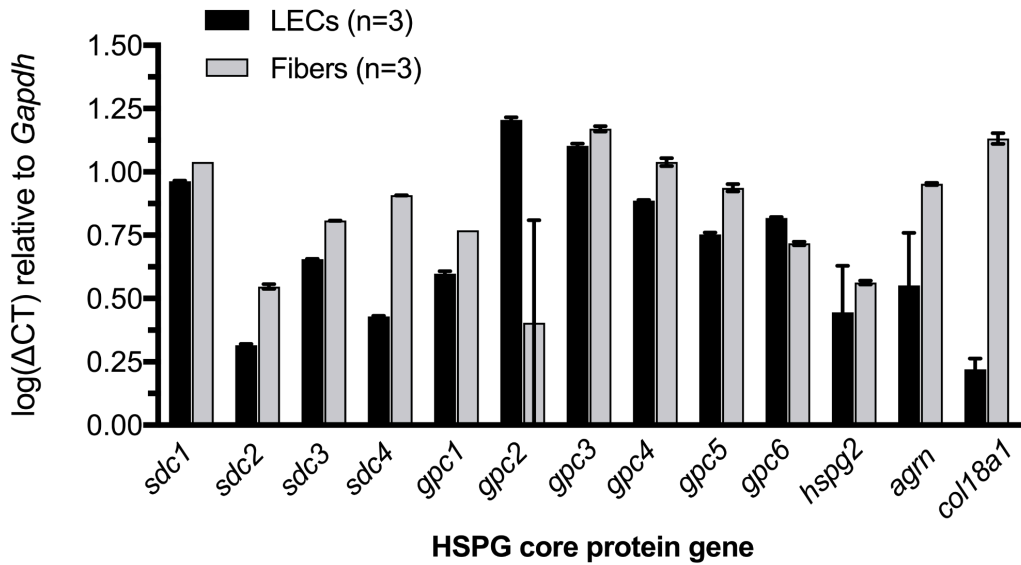


Figure 3.1. Relative expression of HSPG core protein transcripts in 10-day-old rat lens epithelial and fibre cells. Data are represented a log (ΔCt) of HSPG core protein transcript expression in lens epithelial cells (LECs, dark bar) or fibres (grey bar), relative to *Gapdh* $\pm$ SEM, from at least three independent experiments ($n=3$).

3.3.2 Localisation of Sulfated GAGs (HS and CS).

As some HSPGs (i.e. Syndecans -1 (Kokenyesi et al. 1994) and -3 (Gould et al. 1995), agrin (Winzen et al. 2003) and collagen XVIII (Dong et al. 2003) may carry HS or both HS/CS GAG chains, the distribution of HS- and CS-GAG labelling was compared in 10-day-old Wistar rat eye sections (Figure 3.2), and lens epithelial cell whole mounts using antibodies which recognize the functionally “active” sulfated domains on HS (David et al. 1992, VanDenBorn et al. 2005) (Figure 3.2 B1-B11) or CS (Avnur et al. 1984) (Figure 3.2 C1-C11) GAG chains. HS-GAG labelling was prominent in the lens capsule (Figure 3.2 B1, B3, B5), the cytoplasm of lens

epithelial cells (Figure 3.2 B1, B2) and on the cell membranes of newly elongating lens fibre cells (Figure 3.2 B4). HS-GAG reactivity increased at the germinative and transitional zones (Figure 3.2 B3), and adjacent lens capsule (Figure 3.2 B3 inset). A subset of lens epithelial and fibre cell nuclei were positive for HS-GAG immunolabelling (Figure 3.2 B1, B4 inset, yellow arrowheads). Strong HS-GAG immunoreactivity was also observed in the corneal epithelium (Figure 3.2 B6) and endothelium (Figure 3.2 B7), Descemet's membrane (Figure 3.2 B7), and the basement membranes of the iris (Figure 3.2 B8) and the inner limiting membrane of the ciliary body (Figure 3.2 B9). CS-GAG was absent from the lens capsule (Figure 3.2 C1, C3, C5) with infrequent positive labelling for CS-GAG observed in the cytoplasm of lens epithelial cells (Figure 3.2 C1, C2). Relative to the anterior lens epithelium, CS-GAG labelling was more prominent in the equatorial epithelium with a stronger label at the transitional zone (Figure 3.2 C2). This label was further intensified in the cytoplasm of newly elongating lens fibre cells (Figure 3.2 C4). CS-GAG labelled strongly in the corneal epithelium, Bowman's capsule (Figure 3.2 C6) and endothelium (Figure 3.2 C7), with increased reactivity in the iridial stroma (Figure 3.2 C8), and inner limiting membrane and stroma of the ciliary body (Figure 3.2 C9). No CS-GAG was found in the iridial basement membranes (Figure 3.2 C8).

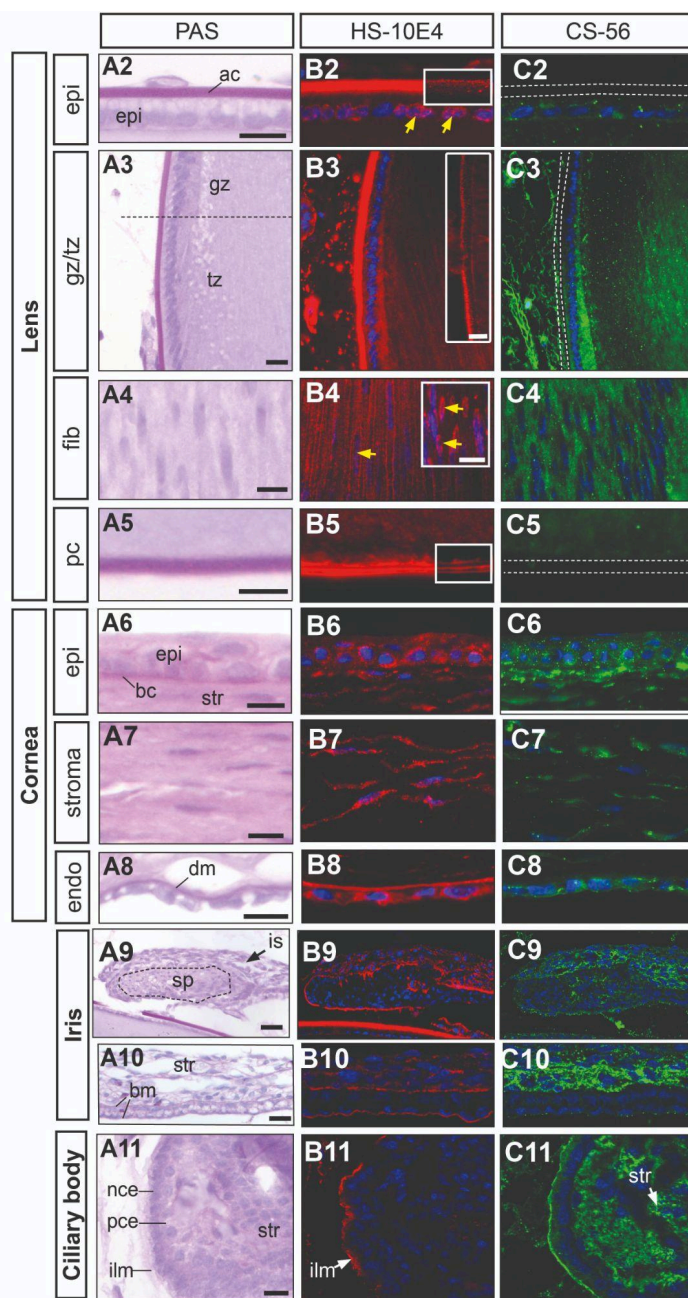
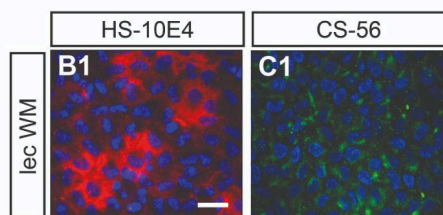
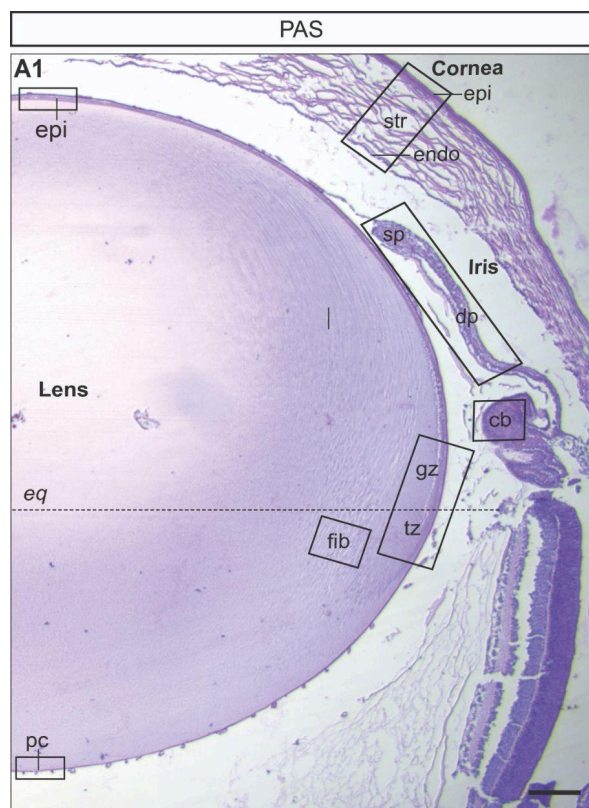


Figure 3.2. *Distribution of sulfated glycosaminoglycans in the 10-day-old rat eye. Mid-sagittal eye sections stained with Periodic acid-Schiffs (PAS, A1-11) show morphology of regions of interest. Corresponding sections (B2-B11, C2-C11) and 10-day-old rat lens epithelial whole mounts (lec WM, B1, C1) immunolabel the highly sulfated domains on heparan sulfate (HS, B1-11) or chondroitin sulfate (CS, C1-11) glycosaminoglycan chains. Yellow arrowheads (B2, B4) indicate nuclear localisation. Dotted white lines demarcate outline of the lens capsule. Insets in B2, B3 and B5 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 fibre 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 μ m (A1), 25 μ m (A2-11, B1-11, C1-11).*

3.3.3 HSPG core proteins are differentially localised key functional regions in the lens.

All HSPG core proteins were detected in the lens (Figure 3.3, 3.4). Expression patterns of individual core proteins were widely varied (Figure 3.3, 3.4), 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).

3.3.4 Lens capsule

The majority of the four transmembrane syndecans (syndecan-1, -2 and -4, Figure 3.3 A, B, D, Figure 3.4, A, B, D), a minority of the six glypican family members (glypican-2 and -4, Figure 3.3 A, B, D, Figure 3.4, F, H), and all three high-molecular weight HSPG core proteins (perlecan, collagen XVIII and agrin, Figure 3.3 A, B, D, Figure 3.4, K, L, M) were localised to the lens capsule. Perlecan and syndecan-2 were the most prominent species labelling the lens capsule. Syndecan-2 (Figure 3.3, K1, Figure 3.4 K1, K3) and perlecan (Figure 3.3, B1, Figure 3.4 B1, B3) labelling was observed throughout the lens capsule, while syndecan-4 (Figure 3.3, D1, Figure 3.4 D1, D3), glypican-2 (Figure 3.3, F1, Figure 3.4 F1, F3), glypican-4 (Figure 3.3, H1, Figure 3.4 H1, H3), collagen XVIII (Figure 3.3, L1, Figure 3.4 L1, L3) and agrin (Figure 3.3, M1, Figure 3.4 M1, M3) were restricted to the outermost layer of the lens capsule. In contrast, syndecan-1 labelling appeared to be uniquely limited to the innermost capsule layer, apposing the cells (Figure 3.3, A1, Figure 3.4 A1, A3). Capsular labelling of syndecan-1 (Figure 3.4 A3), syndecan-2 (Figure 3.4 B3), glypican-2 (Figure 3.4 F3), glypican-4 (Figure 3.4 H3), and to a lesser extent agrin (Figure 3.4 M3), increased in an antero-posterior gradient, while labelling for syndecan-4 (Figure 3.3, D1, Figure 3.4 D1, D3) and collagen XVIII (Figure 3.3, L1, Figure 3.4

L1, L3) 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 and agrin were also localised throughout the corneal stroma (Figure 3.5 B2, D2, H2, K2, L2, M2), iridial stroma, ciliary body stroma and iridial basement membranes (See Figure 3.6). Glypican-2 did not appear in any ocular basement membranes (Figure 3.5 F & 2 E) other than the lens capsule (Figure 3.3, F1, Figure 3.4 F1, F3). Interestingly, syndecan-3 was absent from the lens capsule (Figure 3.3, C1, Figure 3.4 C1, C3), but strongly labelled in the basement membranes and stroma of the iris and ciliary body (Figure 3.6 C).

3.3.5 *Lens epithelium*

Anterior LECs in tissue sections and whole mounts (Figure 3.3) labelled positively for all HSPG core proteins. Expression of glypican-1 (Figure 3.3 E1-2), glypican-3 (Figure 3.3 G1-2) and to a lesser extent syndecan-4 (Figure 3.3 D1-2), glypican-4 (Figure 3.3 H1-2) and perlecan (Figure 3.3 K1-2) staining was diffusely localised to the cytoplasm of LECs. Positive labelling for syndecan-3 (Figure 3.3 C1-2), glypican-2 (Figure 3.3 F1-2) and glypican-5 (Figure 3.3 I1) was distributed in irregular patches within the cytoplasm of LECs. Glypican-5 did not label rat lens epithelial explant cells (Figure 3.3 I2). A similar level of labelling was observed for syndecan-2 (Figure 3.3 B1-2), glypican-6 (Figure 3.3 J1-2), collagen XVIII (Figure 3.3 L1-2) and agrin (Figure 3.3 M1-2), with reactivity concentrated at the basal pole of LECs. Syndecan-1 staining in LECs was weak relative to other syndecans (Figure 3.3 A1-2), but levels increased in mitotic figures of actively proliferating cells (Figure 3.3 A2 inset). Enriched reactivity of the mitotic spindle was also observed for syndecan-4 (Figure 3.3, D2 inset) glypican-2 (Figure 3.3, F2 inset), glypican-6 (Figure 3.3, J2 inset), collagen XVIII (Figure 3.3, L2 inset) and to a lesser

extent glypican-1 (Figure 3.3, E2 inset) and agrin (Figure 3.3, M2 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 Figure 3.5). Syndecan-3 (Figure 3.3 C2) and glypican-3 (Figure 3.3 G2) were localised to apical cell junctions in LEC whole mounts. Syndecan-2 (Figure 3.3 B1-2, Figure 3.4 B1) and glypican-2 (Figure 3.3 F1-2, Figure 3.4 F1) labelled positively in a subset of LEC nuclei. Positive labelling 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 (Figure 3.4 D1), glypican-4 (Figure 3.4 H1), collagen XVIII (Figure 3.4 L1) and agrin (Figure 3.4 M1).

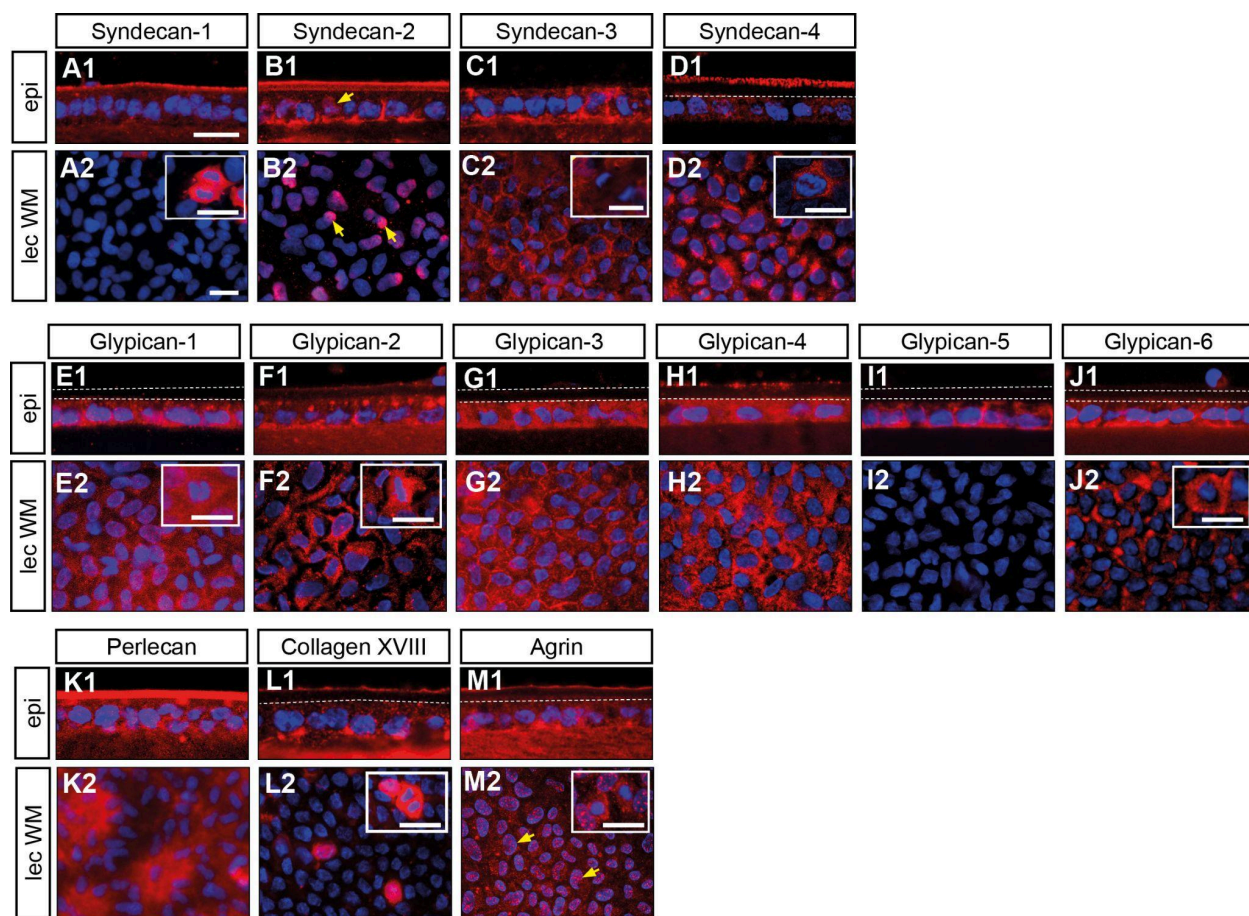


Figure 3.3. *Spatial localisation patterns of HSPG core proteins in the anterior lens capsule and epithelia. Representative immunofluorescence (red) of HSPG core proteins for Syndecan-1 to -4 (A-D), Glypican-1 to -6 (E-J), Perlecan (K), Collagen XVIII/endostatin (L) and Agrin (M) in sections (1-series) or epithelial whole mounts (lec WM, 2-series) of 10-day-old rat lens. Yellow arrowheads indicate nuclear localisation. Dotted white lines demarcate lens capsule. Cell nuclei counterstained with Hoechst (blue). Abbreviations: lens epithelium (epi). Scale bars = 25 μ m.*

3.3.6 *Lens fibre cells*

Posterior to the lens equator, lens epithelial cells in the transitional zone undergoing the early stages of fibre differentiation were labelled intensely for glypicans-1, -3, -5 and -6 (Figure 3.4 E1, G1, I1, J1). To a lesser degree, cells in the transitional zone also labelled for syndecans-3 and -4 (Figure 3.4 C1, D1), glypican-2 (Figure 3.4 F1) and perlecan (Figure 3.4 K1). Nuclear labelling for syndecan-2 (Figure 3.4 B1-2, yellow arrowheads) and glypican-2 (Figure 3.4 F1-2, yellow arrowheads) was also observed in a subset of nuclei in newly differentiating lens fibre cells. HSPG core proteins were observed in four predominant expression patterns in equatorial fibre cells. Syndecan-4 (Figure 3.4 D), and glypicans-1, -3, -5 and -6 (Figure 3.4 E, G, I, J) were uniformly found in the cytoplasm of fibre cells as diffuse punctae. Syndecan-3 (Figure 3.4 E), glypican-2 (Figure 3.4 F) and perlecan (Figure 3.4 K) were also highly labelled in the cytoplasm of fibre cells, with striations of enriched labelling in new secondary fibre cells. Syndecan-2 (Figure 3.4 B) and glypican-4 (Figure 3.4 H) signals in fibre cells were sparse and irregular. Agrin (Figure 3.4 M) and collagen XVIII (Figure 3.4 L) were concentrated at fibre cell membranes, with intensified labelling in the more mature fibres towards the nucleus of the lens. No label was observed for syndecan-1 (Figure 3.4 A) or glypican-5 (Figure 3.4 I) in lens fibre cells. Cell nuclear labelling for syndecan-2 (Figure 3.4 B1-2, yellow arrowheads) and glypican-2 (Figure 3.4 H2, yellow arrowheads) observed in lens epithelia persisted in a subset of fibre cells. Some nuclear labelling for other core proteins, including syndecan-4 (Figure 3.4 D2, yellow arrowheads), glypican-1 (Figure 3.4 E2, yellow arrowheads), collagen XVIII (Figure 3.4 K2, yellow arrowheads) and agrin (Figure 3.4 M2, yellow arrowheads) was observed in early differentiating secondary lens fibre cells. Syndecan-3 (Figure 3.3 C1, Figure 3.4 C1), glypican-2 (Figure 3.3 F1, Figure 3.4 F1) and agrin (Figure 3.3 M1, Figure 3.4 M1) appeared to be enriched

at the apical tips of fibre cells. On the other hand, labelling of syndecan-4 (Figure 3.4 D3), glypican-3 (Figure 3.4 G3) and glypican-6 (Figure 3.4 J3) was stronger at the posterior basal fibre tips. Conversely, glypican-5 staining was absent from both apical and basal fibre tips (Figure 3.3 I1, Figure 3.4 I3).

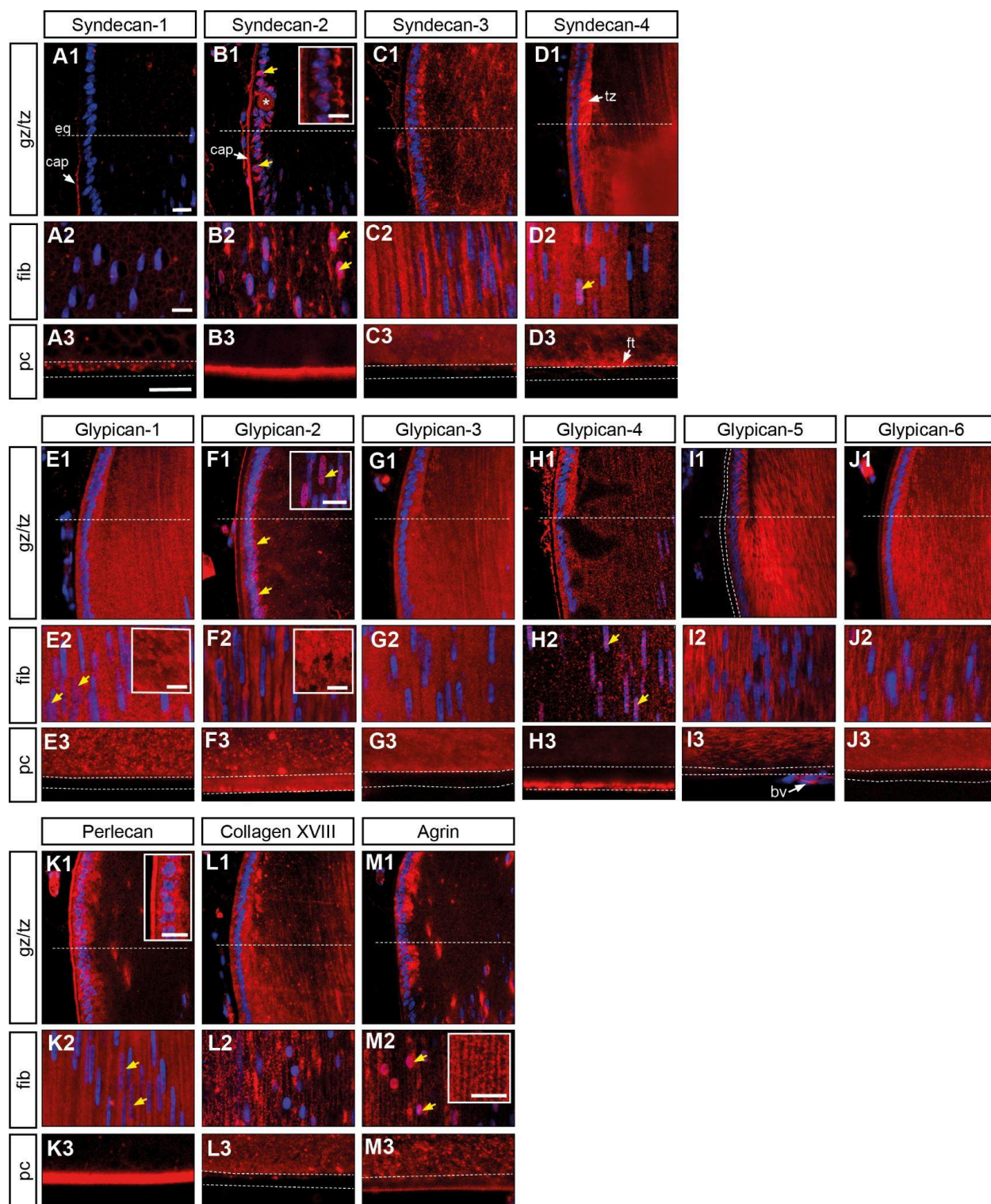


Figure 3.4. *Spatial localisation patterns of HSPG core proteins in the equatorial lens, cortex and posterior capsule. Representative immunofluorescence (red) of HSPG core proteins for Syndecan-1 to -4 (A-D), Glypican-1 to -6 (E-J), Perlecan (K), Collagen XVIII (L) and Agrin (M) in sections of the equatorial germinative/transitional zone (gz/tz, 1-series), outer cortical fibres (fib, 2-series), and posterior capsule (pc, 3-series) of 10-day-old rat lens. Yellow arrowheads indicate nuclear localisation. Dotted white lines demarcate lens capsule. Cell nuclei counterstained with Hoechst (blue). Scale bars = 25 μ m.*

3.3.7 Non-lenticular regions of the eye

In contrast to the lower expression levels for syndecan-1 observed in lens cells, strong labelling was found in the cornea (Figure 3.5 A), iridial stroma (Figure 3.6 A1-2), ciliary non-pigmented epithelium (Figure 3.6 A3), and in the vascular endothelium of the choroid and remnant tunica vasculosa lentis (data not shown). Nuclear labelling of both syndecan-2 and glypican-2 was not restricted to the lens, with both present in a subset of nuclei of corneal epithelial and stromal cells (Figure 3.5 B, F), iris, ciliary body and retina (Figure 3.6 B, E, K). Syndecan-3 and glypican-6 were absent from the corneal epithelium (Figure 3.5 C1, J1). Glypican-6 staining was high in the corneal endothelium (Figure 3.5 J3), with syndecan-3, syndecan-4, glypican-4, perlecan, collagen XVIII and agrin labelling delineating the limiting membranes of the iris (Figure 3.6). Iridial stromal cells were strongly positive for syndecan-2, glypican-2, glypican-4, perlecan and agrin (Figure 3.6 B1-2, E1-2, F1-2, H1-2, I1-2). Syndecan-4 alone appeared to be positively labelled in the myoepithelial cells of the sphincter and dilator pupillae of the iris (Figure 3.6 D1-2). Syndecans-1, -2 and -4 were selectively enriched in the non-pigmented epithelium of the ciliary body (Figure 3.6 A3, B3, D3). 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 (Figure 3.6 C3, E3, I3). Glypican-5 labelling was absent from all tissues investigated (Figure 3.5 I, Figure 3.6 G).

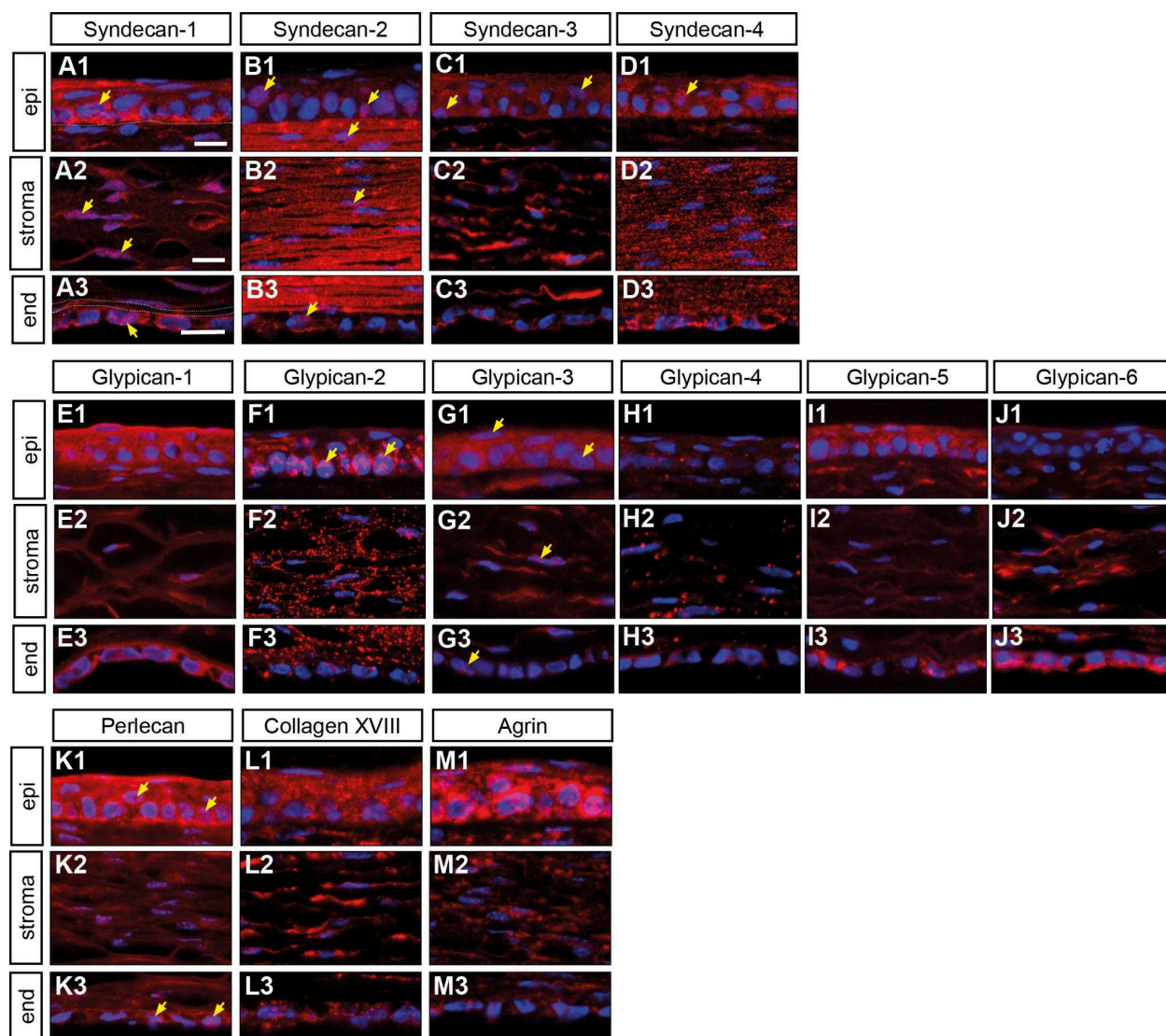


Figure 3.5. *Spatial localisation patterns of HSPG core proteins in the cornea. Representative immunofluorescence (red) of HSPG core proteins for Syndecan-1 to -4 (A-D), Glypican-1 to -6 (E-J), Perlecan (K), Collagen XVIII (L) and Agrin (M) in sections of corneal epithelia (epi, 1-series), stroma (2-series), and endothelium (endo, 3-series) of 10-day-old rat eyes. Yellow arrowheads indicate nuclear localisation. Cell nuclei counterstained with Hoechst (blue). Scale bars = 25 μ m.*

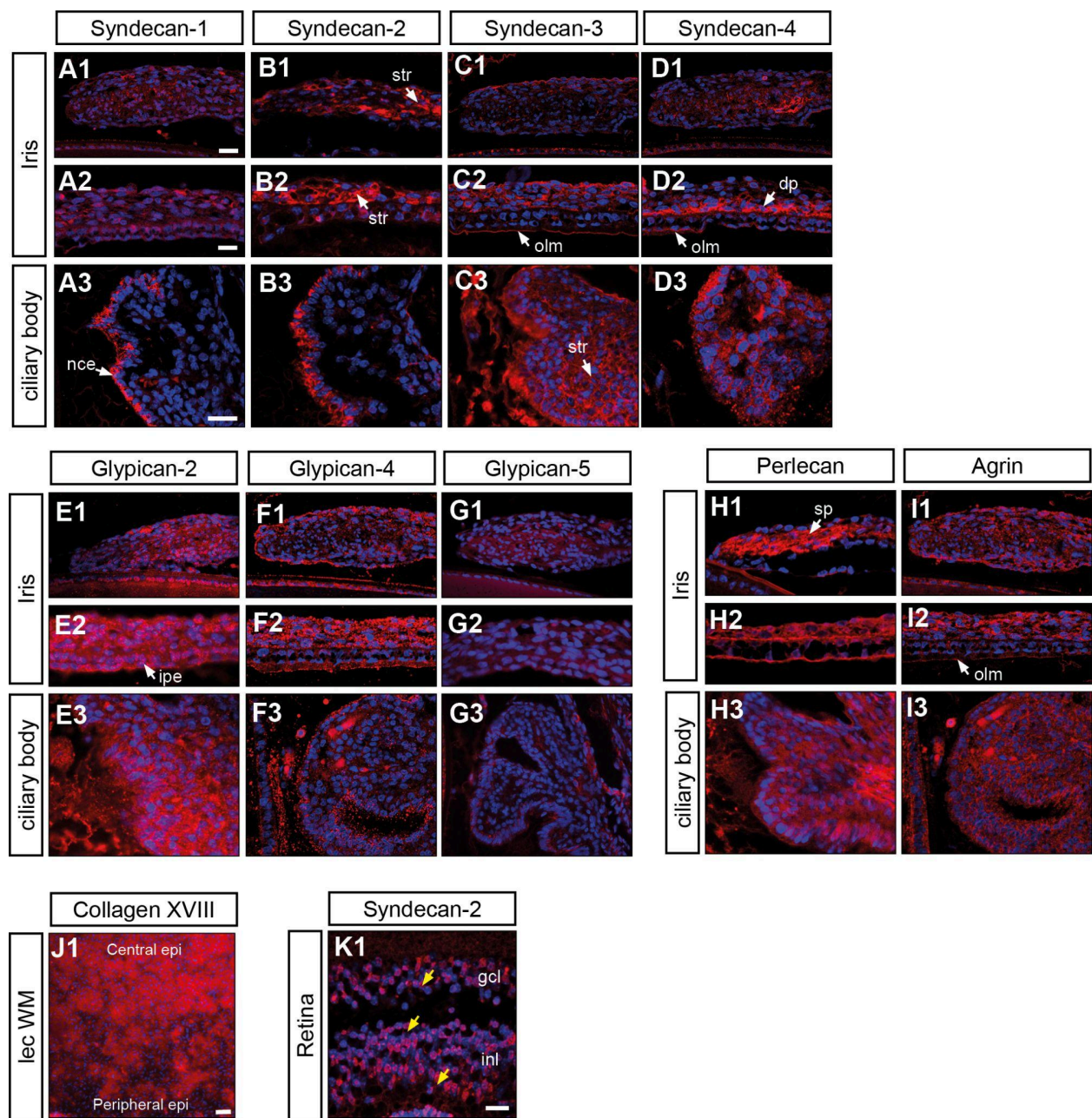


Figure 3.6. *Spatial localisation patterns of HSPG core proteins in the iris and ciliary body. Representative immunofluorescence (red) of HSPG core proteins for Syndecan-1 to -4 (A-D), Glypican-2 (E), Glypican-4 (F), Glypican-5 (G), Perlecan (H), and Agrin (I) in sections of iris (1- and 2-series), and ciliary body (3-series) of 10-day-old rat eyes. Immunolabelling is also shown for Collagen XVIII in lens epithelial whole mounts (lec WM, J) comparing central epithelia (epi) with peripheral epithelia, and for Syndecan-2 in sections of retina of 10-day-old rat eyes (K). Yellow arrowheads indicate nuclear localisation. Cell nuclei counterstained with Hoechst (blue). Abbreviations: stroma (str), outer limiting membrane (olm), posterior iris/dorsal pupillae (dp), non-pigmented ciliary epithelium (nce), anterior iris/sphincter pupillae (sp), iris pigmented epithelia (ipe), ganglion cell layer (gcl), inner nuclear layer (inl). Scale bars = 25 μ m.*

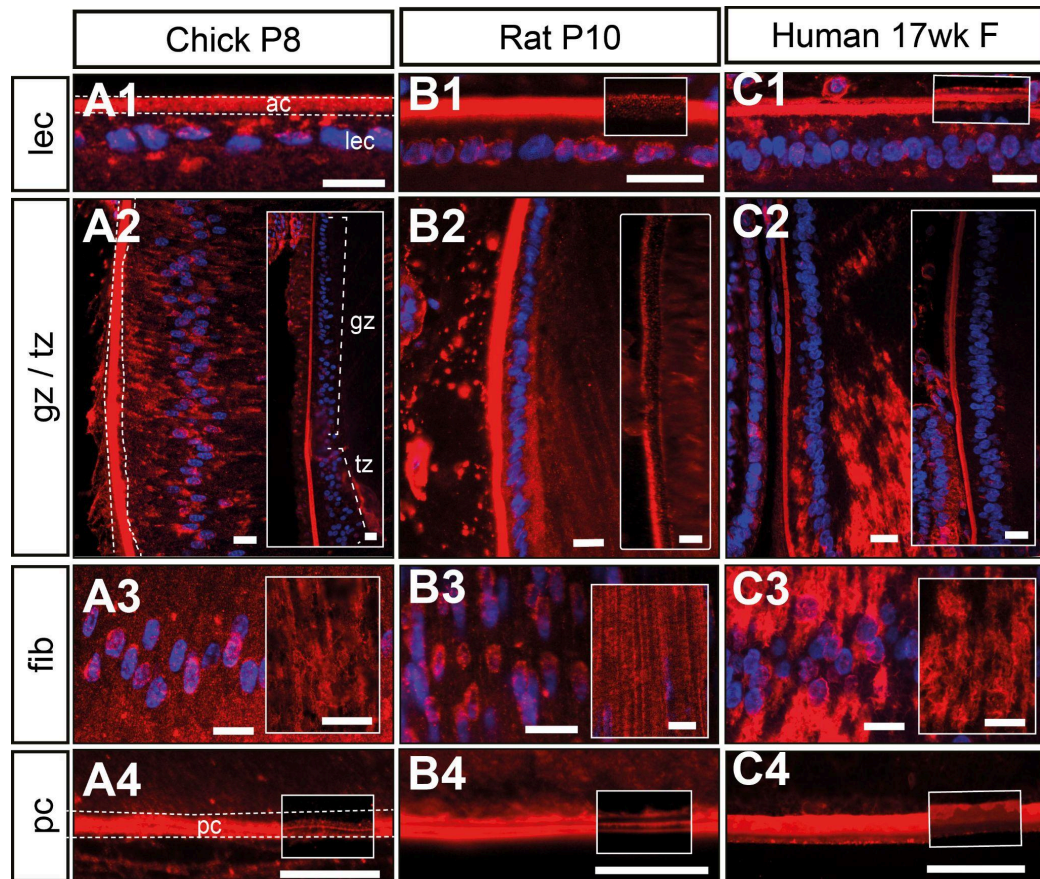


Figure 3.7. Heparan sulfate glycosaminoglycan immunolabelling in the lens across different vertebrate species. Mid-sagittal eye sections from 8-day-old chick (A1-4), 10-day-old Wistar rat (B1-4) and 17-wk-old human foetal eyes (C1-4) were labelled for sulfated heparan sulfate glycosaminoglycan (HS-10E4 antibody) in the lens. Insets represent images that were underexposed to show further detail of the HS-10E4 Ab label in the anterior (B1, C1), equatorial (A2, B2, C2) and posterior (A4, B4, C4) lens capsules. Dotted white lines delineate the lens capsule. Nuclei were counterstained with Hoechst dye (blue). Abbreviations: central anterior epithelium (lec) and capsule (ac), germinative zone (gz), transitional zone (tz), lens fibre cells (fib), posterior lens capsule (pc). Scale bars = 25 μ m (B1-4).

3.4 Discussion

Here we present the most comprehensive analysis examining the spatial localisation of all the different classes of HSPGs in the lens. Our identification of the differential cell compartment, cell type-, and functional regional-specific localisation of key lens HSPGs highlights the lens as an ideal model in which to study the diverse roles of these different proteoglycans. The divergent localisation and expression patterns of HSPG core proteins in the lens suggests unique and diverse roles for different HSPGs in the regulation of lens cell processes, including lens epithelial cell proliferation and fibre differentiation.

3.4.1 The lens expresses all HSPG core proteins

In this study, we found all HSPGs to be expressed in the lens, in agreement with other postnatal HSPG transcript expression profiles from published mouse lens datasets (Hoang et al. 2014, Khan et al. 2015, Audette et al. 2016, Cavaleiro et al. 2017, Kakrana et al. 2018), with the exception of the expression of *Gpc5* (encoding the glypican-5 core protein). Our observation of lack of *Gpc5* in the lens is consistent with other lens transcriptome datasets. Other lens transcriptome/microarray datasets report very low expression (lowest of all HSPGs) of *Gpc5* transcripts at similar ages [GEO series GSE69221] (Khan et al. 2015), [GEO series GSE100136] (Kakrana et al. 2018). Similarly, *Gpc5* mRNA is low or undetected in the developing lens [GEO series GSE69940] (Audette et al. 2016). We detected *Gpc5* mRNA using a predesigned TaqMan Gene Expression Assay against the *Gpc5* sequence and fluorogenic 5' nuclease (TaqMan) chemistry, which has been demonstrated to detect transcript levels with high specificity, sensitivity and reproducibility (Arikawa et al. 2008), however, when a similar analysis was carried out using SYBR Green-based PCR and experimentally validated *Gpc5* primers, we too failed to see any expression in lens (data not shown). Taken together, our findings suggest that all

HSPG core protein transcripts are expressed in the lens, likely with the exception of *Gpc5*. Broad expression profiles of HSPG core proteins are not uncommon and have been reported for various other tissues and cell-types. For instance, human corneal stromal cells express transcripts for all HSPG core proteins, with the exception of *Gpc3* (Garcia et al. 2016).

HS is the predominant sulfated GAG in the postnatal murine lens, in accordance with previous studies (Hay et al. 1974, Mohan et al. 1991). The overlapping and divergent labelling patterns of HS and CS in the lens (e.g. in new differentiating secondary lens fibres) imply that these GAGs may both contribute to the regulation of some functional processes in the lens (e.g. fibre differentiation), while 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 stabilisation of existing synaptic connections, while the primary function of HS is to stimulate the formation of new synapses (Maeda et al. 2011). Given HS and CS each interact selectively with different proteins in a structure-dependent manner (Vallet et al. 2020), it is not surprising that they are often reported to be differentially localised (Smetsers et al. 2003, Clark et al. 2011, Maeda et al. 2011), consistent with their distinct functional roles (Karamanos et al. 2018).

3.4.2 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 MMPs. Both FGF (Schulz et al. 1993, McAvoy et al. 1997, Wu et al. 2014) and HSPGs (Heathcote et al. 1984, Rossi 2003, Wu et al. 2014) have

been localised to the lens capsule. FGF-1 and FGF-2 are co-localised with HSPGs in the capsule in an increasing antero-posterior gradient (McAvoy et al. 1997, Wu et al. 2014). FGF-2 release from the lens capsule by MMPs, which can free HS-bound growth factors (Koyama et al. 2008, Jang et al. 2016), is essential for lens epithelial cell viability and survival (Tholozan et al. 2007). Consistent with previous studies (Wang et al. 2009, Zhao et al. 2018, Zhao et al. 2018), we found that perlecan is the predominant HSPG of the lens capsule. We also localised agrin and collagen XVIII to the lens capsule, though to a lesser extent than perlecan, consistent with recent proteomic analyses of human lens capsules (Uechi et al. 2014). Moreover, the core proteins of these HSPGs are analogous in size to those of two unidentified HSPGs previously isolated from the calf lens capsule (Mohan et al. 1991). These three HSPGs (perlecan, agrin and collagen XVIII) 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 (Groffen et al. 1998ce, Neill et al. 2015). Previous studies have reported an increasing antero-posterior gradient of FGF-2, HS and perlecan in the lens capsule (Chamberlain et al. 1997), 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 (Rossi 2003), indicating that perlecan HS chains may also play an important structural role in the capsule. Our immunolocalisation 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 (Qin et al. 2017), this is the first report of these HSPGs in the lens capsule. Though most often localised 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 (Elenius et al. 1990, Kusano et al. 2000, Tumova et al. 2000), laminin (Lovicu et al. 2001, Iyengar et al. 2006, Wang et al. 2009) and collagen IV (Esko & Selleck 2002, Filla et al. 2004, Esko et al. 2017). Laminin and collagen IV are major components of the lens capsule, and promote the adhesion and migration of lens epithelial cells *in vitro* (Olivero et al. 1993, Parmigiani et al. 2009). Syndecan-2 is co-distributed with fibronectin, laminin and collagen IV in human trabecular meshwork and Schlemm's canal cells (Filla et al. 2004), and plays an important role in regulating the assembly of ECM components (deLongh et al. 1996, Lovicu et al. 2001). Syndecans-1 and -4 have been reported to promote cell adhesion through binding interactions with several ECM proteins native to the lens capsule (Uechi et al. 2014), including collagen IV (Esko et al. 2002, Esko et al. 2017) and laminin (Utani et al. 2003, Carulli et al. 2012), with syndecan-4 recently identified as essential for integrin signalling-dependent cell migration of lens epithelial cells (Qin et al. 2017).

3.4.3 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 labelling for the different HSPGs varied at the level of the subcellular compartment and functional regions of the lens epithelium (anterior vs. equatorial). The subcellular localisation of HSPGs in anterior lens epithelial cells can be categorised broadly as either diffusely cytoplasmic (syndecans-1/-4, glypicans-3/-4, perlecan), or apically polarised (syndecans-2/-3, glypicans-1/-2/-6, collagen XVIII, agrin). Further characterisation in lens epithelial whole mounts revealed more detailed localisation patterns at the apical surfaces of the epithelial cells including accumulation at cell-cell interfaces (syndecan-3, glypican-3), within cell nuclei

(syndecan-2), and to perinuclear regions (syndecan-4, glypicans-2/-6). This differential localisation 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 localisation at the cell surface and within the pericellular matrix, HSPGs can also be found in more unexpected cellular compartments (reviewed in Couchman et al. 2012 and Kolset et al. 2004). Beyond their predictable associations with organelles involved in their biosynthesis (endoplasmic reticulum) and glycosylation (Golgi apparatus), HSPGs have been reported to localise within the cytoplasm (Shworak et al. 2011, Zhu et al. 2002, Miyake et al. 2014, Sun et al. 2019), actin cytoskeleton (Granes et al. 2000, Stigliano et al. 2009), vesicular trafficking networks (Kolset et al. 2004, Matsuo et al. 2014), and cell nuclei (Brockstedt et al. 2002, Chen et al. 2009). The localisation of HSPGs to specific subcellular compartments is functionally relevant (Kolset et al. 2004, Couchman et al. 2012). The strong diffuse cytoplasmic label we observed is most evident for syndecans-1/4 and glypicans-2/3. The cytoplasmic localisation of these HSPGs has previously been reported in other cell types, including skin (Shworak et al. 2011), esophagus (Zhu et al. 2002) and cancer cells of the endometrium (Hakim et al. 2015) and bladder (Miyake et al. 2014). Best characterised, is the diffuse localisation of glypican-3 to the cytosol of liver cancer cells (Akutsu et al. 2010, Li et al. 2019), where it drives the mitogenic activity of these cells by regulating the subcellular localisation of β -catenin (nuclear vs. cytoplasmic) to modulate the activation of canonical Wnt/ β -catenin signalling (Capurro et al. 2014, Kolluri et al. 2019). Other members of the glypican family are also well-known regulators of canonical Wnt signalling (Fico et al. 2011). In the lens, canonical Wnt signalling is involved in both the proliferation and

maintenance of the lens epithelium and in the regulation of fibre cell differentiation (Chen et al. 2008, Dawes et al. 2013).

Syndecans-2/-3 and glypicans -1/-2/-6 are localised predominantly to the apical pole of anterior lens epithelial cells. We also observed regions of the lens epithelium with concentrated basal labelling for syndecan-3 (Figure 3.3, C1), glypican-2 (Figure 3.3, F1) and Perlecan (Figure 3.3, K1). HSPGs are commonly reported to localise specifically to either the apical or basolateral membrane in polarized epithelial cells. For instance, syndecan-1 is often restricted to the basolateral region where it interacts with PDZ-binding proteins such as ZO-1 to maintain an epithelial phenotype. On the other hand, glypicans are usually targeted to the apical surface in polarized epithelial cells (Mertens et al. 1996). The mechanisms by which different HSPGs achieve specific polar distribution are diverse and have only been investigated in detail for a few core proteins. The intracellular localisation of HSPGs may be the result of HSPG-specific protein-binding interactions, secretion, signalling or sorting based on HS-conjugation.

HSPG-protein binding interactions are a primary determinant of HSPG targeting, and these interactions may be unique to specific core proteins, or families of HSPGs (e.g. core protein binding interactions) or ubiquitous among HSPGs (e.g. HS chain binding interactions). For instance, the basal localisation of syndecan-1 in epithelial cells is linked to its interaction with other proteins via its cytoplasmic PDZ-binding domain (Maday et al. 2008). PDZ proteins are also 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 bind several proteins, including integrins¹⁰⁵, and cytoskeletal components. This is a likely explanation for the basal labelling of

syndecan-3. Another possible explanation for the localisation of HSPGs to the apical pole of lens epithelial cells may imply a role in the regulation of events or interactions at the epithelial-fibre interface. For instance, members of the syndecan family are highly expressed at the epithelial-fibre interface and/or anterior and posterior poles of the lens fibre tips, similar in expression to F-actin (Cheng et al. 2017) and $\beta 1$ integrin (Fuerst et al. 2007), that are all known to interact directly with, and/or are regulated by syndecans (Couchman et al. 2012). On the other hand, HS-growth factor receptor complexing/activation and internalisation may target several different HSPGs to different intracellular compartments for signalling, degradation, or recycling (Kolset et al. 2004).

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

Another possible reason for the polar nature of HSPG localisation may be related to HS-chain conjugation. Glypican HSPGs have been reported as the major source of HS localised to the apical pole of epithelial cells (Poulain et al. 2015). The apical localisation of glypican HSPGs in polarised epithelial cells is inversely related to the number of HS chains they carry (Rossi et al. 2003). In MDCK and CaCo2 epithelial cells, glypican HSPGs were transported predominantly to the basolateral membrane, whereas glypican lacking HS attachment sites was sorted to the apical surface (Mertens et al. 1996) .

The intensity of sulfated HS labelling 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 whole mounts, some HSPGs labelled strongly in actively mitotic cells (syndecans-1/-4, glypicans-2, collagen XVIII). 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.

3.4.4 Localisation of HSPGs in fibre cells

Several studies have demonstrated that organisation of the actin cytoskeleton plays an important role in lens fibre differentiation (Weber et al. 2006), as destabilising the actomyosin cytoskeleton or disrupting RhoA/ROCK/Rac-signalling-activity in cultured lens cells with inhibitors leads to impaired fibre cell migration, elongation and survival (Khurana et al. 2002, Maddala et al. 2003, Weber et al. 2006). Syndecan-4 can signal to the cytoskeleton, primarily through PKC α that binds directly to the syndecan-4 core protein (Oh et al. 1997, Horowitz et al. 1998).

Phosphorylation of the syndecan-4 core protein is also sufficient for the assembly of focal adhesions and actin stress fibres (Echtermeyer et al. 1999). Integrin signalling is key in several lens processes in morphogenesis and for lens maintenance throughout life (Walker et al. 2009). In particular, β 1 integrin-signalling, that has been shown to be regulated by syndecans-2 and -4 (Couchman et al. 1999, Kusano et al. 2000, Filla et al. 2006) and potentially other syndecans (Morgan et al. 2007), is essential for maintenance of the lens epithelium and for the structural maintenance and homeostasis of differentiating fibre cells (Simirskii et al. 2013).

Downregulation of syndecan-4 suppressed β 1 integrin-mediated activation and cell adhesion in

human lens epithelial cells *in vitro*, and in syndecan-4-deficient murine lenses (Qin et al. 2017). The association of syndecan-3 with fibre cells may relate to the association of this syndecan core protein with Src kinase and cortactin (Wu et al. 2014hb), that regulate fibre differentiation in concert with Ephrin-signalling (Dealy et al. 1997, Galli et al. 2003, Zhang et al. 2003, Shimokawa et al. 2011). Moreover, *in vitro* the syndecan-3 core protein has been reported to be phosphorylated by Ephrin receptor activation (Bernfield et al. 1992).

3.4.4 HSPGs in the lens cell nuclei

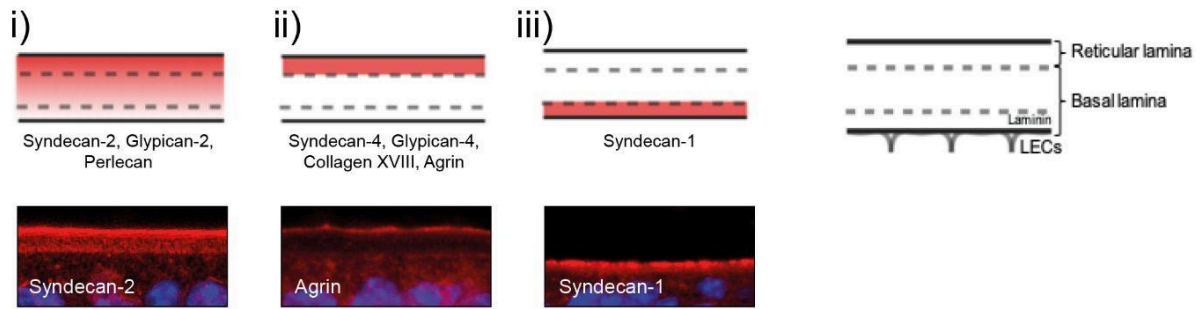
In intact rat eyes, we observed nuclear localisation of HS in newly differentiating secondary lens fibres. In agreement with this, *in vitro* we observed HS translocating to the nuclei of lens epithelial cells undergoing FGF-induced lens fibre differentiation. Nuclear HS was not observed in untreated 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 localise to the nuclear compartment of various cell-types, although the functions of HS/HSPGs in the nucleus are yet to be completely understood (Kovalszky et al. 2014, Stewart et al. 2014). 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 et al. 2014). 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 (Maher et al. 1996), where it can influence both proliferation and differentiation in a cell type-specific manner (Amalric et al. 1994). FGFR-1 has been shown to be localised to the membrane and nuclei of epithelial lens cells and differentiating fibre cells (deLongh et al. 1996), suggesting possible internalisation and trafficking of the receptor to the nucleus.

HSPGs also can co-localise with FGF-2 in the nuclei of cells. Syndecan-1 and FGF-2, but not FGFR-1 co-localise in the nuclei of mesenchymal cancer cells (Zong et al. 2009). CHO cells defective in the ability to properly synthesise heparan sulfate chains showed reduced nuclear FGF-2 (Vallet et al. 2020). 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 (David et al. 1992). Nuclear HS/HSPGs have also been reported to negatively regulate cell proliferation by initiating cell-cycle arrest (Fedarko et al. 1986, Fedarko et al. 1989, Szatmari et al. 2017). Syndecan-4 regulates FGF-2 signalling not only through PI3K/Akt and ERK1/2 activation, but also via internalisation and nuclear localisation (Jang et al. 2012). Syndecan-1 nuclear localisation of FGF-2 in corneal stromal fibroblasts is mediated by HSPGs through a protein kinase C α -dependent-signalling mechanism (Ito et al. 2005). During muscle cell differentiation, cell surface syndecan-4 is internalised and trafficked to cell nuclei, where it is suggested to play a regulatory role in the expression of β 1-integrin and actin (Ronning et al. 2015). 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 localisation sequence in its cytoplasmic domain, however, syndecan-2 has previously been reported in the nuclei of chondrosarcoma cells (Schrage et al. 2009), and co-localised with nuclear FGF-2 in neural cells (Leadbeater et al. 2006). Taken together, these observations suggest that the nuclear localisation of syndecan-2 is likely mediated through its interaction with other proteins (e.g. FGF-2, FGFR-1).

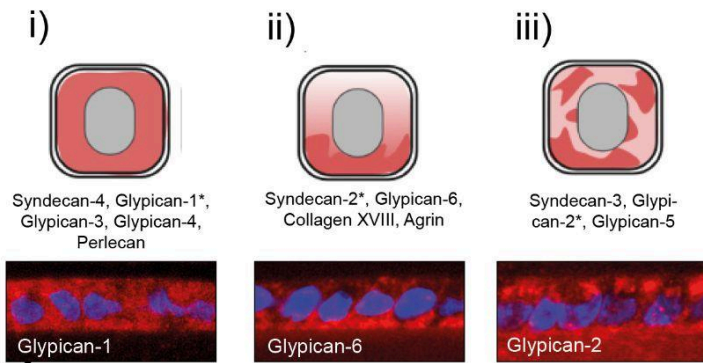
3.4.5 Conclusions

The lens epithelium, fibre cells and lens capsule display unique and distinct labelling 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 localisation (lens epithelium and fibre 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 work highlight that different HSPGs may play distinct roles in lens function and growth processes.

A



B



C

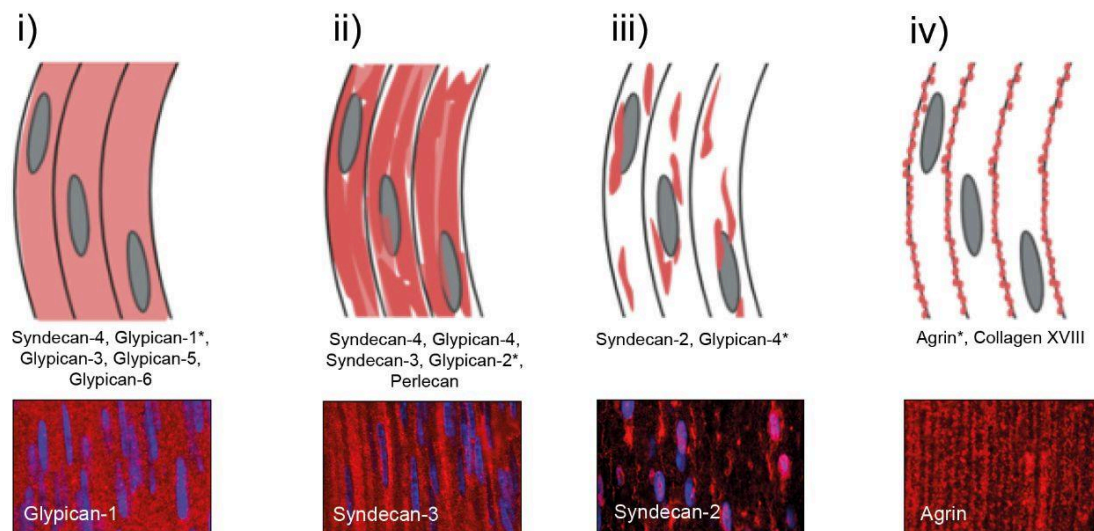


Figure 3.8. *Spatial localisation patterns of HSPG core proteins in lens cells. Comparative schematic of immunolocalisation patterns of HSPG core proteins in the capsule (A), anterior epithelium (B) and equatorial fibre cells (C) of the 10-day-old Wistar rat lens. * indicates nuclear labelling (Adapted from Wishart and Lovicu, 2021).*

Table 3.1. Summary of postnatal HSPG immunolocalisation.

Lens	Lens capsule	Syndecan-1, Syndecan-2, Syndecan-4, Glypican-2, Glypican-4, Perlecan, Collagen XVIII, Agrin
	Anterior lens epithelial cells	(Syndecan-1), Syndecan-2, Syndecan-3, Syndecan-4, Glypican-1, Glypican-2, Glypican-3, Glypican-4, Glypican-5, Glypican-6, Perlecan, Collagen XVIII, Agrin
	Germinative zone / transitional zone	Syndecan-2, Syndecan-3, Syndecan-4, Glypican-1, Glypican-2, Glypican-3, Glypican-4, Glypican-5, Glypican-6, Perlecan, Collagen XVIII, Agrin
	New lens fibre cells	Syndecan-2, Syndecan-3, Syndecan-4, Glypican-1, Glypican-3, Glypican-4, Glypican-5, Glypican-6, Perlecan, Agrin
	Lens fibre cell nuclei	Syndecan-2, Syndecan-4, Glypican-1, Glypican-2, Glypican-4, Agrin
	Mature lens fibre cells	Syndecan-3, Syndecan-4, Glypican-1, Glypican-2, Glypican-3, Glypican-5, Glypican-6, Collagen XVIII, Agrin

Cornea	Corneal epithelium	Syndecan-1, Syndecan-2, Syndecan-4, Glypican-1, Glypican-2, Glypican-3, Glypican-5, Perlecan, Collagen XVIII, Agrin
	Bowman's capsule	Syndecan-2
	Stroma (*Keratocytes)	Syndecan-1*, Syndecan-2, Syndecan-3*, Syndecan-4, Glypican-1*
	Descemet's membrane	Perlecan
	Corneal endothelium	Syndecan-1, Syndecan-2, Syndecan-4, Glypican-1, Glypican-5, Glypican-6
Iris	Iridial stroma	Syndecan-2, Syndecan-3, Glypican-2, Perlecan, Agrin
	Iridial basement membrane	Syndecan-3, Syndecan-4, Gpc2, Glypican-4, Perlecan, Agrin
	Sphincter pupillae / sphincter dilator	Syndecan-4
	Iridial epithelium	Glypican-2

Ciliary body		Syndecan-3, Syndecan-4, Glypican-2, Perlecan, Agrin
	Non-pigmented ciliary epithelium	Syndecan-1, Syndecan-2
	Inner limiting membrane	Glypican-4

Syndecan (sdc), glypican (gpc), perlecan (Perl), collagen XVIII (Col18a1), agrin (Agrn)

CHAPTER 4: RESULTS II

SPATIOTEMPORAL LOCALISATION OF HSPGS THROUGHOUT MURINE LENS MORPHOGENESIS

4.1 Preface

The following manuscripts comprise the majority of the following results chapter. Some sections of the following manuscript have been reordered and/or edited for the purposes of elaboration or continuity. Original unedited manuscripts are attached in appendix 1.

TFL Wishart and FJ Lovicu (2023). Spatiotemporal Localisation of Heparan Sulphate Proteoglycans throughout Mouse Lens Morphogenesis. *Cells* (DOI: 10.3390/cells12101364).

This manuscript was published in *Cells* in 2023. The work comprising these manuscripts was completed entirely during my PhD candidature under the guidance of my primary supervisor Professor Frank Lovicu who contributed to this manuscript by facilitating experimental design and manuscript editing.

4.2 Introduction

The development and maintenance of the vertebrate lens throughout life is orchestrated by the interplay of a complex network of signalling molecules within the native ocular environment (Lovicu et al. 2005). Decades of research have identified these key molecules and characterised their differential and complementary roles in the coordination of specific morphogenetic events and cell behaviours (Cvekl et al. 2014, Cvekl et al. 2017). Secreted primarily from the different ocular tissues, including the lens, retina and ciliary body, these diffusible growth factors,

including members of the FGF (McAvoy et al. 1991,, Faber et al. 2001, Robinson et al. 2006), Wnt (DeIongh et al. 2006, Chen et al. 2008, Sugiyama et al. 2011), BMP (Faber et al. 2002, Shu et al. 2021) and Hedgehog (Hh) (Tsonis et al. 2004) families found in the ocular milieu influence lens cell behaviour (i.e. proliferation, migration, differentiation, and cell death(Lovicu et al. 2005jg). For the defined stages of lens morphogenesis, these factors need to be tightly controlled in a spatial and temporal manner to regulate the induction, and anteroposterior, left–right and dorsoventral patterning of the lens (Cvekl et al. 2014, Cvekl et al. 2017). Although the many distinct and overlapping effects of these factors on lens cell behaviour are well-characterised, the mechanisms underlying how these growth factors traverse the extracellular space, arrive precisely at, and transmit signalling within target cells at the appropriate level, remain elusive. Recent studies in mice and other model animals have revealed the importance of HSPGs, that are localised to the cell membrane and in the ECM of tissues, to promote the signalling and extracellular distribution of growth factors, respectively (Sarrazin et al. 2011).

Downstream effects of HSPG-protein interactions include the activation of high affinity receptor signalling complexes, and mediation of intracellular signalling cascades leading to the activation of key effector genes and changes in cell behaviour and phenotype (Bishop et al. 2007, Sarrazin et al. 2011). The pleiotropic modulation of growth factor receptor signalling by HSPGs (altering ligand density and activity), can stabilise mitogen and morphogen gradients locally and at the tissue level (Bishop et al. 2007). These gradients are critical to organogenesis and for the maintenance of normal tissue homeostasis (Hufnagel et al. 2006). As the activity of HSPGs depends primarily on its fine structure (i.e. sulfation) and location (i.e. the cell surface or ECM) of their HS glycosaminoglycan chains, the specific activity of HSPGs in a given cell-type or

tissue at a given time can be determined by its HSPG profile et al. localisation of HSPG core proteins and its complement of HS sulfation enzymes (Bishop et al. 2007, Sarrazin et al. 2011). Previously, we have shown all HSPG core proteins are present in the postnatal rat lens, localised to its epithelia, fibres and basement membrane, the lens capsule, in unique and distinct expression patterns that align with key functional regions (See chapter 3). This differential spatial and temporal expression of HSPG core proteins in the lens suggests that lens cells may acquire a distinctive repertoire of HSPGs as they differentiate, resulting in functional patterns of HS-protein interactions and activity during lens morphogenesis. Here we report for the first time the comprehensive spatial and temporal localisation of all HSPGs in the developing murine lens, highlighting putative roles for specific HSPGs during key stages of lens development.

While we know much of vertebrate lens morphogenesis, less is understood about the regulation of the growth factors driving this development. Given the important role of HSPGs in regulating diverse growth factors, and with recent studies implicating HSPGs as essential for the induction and development of the lens (Rossi et al. 2003, Pan et al. 2006) and accessory glands (Qu et al. 2011), to identify the specific roles of the diverse HSPG core proteins, here, we characterise the most comprehensive spatiotemporal localisation of key HSPGs throughout lens development, the first known study to map the HSPG expression and localisation profile of a developing tissue over the course of organogenesis.

The differential spatial and temporal expression of HSPG core proteins suggests that lens cells acquire a distinctive repertoire of HSPGs as they differentiate, resulting in selective patterns of HS-protein interactions and activity. This implies differential roles for HSPGs and HS sulfation

enzymes at key stages of lens induction and morphogenesis. Taken together with the current literature, our findings support a requirement for HS sulfation in growth factor-induced cellular processes in the lens.

4.3 Results

A complete summary of the labelling for HS-GAG, CS-GAG, PAPSS2 and all thirteen HSPG core proteins during murine lens development is outlined in Table 4.1.

4.3.1 HS-sulfation enzymes in the developing and postnatal lens.

We immunolabelled for PAPSS2 (HSPG sulfation) in the developing mouse lens (Figure 4.1), and HS-2-/3-6- O-sulfotransferases in the 10-day-old rat lens (Figure 4.2). PAPSS2 reactivity was absent from all early eye structures, including cells comprising the lens placode (E9.5, Figure 4.1 A1), and lens pit (E10.5, Figure 4.1 A2). It was not until E11.5, in the lens vesicle that PAPSS2 immunolabeling appeared, strongly localised to the elongating primary lens fibre cells (Figure 4.1 A3). This fibre-specific label persisted and was also observed in the early secondary lens fibre cells at E14.5 (Figure 4.1 A4-7) and E16.5 (Figure 4.1 A8-10). At E16.5, PAPSS2 reactivity was also evident in the anterior lens epithelium but was not present in the lens capsule (Figure 4.1 A9).

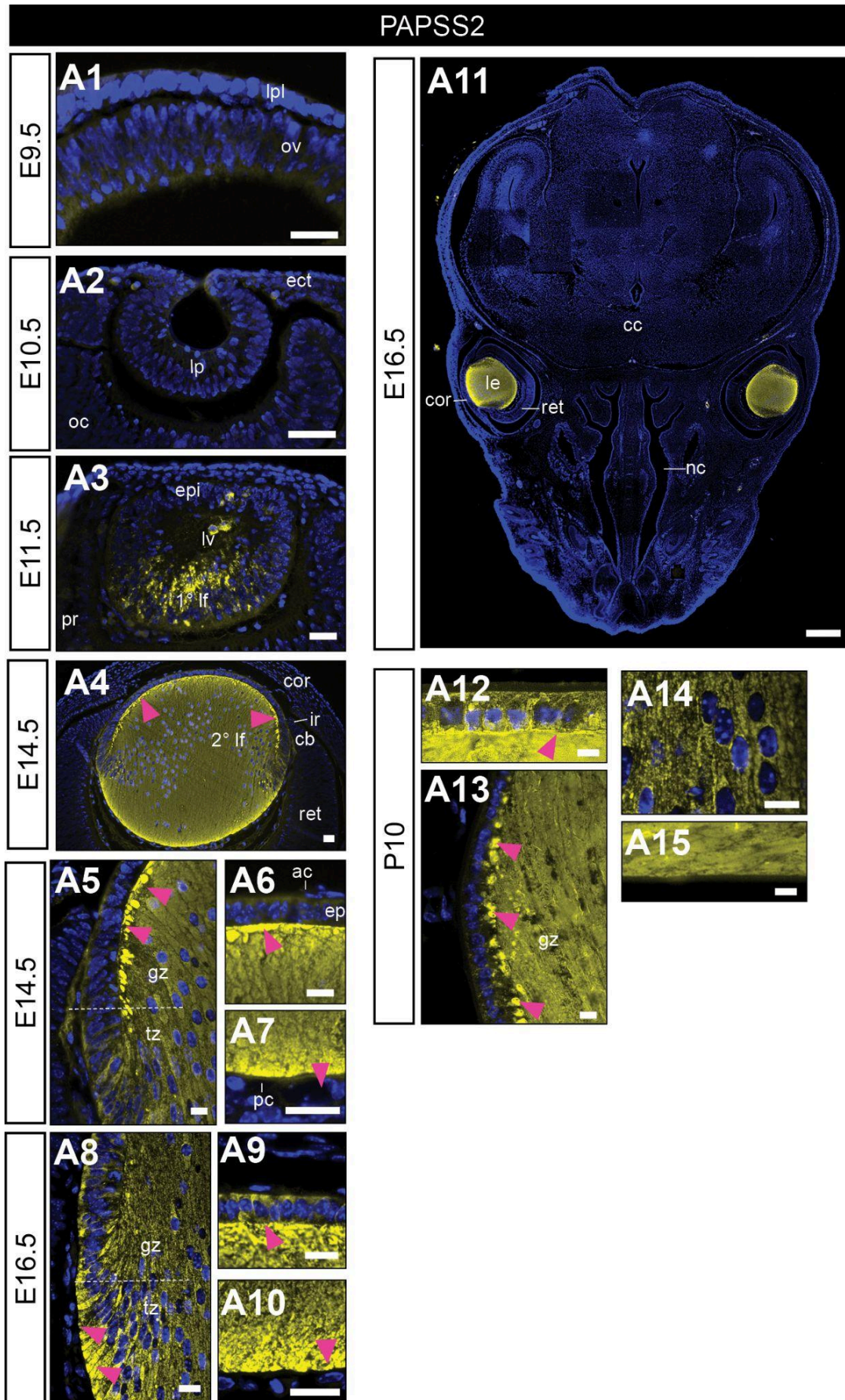


Figure 4.1. Immunolocalisation of PAPS Synthetase-2 (PAPSS2) in the murine eye. Mid-sagittal mouse (FvB/N) eye sections from embryonic day-9.5 (E9.5, A1), E10.5 (A2), E11.5 (A3), E14.5 (A4-7) and E16.5 (A8-11), and 10-day-old (P10) Wistar rat lens (A12-15) labelled for 3'-Phosphoadenosine 5'-Phosphosulfate Synthase 2 (PAPSS2, yellow). Nuclei counterstained with Hoechst (blue). Dotted white lines indicate lens equator (A5, A8). Magenta arrowheads indicate localisation at lens fibre tips (A5-10). Cornea (cor), lens (le), retina (ret), cerebral cortex (cc), lens placode (lpl), optic vesicle (ov), head ectoderm (ect), lens pit (lp), optic cup (oc), lens vesicle (lv), anterior lens epithelium (epi), primary lens fibre cells (1° lf), secondary lens fibre cells (2° lf), cornea (cor), retina (ret), ciliary body (cb), iris (ir), germinative zone (gz), transitional zone (tz), anterior lens capsule (ac), posterior lens capsule (pc). Scale bars = 200 μ m (A11), 25 μ m. Images in figures are oriented with ventral side positioned to the right and the dorsal side to the left.

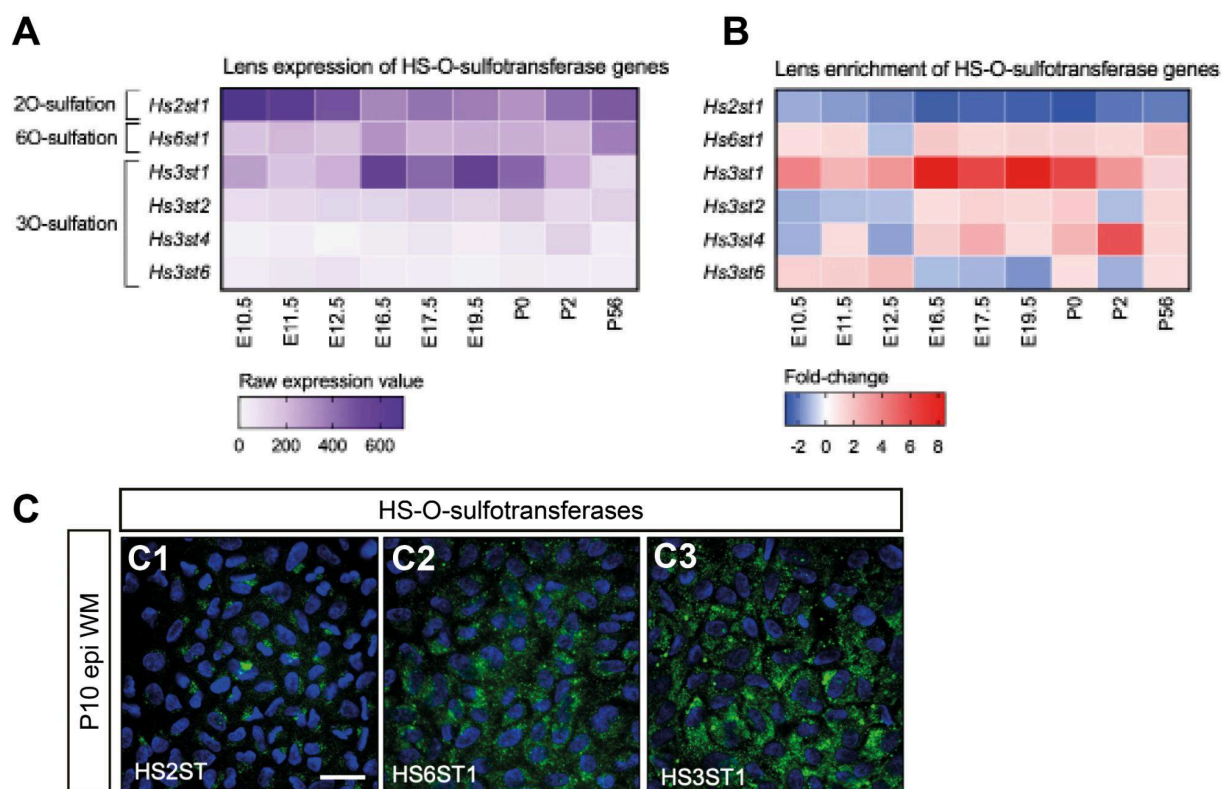


Figure 4.2. Heparan sulfate O-sulfotransferase expression in the murine and rat lens. Transcript expression (A) and lens-enrichment (B) profiles of genes encoding heparan sulfate- 2O- (Hs2st1), 6O- (Hs6st1) and 3O- (Hs3st1, Hs3st2, Hs3st4, Hs3st6) sulfotransferase genes in Affymetrix microarrays from mouse lenses at embryonic stages 10.5 (E10.5), E11.5, E12.5, E16.5, E17.5 and E19.5, and postnatal days 0 (P0), P2 and P56. Lens expression and enriched expression data represent processed normalised microarray data from Affymetrix Mouse 430 2.0 platform sourced from the iSyTE (integrated Systems Tool for Eye gene discovery) web-tool (Kakrana et al., 2018). A) Heat map intensity (purple) represents high to low gene expression. B) Lens-enrichment heat map reflects high (red) to low (blue) fold-change in gene expression relative to age-matched aphakic whole embryo control (in silico subtraction). C) Localisation of heparan sulfate O-sulfotransferases heparan sulfate 2-O-sulfotransferase (HS2ST, C1), heparan sulfate 6-O-sulfotransferase-1 (HS6ST1, C2), and heparan sulfate 3-O-sulfotransferase-1 (HS3ST1, C3) in 10-day-old (P10) Wistar rat lens epithelial (epi) whole mounts (WM). Nuclei were counterstained with Hoechst dye (blue). Abbreviations: Scale bar = 25 μ m (C1-3).

4.3.2 Heparan sulfate is the predominant sulfated glycosaminoglycan expressed throughout murine lens development.

HSPG core proteins can carry HS-GAG chains, or a combination of both HS and CS chains. To determine which of these two GAG species represents the major “active” sulfated fraction of HSPGs in the developing lens, the distribution of HS and CS was compared during major stages of lens development (Figure 4.3 A1-8, 3 B1-8) using antibodies specific for the sulfated domains on HS (HS-10E4, Figure 4.3 C1-8) or CS (CS-56, Figure 4.3 D1-8) GAG chains. HS-GAG chain labelling was first observed at very low levels in the cells of the lens placode (E9.5, Figure 4.3C1) with stronger labelling in the lens pit (E10.5, Figure 4.3 C2). At E11.5 (Figure 4.3 C3) HS-GAG labelling was prominent in the developing lens capsule, anterior lens epithelium and ubiquitously in elongating primary lens fibre cells. HS-GAG reactivity was maintained in the lens capsule and cytoplasm of anterior epithelial cells at E14.5 (Figure 4.3 C4) and E16.5 (Figure 4.3 C5-8). HS-GAG chain labelling was strongest throughout the cytoplasm of secondary lens fibre cells (Figure 4.3 C4), with decreased reactivity at the germinative and transitional zones (Figure 4.3 C4, C8). Concentrated immunolabelling of HS-GAG was also observed at the posterior tips (E14.5, Figure 4.3 C4) and in the nuclei (Figure 4.3 C4, C7) of elongating secondary fibre cells. Although absent from the matrix of the early developing eye (E9.5 and E10.5, Figure 4.3 B1-2), HS-GAG was strongly associated with the matrix connecting the posterior lens vesicle and early retina (Figure 4.3 C3), and the vitreous humour from E14.5 (Figure 4.3 C4). In contrast to the localisation of active HS-GAG chains in early lens development, CS-GAG- chain labelling was primarily observed in the pericellular matrix (Figure 4.3 D1-3). CS immunolabelling was completely absent from the lens placode (Figure 4.3 D1), lens pit (Figure 4.3 D2) and lens vesicle (E11.5, Figure 4.3 D3). Similarly, CS-GAG reactivity

was first observed at low levels in the E14.5 lens capsule, anterior lens epithelium, and anterior pole of secondary lens fibre cells (Figure 4.3 D4). CS-GAG immunostaining was strongly correlated with the extracellular matrix at all early stages of eye development, particularly in the preplacode matrix (E9.5, Figure 4.3 D1), and the interstitial matrix connecting the invaginating lens pit and optic cup (E10.5, Figure 4.3 D2) and surrounding the lens vesicle (E11.5, Figure 4.3 D3). CS-GAG was weakly evident in the ocular humours in the later stages of eye development (E14.5 and E16.5, Figure 4.3 D4, D5, D6). CS GAG-immunolabelling was absent from the lens capsule at E16.5 (Figure 4.3 D5-6, D8), and decreased in the fibre cells relative to E14.5 (Figure 4.3 D7-8). The CS-GAG labelling remained in the anterior lens epithelium (Figure 4.3 D5), that decreased at the germinative and transitional zones (Figure 4.3 D8).

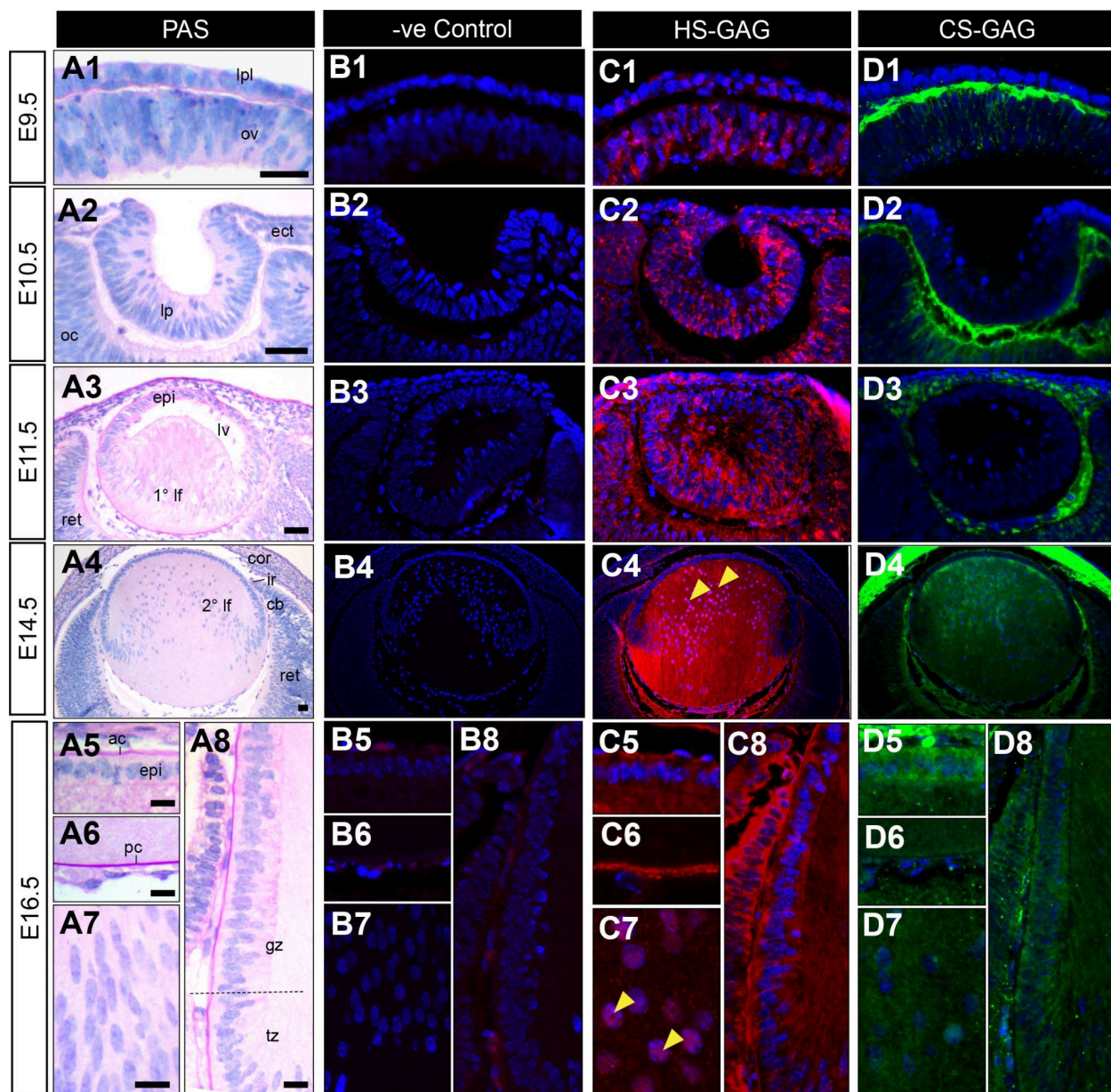


Figure 4.3. *Distribution of sulfated GAGs in mouse eye development. Eye sections from embryonic day-9.5 (E9.5, A1, B1, C1, D1), E10.5 (A2, B2, C2, D2), E11.5 (A3, B3, C3, D3), E14.5 (A4, B4, C4, D4) and E16.5 (A5-8, B5-8, C5-8, D5-8) FvB/N mice. Mid-sagittal eye sections stained with Peri-odic acid-Schiffs (PAS, A1-8) show cell morphology. Sections stained with secondary antibody only show representative negative controls (-ve control, B1-8). Sections labelled the highly sulfat-ed “active” forms of heparan sulfate (HS-GAG, red, C1-8) or chondroitin sulfate (CS-GAG, green, D1-8) glycosaminoglycan chains. Nuclei counterstained with Hoechst (blue). Yellow arrowheads indicate nuclear localisation (C4, C7). Dotted lines indicate lens equator (A8). Lens placode (lpl), head ectoderm (ect), preplacodal matrix (ppm), optic vesicle (ov), lens pit (lp), optic cup (oc), lens vesicle (lv), anterior lens epithelium (epi), primary lens fibre cells (1° lf), secondary lens fibre cells (2° lf), cornea (cor), retina (ret), ciliary body (cb), iris (ir), germinative zone (gz), transitional zone (tz), anterior lens capsule (ac), posterior lens capsule (pc). Scale bars = 25 µm. Images in figures are oriented with ventral side positioned to the right and the dorsal side to the left.*

4.3.3 Syndecan HSPGs are differentially spatially localised in the lens throughout development.

Syndecan HSPGs differ in their expression both temporally and spatially at all major stages of eye development. Syndecan-1 remains absent from the developing lens until E16.5, where it is localised strongly to the lens capsule and posterior vessels of the tunica vasculosa lentis (tv1). Weak syndecan-1 staining is also observed in a subset of anterior lens epithelial cells and anterior secondary fibre cell tips (Figure 4.4 A5). Syndecan-2 (Figure 4.4 B1), -3 (Figure 4.4 C1) and -4 (Figure 4.4 D1) are expressed at E9.5 in both the optic vesicle and the lens placode. The expression of these syndecans persist at embryonic day 10.5 with strong labelling in the lens pit and optic cup (Figure 4.4 B2, C2, D2). Syndecan-2 stained strongly throughout the invaginating lens pit, while syndecans-3 and -4 labelled particularly strongly in the posterior cells of the lens pit (Figure 4.4 C2, D2). At E11.5, prospective lens epithelial cells and elongating primary lens fibre cells showed strong labelling of syndecans-2 (Figure 4.4 B3), and -3 (Figure 4.4 C3). For syndecan-2, this label was restricted to primary lens fibres and absent from the anterior aspect of the lens (Figure 4.4 D3). Syndecan-1 was absent from the lens up to E11.5, where it first appears to localise to the inner aspect of the retina (Figure 4.4 A3). At E14.5 the localisation of each of the syndecans was distinct from each other. Syndecan-1 was localised primarily to the retinal ganglion cells, and lens capsule and blood vessels (Figure 4.4 A4). Syndecan-2 was strongly localised to the lens capsule, tunica vasculosa lentis (tv1), lens epithelial cells, and nuclei of lens fibre cells. It also showed strong localisation to the cornea and developing iris (Figure 4.4 B4). Syndecan-3 (Figure 4.4 D3) and -4 (Figure 4.4 D4) were both present in the lens epithelium and fibres in different patterns. Syndecan-3 staining was primarily observed in the secondary lens fibres, concentrated at the anterior fibre tips. It was absent from newer secondary lens fibre cells

(Figure 4.4 C4). On the other hand, syndecan-4 staining was observed primarily in newer secondary fibre cells, with distinct staining at the anterior and posterior fibre cell tips (Figure 4.4 D4). These expression patterns were maintained at E16.5 for syndecan-2 (Figure 4.4 B5-8), -3 (Figure 4.4 C5-8) and -4 (Figure 4.4 D5-8). At E16.5, syndecan-1 was absent from lens cells, but localised strongly to the lens capsule (Figure 4.4 A5-8).

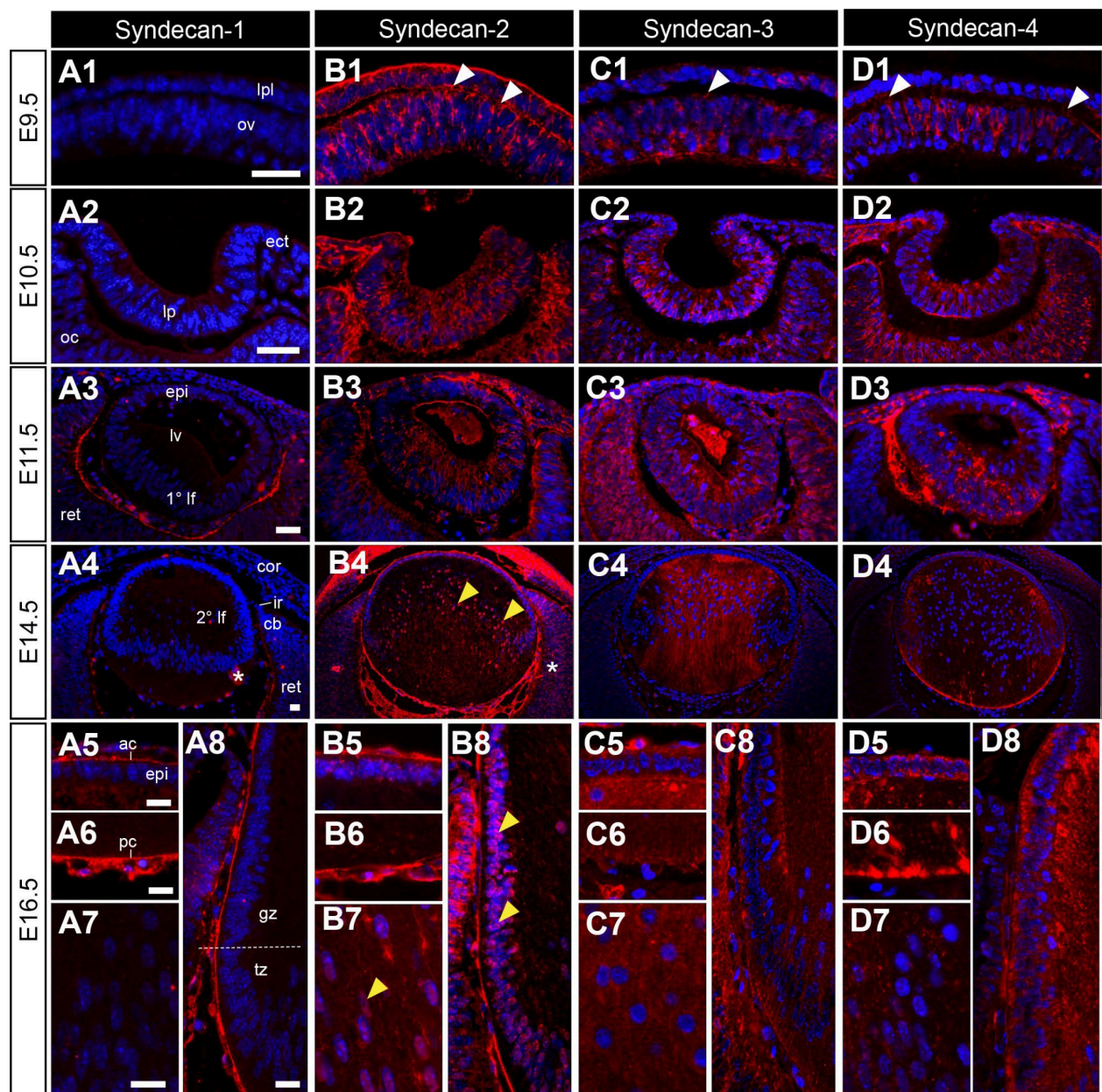


Figure 4.4. *Distribution of syndecan core proteins in the developing mouse eye. Mid-sagittal sections from embryonic day-9.5 (E9.5, A1, B1, C1, D1), E10.5 (A2, B2, C2, D2), E11.5 (A3, B3, C3, D3), E14.5 (A4, B4, C4, D4) and E16.5 (A5-8, B5-8, C5-8, D5-8) FvB/N mice. Sections labelled for syndecan-1 (A1-8), syndecan-2 (B1-8), syndecan-3 (C1-8) or syndecan-4 (D1-8) HSPG core proteins (red). Nuclei counterstained with Hoechst (blue). Yellow arrowheads indicate nuclear localisation (B4, B7, B8). White arrowheads indicate staining of lens fibre cell tips (C4-6, C8, D3-4, D6-8). Dotted line indicates the lens equator (A8). Lens placode (lpl), head ectoderm (ect), preplacodal matrix (ppm), optic vesicle (ov), lens pit (lp), optic cup (oc), lens vesicle (lv), anterior lens epithelium (epi), primary lens fibre cells (1° lf), secondary lens fibre cells (2° lf), cornea (cor), retina (ret), ciliary body (cb), iris (ir), germinative zone (gz), transitional zone (tz), anterior lens capsule (ac), posterior lens capsule (pc). Scale bars = 25 µm. *denotes artefact. Images in figures are oriented with the ventral side positioned to the right and the dorsal side to the left.*

4.3.4 Glypican HSPGs differ in temporal expression at key stages of lens/eye development.

At E9.5, glypican-4 is localised exclusively to pre-placodal matrix (Figure 4.5 D1), glypican-2, -3, -5 and -6 are present in both lens placode cells and optic vesicle (Figure 4.5 B1, C1, E1, F1). Glypican-1 was absent (Figure 4.5 A1) at this early stage. By E10.5, all glypicans were present in the lens pit and optic cup, apart from glypican-4, that was localised to the extracellular matrix between the lens pit and optic cup (Figure 4.5 D2). The label for glypican-3 and -5 was concentrated at the basal aspect of the lens pit in the presumptive primary lens fibre cells (Figure 4.5 C2, E2). Glypican-1, -2, -5 and -6 were also present in the surface head ectoderm (Figure 4.5 A2, B2, E2, F2). All glypicans were present in the lens vesicle (Figure 4.5 A2, B2, C2, E2, F2), except for glypican-4, that was localised primarily to the lens capsule, retinal ganglion cells, and posterior lens blood vessels (Figure 4.5 D3). At E11.5, glypican-3 stained all lens vesicle cells diffusely, with more intense staining in the presumptive lens epithelial cells (Figure 4.5 C3). Glypican-1, -2, -5 and -6 were localised predominantly to the posterior elongating primary lens fibre cells (Figure 4.5 A3, B3, E3, F3). These glypicans primarily were localised in a concentrated region at the posterior tips of the primary lens fibres, apart from glypican-6, that strongly labelled all fibre cells (Figure 4.5 F3). From E14.5, the expression of glypicans was generally consistent. Glypican-1 (Figure 4.5 A4-8), -2 (Figure 4.5 B4-8), -3 (Figure 4.5 C4-8) and -5 (Figure 4.5 E4-8) were predominantly localised to the lens epithelium, and newly differentiating secondary lens fibre cells, where perinuclear staining was observed for all four glypicans (Figure 4.5 A7, B7, C7, E7). Glypican-4 was absent from lens cells, with some punctate staining in the anterior lens epithelium, and a strong localisation to the lens capsule (Figure 4.5 D4-8). Glypican-6 was absent from the epithelium and transitional zone, but strongly labelled the cytoplasm of fibre cells (Figure 4.5 F4-F8).

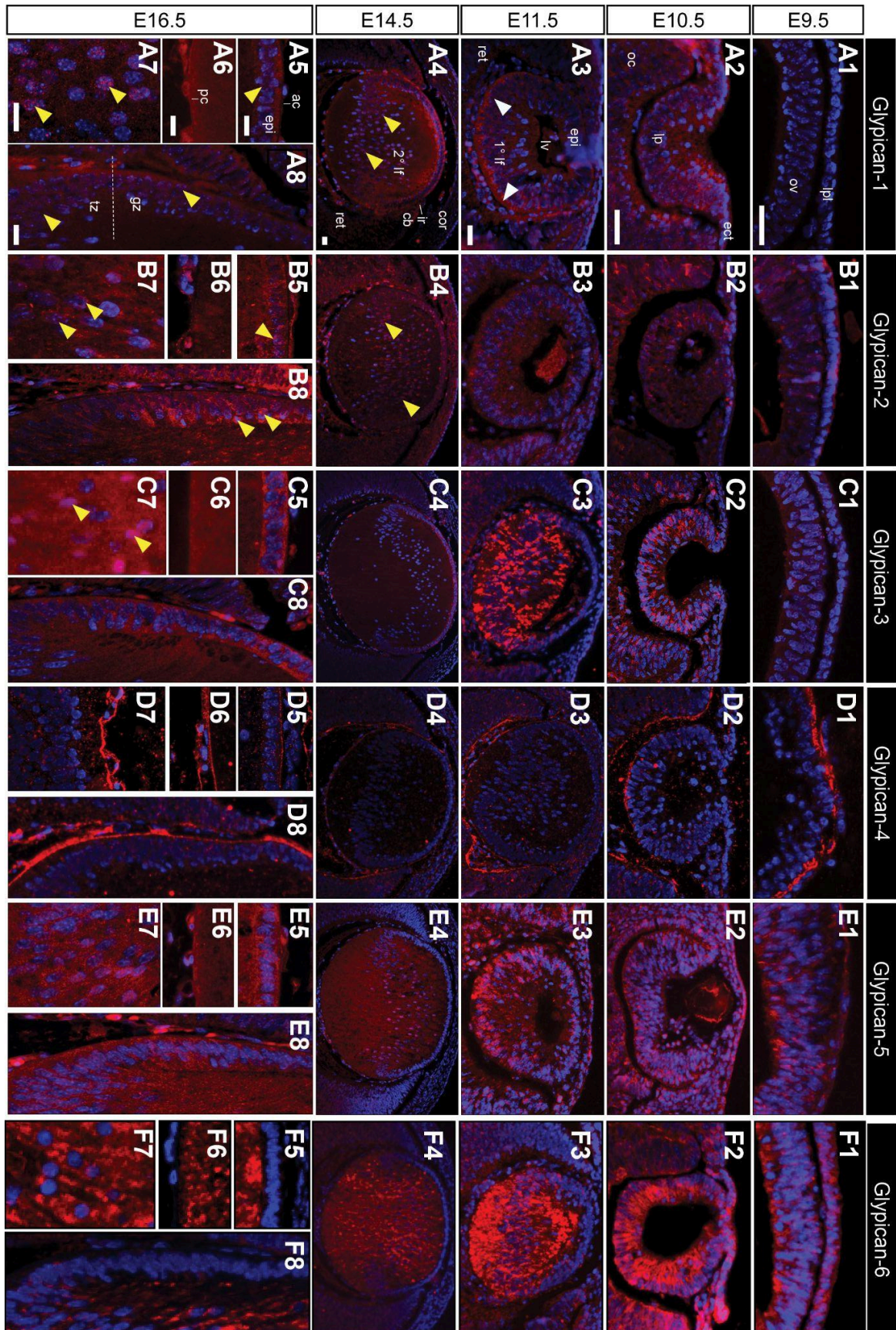


Figure 4.5. *Distribution of glypican core proteins in the developing mouse eye. Mid-sagittal sections from embryonic day-9.5 (E9.5, A1-F1), E10.5 (A2-F2), E11.5 (A3-F3), E14.5 (A4-F4) and E16.5 (A5-8, B5-8, C5-8, D5-8, E5-8, F5-8) FvB/N mice. Sections labelled for glypican-1 (A1-8), glypican-2 (B1-8), glypican-3 (C1-8), glypican-4 (D1-8), glypican-5 (E1-8) or glypican-6 (F1-8) HSPG core proteins (red). Nuclei counterstained with Hoechst dye (blue). Yellow arrowheads indicate nuclear localisation (A4-5, A7-8, B4-5, B7-8, C7). White arrowheads indicate staining of lens fibre cell tips (A3). Dotted lines indicate lens equator (A8). Lens placode (lpl), head ectoderm (ect), preplacodal matrix (ppm), optic vesicle (ov), lens pit (lp), optic cup (oc), lens vesicle (lv), anterior lens epithelium (epi), primary lens fibre cells (1° lf), secondary lens fibre cells (2° lf), cornea (cor), retina (ret), ciliary body (cb), iris (ir), germinative zone (gz), transitional zone (tz), anterior lens capsule (ac), posterior lens capsule (pc). Scale bars = 25 µm. Images in figures are oriented with ventral side positioned to the right and the dorsal side to the left.*

4.3.5 Localisation of high molecular weight HSPGs extends beyond the lens capsule.

Immunoreactivity for perlecan is weak in early lens structures (Figure 4.6 A1-2) but appears strongly labelled in the matrix and basement membranes of the E11.5 eye (Figure 4.6 A3). At this stage, perlecan is also observed in the anterior cells of the lens vesicle (Figure 4.6 A3). This pattern of expression is maintained in the fully formed lens at E14.5 and E16.5, with perlecan mostly restricted to the lens capsule and anterior lens epithelium (Figure 4.6 A4-8). Diffuse perlecan staining was observed in the nuclei of some differentiating lens fibre cells (Figure 4.6 A7). Both collagen XVIII (Figure 4.6 B1-2) and agrin (Figure 4.6 C1-2) were observed in the cells of the lens placode and lens pit. Collagen XVIII/endostatin reactivity was restricted to a punctate label in the primary lens fibres of the lens vesicle (Figure 4.6 B3). This label persisted in the E14.5 lens (Figure 4.6 B4). At E16.5, collagen XVIII labelled in condensed patches throughout the cytoplasm of both lens epithelial and fibre cells (Figure 4.6 B5-8). On the other hand, agrin stained strongly throughout the cells of the lens vesicle, concentrated apically in the presumptive anterior lens epithelial cells, and basally in the new elongating primary lens fibre cells (Figure 4.6 C3). From E14.5, agrin immunoreactivity was strongly visible throughout the cytoplasm of central lens fibre cells (Figure 4.6 C4), and this label persisted in the E16.5 lens (Figure 4.6 C5-8). Agrin was clearly associated with a subset of fibre cell nuclei (Figure 4.6 C4, C7). Agrin staining was almost completely absent from the anterior lens epithelium and transitional zone (Figure 4.6 C4, C5, C8).

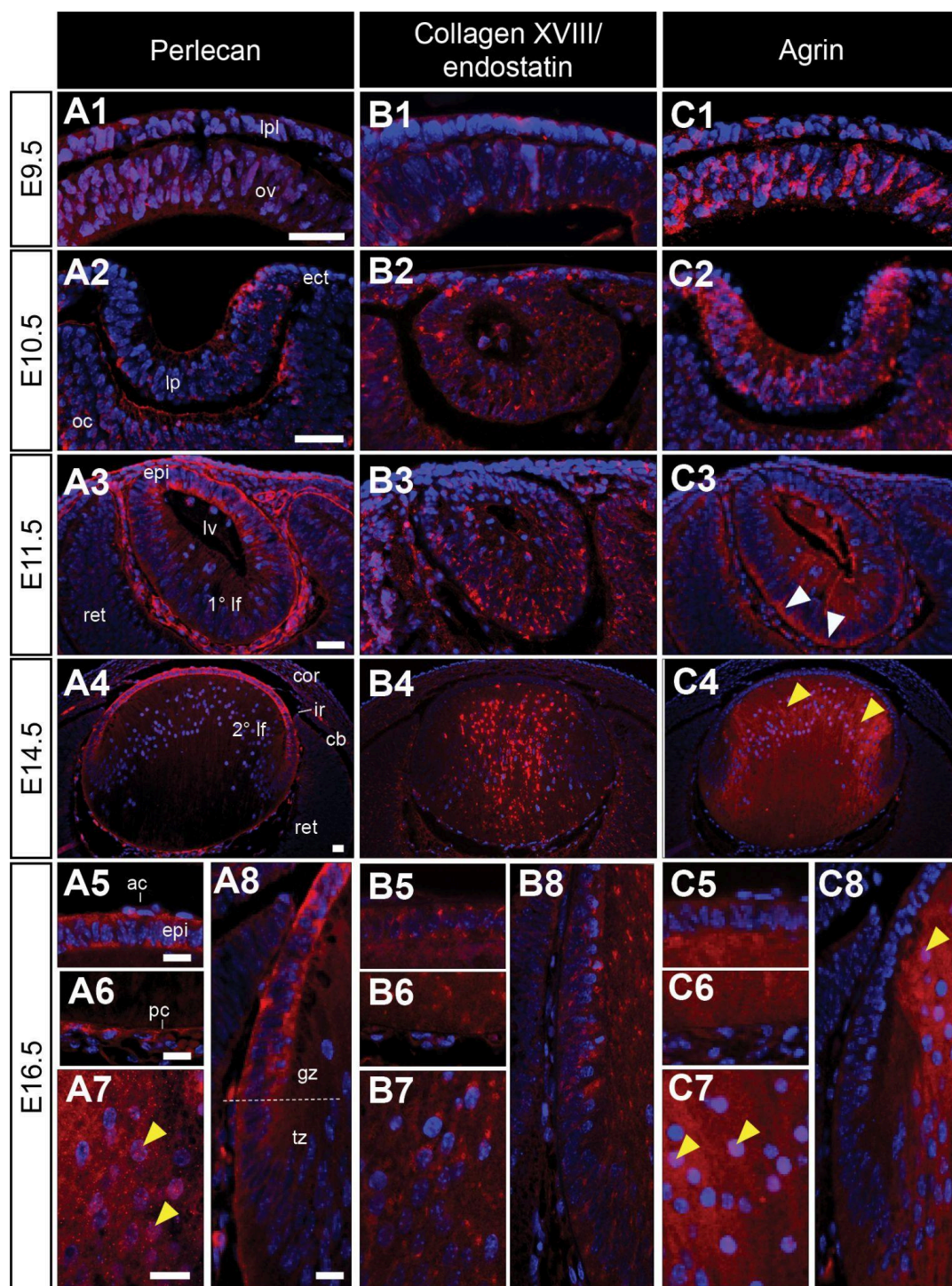


Figure 4.6. *Distribution of high molecular weight HSPG core proteins in the developing mouse eye. Mid-sagittal sections from embryonic day-9.5 (E9.5, A1-C1), E10.5 (A2-C2), E11.5 (A3-C3), E14.5 (A4-C4) and E16.5 (A5-8, B5-8, C5-8) FvB/N mice. Sections labelled for perlecan (A1-8), colla-gen-XVIII/endostatin (B1-8) or agrin (C1-8) HSPG core proteins (red). Nuclei counterstained with Hoechst dye (blue). Yellow arrowheads indicate nuclear localisation (A7, C4, C7-8). White arrowheads indicate staining of lens fibre cell tips (C3). Dotted lines indicate lens equator (A8). Lens placode (lpl), head ectoderm (ect), optic vesicle (ov), lens pit (lp), optic cup (oc), lens vesicle (lv), anterior lens epithelium (epi), primary lens fibre cells (1° lf), secondary lens fibre cells (2° lf), cornea (cor), retina (ret), ciliary body (cb), iris (ir), germinative zone (gz), transitional zone (tz), anterior lens capsule (ac), posterior lens capsule (pc). Scale bars = 25 µm. Images in figures are oriented with ventral side positioned to the right and the dorsal side to the left.*

4.4 Discussion

While there are many reports to date studying vertebrate lens morphogenesis and the growth factors involved, less is understood of how these different growth factors are positioned to drive this development. Given the important role of HSPGs in regulating many growth factors, together with recent studies implicating HSPGs as essential for the induction and development of the eye, here we present the most comprehensive spatiotemporal labelling of all the different HSPG core proteins and their associated GAGs throughout murine lens development. This study complements and extends on from our earlier report of the localisation of HSPGs in the postnatal rat lens (See chapter 3), where we described how the defined patterns of all the HSPGs associate with lens cellular activity. Given that all HSPGs are expressed in the lens, by linking the defined localisation of their respective HSPG core proteins, along with their reported mode of activity, it can provide valuable insights into their functional roles.

4.4.1 HSPG sulfation enzymes in the lens

As the importance and localisation of EXT (regulating HS-GAG chain elongation) and NDST (regulating HS-GAG chain post-translational N-sulfation) enzymes in lens development has been established, here we sought to investigate two important classes of enzymes contributing to HS-GAG sulfation: PAPSS2, and HS-O-sulfotransferases.

The activity of HSPGs is largely dependent on the distinct sulfotransferases of their HS chains, which source sulfate from PAPS produced by PAPSS1/2 synthetases. Transcriptome data identifies both *papss1* and *papss2* in the lens (Kakrana et al. 2018), with studies demonstrating strong labelling for PAPSS2 in the developing murine and postnatal rat eye, with high specificity to lens in both prenatal and postnatal ages (See chapter 3). Here, we found that PAPSS2 was

absent in very early lens induction (i.e. lens placode, lens pit) with strong reactivity first appearing in the lens vesicle, distinctly labelling the elongating primary lens fibre cells. This fibre-specific label persisted and was observed later in secondary lens fibres at E14.5 and E16.5 when the differentiating lens epithelium also began to label strongly for PAPSS2. The weak to no immunolabelling for PAPSS2 in the lens placode and pit is of interest as we see strong labelling of not only HS-GAG but many HSPGs that require PAPSS for their sulfation. This absence of PAPSS2 suggests that the activity of HSPGs in early lens induction may be mediated largely by HSPG core protein-protein interactions, and is independent of GAG-protein interactions, implying an expected low sulfation and binding potential of these HSPGs with ocular growth factors. An example of this is glypican-4, which only carries HS chains, but does not overlap in expression with HS-GAG. Alternatively, it is also plausible that the other PAPSS isoform, PAPSS1 (encoded by the *papss1* gene), is the predominant sulfate donor at this early stage of lens development, consistent with transcript expression profiles of *papss1* and *papss2* from published embryonic mouse lens datasets (Khan et al. 2015, Hoang et al. 2014, Cavaleiro et al. 2017, Audette et al. 2016).

Here we also found that three isoforms of HS-O-sulfotransferase are expressed by rat lens epithelial cells in the postnatal rat lens. Given that the lenses of *Hs2st*^{-/-}/*Hs6st*^{-/-} double mutants have small lenses it is likely that these are important sulfotransferases responsible for HS-GAG sulfation (Qu et al. 2011a). For the first time, here we confirm the presence of a HS-3-O-sulfotransferase in lens cells. Compared with 2-O- and 6-O-sulfation, 3-O-sulfation of HS is relatively rare, and typically comprises less than 1% of overall HS sulfation (De Agostini et al. 1990). Hs3sts are also expressed by fewer cell types (De Agostini et al. 1990). Out of the

seven HS 3-O-sulfotransferase genes present in the mouse, transcripts for Hs3st1 represent 66% of all the HS 3-O-sulfotransferase transcripts in the newborn lens (Hoang et al. 2014). The *Hs3st1*^{-/-} genotype is early postnatal lethal on a C57BL/6 inbred background where homozygous embryos exhibit intrauterine growth retardation (HajMohammadi et al. 2003), and no studies have investigated impact on lens development to date.

4.4.2 HS-GAG is the predominant GAG associated with lens development.

The specificity of HSPG-protein interactions does not only depend on the core protein type (e.g. syndecan, glypican), or their localisation (e.g. the cell surface or ECM), but also on the fine structure (i.e. sulfation) of their HS-GAG chains. HSPGs are predominantly HS-GAGs, however, certain species such as syndecans-1/-3 (Kokenyesi and Bernfield, 1994, Gould et al. 1995), agrin (Winzen et al. 2003) and collagen XVIII (Dong et al. 2003) may also carry CS-GAGs. Lens cells produce some proteoglycans carrying both HS-GAG and CS-GAG, although HS-GAG is more common (Heathcote and Orkin, 1984, Mohan and Spiro, 1991, Wishart and Lovicu, 2021).

Consistent with this, here we report that HS-GAG, and not CS-GAG, is the predominant GAG associated with HSPGs throughout murine lens development. Unlike HS-GAG, CS-GAG was absent from the lens until E14.5, indicating that HS-GAGs are likely the major species regulating the early events of lens induction, invagination, and cell fate specification. The presence of CS-GAG in the pre-placodal and peri-lenticular matrix throughout development may suggest a role in cell-matrix interactions or morphogen gradient formation. In contrast, HS-GAG was absent from the ocular matrix throughout the early stages of lens development, instead appearing and localising predominantly to the presumptive and differentiating lens cells. These patterns were often associated with regions of lens cell activity (i.e. lens placode, posterior aspect of lens

pit/vesicle and lens transitional zone). The overlapping and divergent patterns of HS-GAG and CS-GAG in the lens (e.g. in newly differentiating secondary lens fibres) implies that these GAGs may both contribute to the regulation of some functional processes in the lens (e.g. fibre differentiation), while other processes are likely to be regulated predominantly by HS (e.g. morphogen gradient formation in the lens capsule). HS-GAG and CS-GAG serve functionally distinct roles in other tissues, such as the developing brain, CS-GAG is important for the stabilisation of existing synaptic connections, while the primary function of HS-GAG is to stimulate the formation of new synapses [51]. Given HS-GAG and CS-GAG each interact selectively with different proteins in a structure-dependent manner (Vallet et al. 2020), it is not surprising that they are often reported to be differentially localised (Clark et al. 2011, Maeda et al. 2011, Smetsers et al. 2003), consistent with their reported distinct functional roles (Karamanos et al. 2018).

4.4.3 HSPG core proteins are differentially spatiotemporally regulated throughout lens development

Most of the HSPG core proteins that we examined localised to the cells of the surface head ectoderm and optic vesicle, however, syndecan-1 and glypican-1 were both absent at this stage. Despite being a traditionally cell-associated HSPG, glypican-4 was absent from the head ectoderm and optic vesicle but was strongly localised to the pre-placodal matrix. Similarly, syndecan-2, that is traditionally a cell-associated HSPG, was also observed in the pre-placodal matrix, along with the surface head ectoderm and optic vesicle, consistent with previous reports of syndecan-2 as a matrix-associated HSPG in ocular tissues (Filla et al. 2004, See chapter 3). The pre-placodal matrix is thought to prevent this defined region of proliferative head

ectodermal cells from spreading, so that their continued proliferation and progressive cell crowding thickens this region of ectoderm to form the lens placode (Huang et al. 2011). The regulated signalling activity of several mitogens and morphogens, such as BMPs, Hedgehog (Hh), FGFs and Wnts, orchestrate the inductive events associated with the optic vesicle-presumptive lens interaction (Lang 2004, Gunhaga 2011), and all these growth factors have been shown to be regulated by the HSPGs that we localise to the lens placode (Rapraeger et al. 2000, Essner et al. 2006, Fimus et al. 2008, Bányai et al. 2010, Seppinen and Pihlajaniemi 2011, Kerever et al. 2014). FGF, Hh, BMP and Wnt-signalling are each essential regulators in eye development, with the switching on/off of these morphogens essential for different stages of lens development (Cvekl and Zhang, 2017), with defects in their signalling activity shown to compromise lens development. While sustained inductive signalling of BMP and FGF are essential for lens placode development and early stages of lens specification (Jin et al. 2012), specification of these early stages requires the inhibition of Hh signalling, with heightened Hh-signalling activity suppressing lens formation in zebrafish (Barth and Wilson, 1995). Later in lens morphogenesis, Wnt signalling is activated to promote epithelial cell adhesion, integrity, and polarity, as well as fibre differentiation (de Jongh et al. 2006). Constitutively active Hh signalling through its receptor, patched, promotes epithelial maintenance at the expense of fibre differentiation (Kerr et al. 2012). Thus, the inhibition of Hh-signalling, once again with fibre cell differentiation, is essential for normal lens development. BMP-signalling promotes apical constriction and pro-survival signals during lens placode invagination and drives cell proliferation of lens epithelial cells and cell cycle exit at the transitional zone, which is bolstered by FGF-signalling that drives lens epithelial cell proliferation and fibre differentiation (Faber et al. 2002, Wederell et al. 2006, Shu et al. 2021).

The spatiotemporal regulation and of the ocular morphogens, together with other extracellular signalling proteins, forms the basis of lens patterning, polarity, growth, and cell fate specification (McAvoy et al. 1999, Wederell et al. 2006, Cvekl et al. 2017), that are regulated by the many HSPGs we report to be expressed here. Many of these same HSPGs regulate other matrix related proteins, including β 1-integrins and fibronectin (Richardson et al. 2001, Morgan et al. 2007, Kim et al. 2011, Roper et al. 2012), that are also co-localised to the early lens placode cells and play an essential role in this inductive process (Parmigiani and McAvoy 1984, Zelenka et al. 2004, Simirskii et al. 2007, Pathania et al. 2016).

Glypicans are predominantly expressed during embryonic development, and increasing evidence suggests they are major regulators of morphogenic-signalling and gradient events in development controlling left/right and dorsal/ventral patterning, as well as controlling tissue size and growth cues (Filmus, 2022). Genetic and biochemical studies have shown that glypicans can stimulate or inhibit the signalling pathways triggered by Wnts, Hh, BMPs and FGFs responsible for patterning and tissue formation during embryogenesis depending on the context of the tissue to drive growth patterning events (Filmus, 2022). Not surprisingly, mutations in genes encoding glypicans often result in developmental abnormalities or overgrowth (Filmus, 2022). For instance, Glypican-3 has been shown to inhibit hedgehog signalling during development by competitively binding to the ligand, preventing it from binding to patched, its receptor, through a HS-GAG independent process (Capurro et al. 2008). Similarly, Glypicans-1, -3 and -4 negatively regulate BMP-signalling by competing with BMP receptors for ligand binding in a similar fashion (Strate et al. 2015, Pan and Ho, 2021). On the other hand, glypicans seem to both

positively and negatively regulate Wnt-signalling in different developmental systems.

Glypican-4 promotes cardiac specification and differentiation by attenuating canonical Wnt- and BMP-signalling (Strate et al. 2015), while glypican-1 can act as a Wnt-coreceptor to activate Wnt-signalling (Pan and Ho, 2021). For different morphogens, glypicans seem to mainly activate or enhance signalling. Glypicans can interact with FGFs and their receptors to stabilise their assembly and enhance downstream ERK1/2 or PI3K/AKT, as well as bind to TGF- β and its receptors to promote SMAD-signalling (Pan and Ho, 2021).

As the mouse lens placode and optic vesicle invaginate at E10.5, we find that most of the HSPG core proteins localised to the cells of the lens pit, with syndecan-1 and glypican-4 are yet to appear. Glypican-4 was predominantly localised to the surrounding matrix together with syndecan-2 and perlecan. Here, we begin to observe subtle changes between some of the core proteins, such as syndecan-4, that appears to distinctly label the posterior tips of lens pit cells, with the majority of the other core proteins localising diffusely throughout all lens pit cells. BMP-4/-7 activity (Jidigam et al. 2015) and downstream activation of GTPases (RhoA, Rac1, ROCK) drive changes in cytoskeletal architecture essential for cell elongation, apical constriction and epithelial invagination during lens pit formation (Borges et al. 2011, Chauhan et al. 2011). Glypican-3 is well established as a primary regulator of cellular responses to BMP-4/-7 in developing tissues (Paine-Saunders et al. 2000, Grisaru et al. 2001), although emerging evidence suggests other HSPGs, such as agrin (Souza et al. 2021) and syndecan-3 (Fisher et al. 2006) may also play tissue-specific roles in BMP regulation. On the other hand, the syndecans are major regulators of RhoA/Rac1/ROCK GTPase-signalling and cytoskeletal modifications

through interactions with intracellular domains of their core proteins (Bass et al. 2007, Okina et al. 2009, Tsoyi et al. 2018).

As the mouse lens acquires more of an antero-posterior polarity with the formation of a lens vesicle at E11.5, we see that the acute change in posterior lens vesicle cell elongation to form the first primary fibres is associated with much HSPG activity. In the first instance we not only have PAPSS2 appearing in these cells, but also co-localising with HS-GAG and core proteins of most HSPGs, suggesting their putative roles in the differentiation, attachment, and elongation of primary lens fibre cells. Syndecan-1 continues to remain absent not only from lens cells but from all developing eye structures. Given PAPSS2 appears distinctly at this developmental stage, overlapping with many HSPGs and HS-GAGs, this suggests a marked rise in HS sulfotransferase activity that may be essential to drive primary lens fibre differentiation. With the differentiation of the primary fibres we also have the concomitant differentiation of the anterior lens vesicle cells to form the epithelium. Lens epithelial cell differentiation is known to be regulated by Wnt/beta-catenin signalling (Stump et al. 2003) that in turn is regulated by glypican-3 (Kolluri and Ho 2019), that is perfectly positioned for this, with strong reactivity in the anterior lens vesicle cells.

While we show that glypicans appear to generally localise in similar patterns to syndecans, the glypicans display temporal differences in their onset. This may be indicative of different core proteins regulating different developmental/cell fate specification/patterning events. It may be the case that the variable spatiotemporal localisation of the different HSPGs, particularly the glypicans, throughout lens development is representative at least in part by their differential

regulation of the morphogenetic switches in signalling events (Filmus 2022). Although no lens-specific phenotypes have been reported for animals deficient in glypicans, compensatory regulation between closely related HSPGs (i.e. glypicans-2/-4 and -3/-5) are likely. Our understanding of this family of HSPGs and how they function in different tissues is still very limited.

The larger secreted HSPGs, perlecan, collagen XVIII, and agrin are recognized as the predominant HSPGs in the postnatal lens capsule (Couchman and Pataki 2012, Schaefer 2014), however, we have previously reported different patterns of other HSPG core proteins (syndecans-1/-2/-4 and glypicans-2/-4) in the postnatal rat lens capsule: syndecan-2 and perlecan are abundant throughout the lens capsule, while syndecan-4, glypican-2, glypican-4, collagen XVIII, and agrin are restricted to the outer reticular lamina of the lens capsule (Schaefer 2014). In contrast, syndecan-1-labelling appeared to be uniquely limited to the basal lamina. In the developing mouse eye, we observed only a subset of these HSPGs in the capsule (syndecans-1/-2/-4, glypican-4 and perlecan), suggesting that some HSPGs (i.e. glypican-2, collagen XVIII and agrin) are deposited in the lens capsule at later stages. Of the minor HSPG core proteins we observed in the developing lens, three are syndecans, that bind to several structural and matrix regulatory proteins found in the lens capsule (e.g. collagen IV, laminin, fibronectin and integrins), and have previously been implicated as key regulators of ECM assembly, cell-matrix interactions, and cell adhesion (Bernfield, 1992, Couchman et al., 2015, Afratis et al., 2017).

Perlecan exists in an antero-posterior gradient in the lens capsule, consistent with complimentary antero-posterior gradients of FGF bioavailability and signalling activity (Chamberlain & McAvoy 1997, Hubka et al. 2019). Perlecan HSPGs are also required for structural integrity of the lens capsule (Rossi 2003), as it is shown here to progressively be deposited to the capsule over the course of lens development, consistent with its presence in the embryonic chick (Soulintzi et al. 2007). While the other large secreted high molecular weight HSPGs, Collagen XVIII and agrin, are often reported to be associated with ECM and basement membranes, here we report that both these core proteins are present from the earliest stages of lens development primarily within the cells of the presumptive and developing lens, and are not observed in the lens capsule, as we previously reported in the postnatal rat lens (See chapter 2). Rather, they are primarily associated with the lens epithelium, and show particularly strong staining in lens fibre cells. Mice deficient for *coll18a1*, encoding the collagen XVII core protein, have widespread malformation of ocular tissues (Marneros et al. 2004, Elamaa et al. 2005), with overexpression of the endostatin domain of Collagen XVIII leading to cataract and structurally compromised basement membranes (Liu et al. 2008, Aikio et al. 2013). Overexpression of agrin also produces a dysgenic phenotype that is specific to the eye (Chamberlain & McAvoy 1997). Similarly, knock-down of agrin in zebrafish embryos results in a small eye and lens phenotype, leading to decreased HS-GAG staining in the lens, but not retina, suggesting that other HSPGs may be upregulated in response to this loss of agrin (Zhao et al. 2008). The knockdown of agrin in zebrafish also leads to selectively decreased FGF-signalling and downstream activity of MAPK/ERK in the lens, providing strong evidence that agrin is likely regulating FGF-ERK signalling activity in the developing lens (Zhao et al. 2008). Interestingly, the strong pattern of HSPG-labelling in the posterior lens vesicle cells mirrors that of phospho-ERK1/2 staining

(Lovicu et al. 2001). Since FGF/ERK signalling is required for lens fibre cell elongation (Lovicu et al. 2001), and agrin is localised strongly to both the rapidly elongating primary and secondary lens fibres, it may be playing an important role in FGF-induced lens fibre differentiation.

Notably, agrin is absent from the transitional zone of the lens, where cells exit the cell cycle and begin to differentiate into fibres, instead selectively staining the elongated/ing lens fibres.

Here we report on the immunolabelling of HS-GAG and specific core proteins in the nuclei of lens epithelial cells (syndecan-2) and fibre cells (syndecan-2, glypican-1, glypican-5, perlecan, and agrin). Some of these HSPGs have previously been reported in the cell nuclei of other tissues, such as glypican-1 (neurons, glial cells, Liang et al. 1997) and agrin (developing rat brain cells, Cohen et al. 1997), while other HSPGs (i.e. glypican-5), to the best of our knowledge, are reported here for the first time in the nuclei of lens cells. HS-GAG and HSPG core proteins in select cell types, have been proposed to play roles in the transport of cargo or growth factor/receptor complexes to the cell nucleus (e.g. FGF/FGFR) to regulate gene expression (Stewart et al. 2014). The presence or absence of HSPGs correlates with cellular function, phenotype, and changes in cell cycle phases, suggesting their placement in the nucleus may control cellular function (Stewart et al. 2014). Here, we report that most HSPGs associated with lens cell nuclei are present in the transitional zone or in new fibre cells, where the lens epithelial cells exit cell cycle and undergo major morphological changes to differentiate into fibre cells, suggesting these specific HSPGs may play a regulatory role in these processes. Further investigations are necessary to elucidate the specific functional roles of HSPGs in lens cells.

4.4.3 Conclusions

The current study is the first to comprehensively map all HSPG localisation in a developing tissue during organogenesis. The differential spatial and temporal labelling of HSPG core proteins suggests that lens cells acquire a distinctive repertoire of HSPGs as they differentiate, resulting in selective patterns of HS-protein interactions and activity. This implies differential roles for HSPGs and HS sulfation at key stages of lens induction and morphogenesis. Taken together with the current literature, our findings support a requirement for HS sulfation in growth factor-induced cellular processes in the lens, with the sulfate donor PAPSS2 playing a unique temporal role. By further characterising and specifically modulating the different sulfated HSPGs expressed in the lens, we hope to better understand how HSPGs regulate different growth factor-mediated signalling events and cell behaviours in the lens. Moreover, gaining a better understanding of how specific HSPGs regulate the ways cells respond to their environment (e.g. response to growth factors) may allow us to develop novel strategies of regulating cell behaviour, that will not only be relevant to lens biology and disease, such as cataract, but may extend to other systemic growth factor-mediated pathologies.

Table 4.1. Summary of HSPG core protein immunolocalisation in mouse eye lens development.

Embryonic day		GAG	Other	Core proteins
E9.5	<i>Lens placode</i>	HS		Sdc2, Sdc3, Sdc4, Gpc2, Gpc3, Gpc4, Gpc5, Gpc6, perlecan, collagen XVIII, agrin
	<i>Optic vesicle</i>	HS, CS		Sdc2, Sdc3, Sdc4, Gpc2, Gpc3, Gpc5, Gpc6, perlecan, collagen XVIII, agrin
	<i>Pre Placodal matrix</i>	CS		Sdc2, Sdc4, Gpc4, Agrin
E10.5	<i>Lens pit</i>	HS		Sdc2, Sdc3, Sdc4, Gpc1, Gpc2, Gpc3, Gpc5, Gpc6, perlecan, collagen XVIII, agrin

	<i>Head ectoderm</i>	HS		Sdc2, Sdc3, Sdc4, Gpc1, Gpc2, Gpc3, Gpc4, Gpc5, Gpc6, perlecan, agrin
	<i>Optic cup</i>	HS, CS		Sdc2, Sdc3, Sdc4, Gpc1, Gpc2, Gpc3, Gpc4, Gpc5, Gpc6, perlecan, collagen XVIII, agrin
	<i>Extracellular matrix (lens pit-optic cup)</i>	CS		Sdc2, Gpc4, perlecan?
E11.5	<i>Anterior lens epithelium</i>	HS	PAPSS 2	Sdc2, Sdc3, Sdc4, Gpc1, Gpc2, Gpc3, perlecan, collagen XVIII, agrin
	<i>1° lens fibre cells</i>	HS	PAPSS 2	Sdc2, Sdc3, Sdc4, Gpc1, Gpc2, Gpc3, Gpc5, Gpc6, perlecan, collagen XVIII, agrin

	<i>Lens capsule</i>	HS		Sdc1, Sdc2, Gpc4, perlecan
E14.5	<i>Anterior lens epithelium</i>	HS*, CS		Sdc2*, Sdc3, Sdc4, Gpc1, Gpc2, Gpc3, Gpc4, perlecan, collagen XVIII
	<i>2° lens fibre cells</i>	HS*, CS	PAPSS 2	Sdc2*, Sdc3, Sdc4, Gpc1, Gpc2, Gpc3, Gpc5, Gpc6, collagen XVIII, agrin
	<i>Lens capsule</i>	HS, CS		Sdc1, Sdc2, Gpc2, Gpc4, perlecan
	<i>Tunica vasculosa lentis</i>	HS, CS		Sdc1, Sdc2, Gpc1, Gpc2, Gpc4, Gpc5, perlecan
E16.5	<i>Anterior lens epithelium</i>	HS*, CS		Sdc2*, Sdc3, Sdc4, Gpc1*, Gpc2*, Gpc3*, Gpc5, perlecan, collagen XVIII, agrin

	<i>2° lens fibre cells</i>	HS*, CS	PAPSS 2	Sdc2*, Sdc3, Sdc4, Gpc1*, Gpc2*, Gpc3*, Gpc5, Gpc6, perlecan*, collagen XVIII, agrin*
	<i>Lens capsule</i>	HS		Sdc1, Sdc2, Gpc4, perlecan
	<i>Tunica vasculosa lentis</i>	HS, CS		Sdc1, Sdc2, Gpc1, Gpc2, Gpc4, Gpc5, perlecan

*Nuclear localisation in cells. Sdc (syndecan), gpc (glypican)

CHAPTER 5: RESULTS III

HS-GAG SULFATION ORCHESTRATES LENS EPITHELIAL CELL PROLIFERATION AND FIBRE DIFFERENTIATION

5.1 Preface

The following manuscripts comprise part of the following results chapter. Some sections of the following manuscript have been reordered and/or edited for the purposes of elaboration or continuity. Original unedited manuscripts are attached in [appendix 1](#).

TFL Wishart and Frank J. Lovicu (2021). An Atlas of Heparan Sulfate Proteoglycans in the Postnatal Rat Lens. *Investigative Ophthalmology & Visual Science*, (DOI: 10.1167/iovs.62.14.5)

This manuscript was published in *Investigative Ophthalmology & Visual Science* in 2022. The work comprising these manuscripts was completed entirely during my PhD candidature under the guidance of my primary supervisor Professor Frank Lovicu who contributed to this manuscript by facilitating experimental design and manuscript editing.

Part of the experimental work in this chapter involved the technical assistance of a student under my co-supervision, Miss Ye (Linzy) Zhao.

5.2 Introduction

The lens epithelium can be separated into three regions: the anterior-most central zone, comprised of a quiescent population of lens epithelial cells, the mitotically active germinative zone just anterior to the equator, and finally, cells which enter the transitional zone posterior the equator exit from the cell cycle, migrate and differentiate to form elongated anucleate fibre cells ([Figure 1.3](#)). The refined architecture and well characterised biology of the vertebrate lens makes it an excellent model to study how these basic cellular processes are coordinated. The transitional

cellular processes of proliferation, migration and fibre cell differentiation are distinct, and each is orchestrated by the interplay of a complex array of signalling pathways regulated by growth factors native to the surrounding ocular media, the aqueous and vitreous humours (Lovicu and McAvoy, 2005). Mitogens present in the aqueous humour, bathing the posterior aspect of the lens, promote the proliferation, survival, and maintenance of the anterior epithelial monolayer. Posterior to the lens equator, factors found in the vitreous humour drive the differentiation of lens epithelial cells into elongated lens fibre cells (Lovicu and McAvoy 2005).

Numerous studies have been conducted to investigate and characterise the various factors present in the ocular environment and their individual and collective effects on lens cell behaviour. Key among the factors found in the ocular humour are members of the FGF, PDGF, EGF, and IGF families. These growth factors bind to and activate associated receptor tyrosine kinases found on the surface of the lens cells, initiating the activation of downstream signalling pathways, including Ras/Raf/MEK/ERK, PI3K/AKT, Wnt/ β -catenin and Hippo/YAP pathways, essential for lens epithelial cell proliferation and fibre differentiation.

Despite the well-characterised effects of various growth factors on lens cell behaviour, the precise mechanisms by which these factors navigate the extracellular space, reach their intended target cells, and initiate signalling at the appropriate intensity remain largely unknown. The specific signalling pathways and downstream events activated can be influenced by growth factor bioavailability, receptor expression, and even interactions with ECM proteins. Modulators of growth factor signalling also play a key role. Two primary types of modulators play a role: first, are regulatory pathways and molecules able to repress the activity of specific growth factors

(e.g. negative regulation of FGF-2 by the Sef and Sprouty families of genes). The second mechanism proposed is that of regulation of lens growth factor bioavailability and receptor activation by ECM proteins native to the lens capsule.

The interplay of lens growth factors and their interactions with ECM proteins influence the precise timing and location of cellular events such as proliferation, migration, and differentiation along the anterior-to-posterior axis of the lens. Given the complexity of these processes, identifying factors that coordinate these signalling events is crucial for understanding normal lens physiology and pathologies arising from the disruption of these pathways. Research conducted in mice and other model organisms points to a key regulatory role for HSPGs in the signalling and extracellular distribution of growth factors in the lens.

HSPGs can bind to and influence the signalling activity and distribution of a wide range of growth factors. At the cell surface, HSPGs can serve as receptors or co-receptors for various signalling molecules, including growth factors, cytokines, and chemokines. In this role, they directly and indirectly impact the regulation of intracellular signalling cascades, leading to the activation of key genes that govern cell behaviour and cellular phenotype. Within the ECM, HSPGs serve as molecular scaffolds capable of binding and sequestering growth factors, stabilising dynamic gradients of mitogens and morphogens at the tissue level, and orchestrating the bioavailability of these factors at the cell surface. HSPGs regulate the binding and activation of proteins involved in cell-matrix interactions, including integrins, laminin, and collagens. This regulation influences important cellular processes such as attachment, migration, differentiation, and polarity.

The lens provides a platform to dissect the molecular mechanisms and functional significance of HSPGs, shedding light on their impact on lens development, maintenance, and disease progression. Investigating the intricate interplay between growth factors and HSPGs holds promise for uncovering the elusive mechanisms that govern their precise actions within the lens and other tissues. Although previous findings lend strong evidence to suggest an important role for HSPGs in the regulation of mitogen-induced epithelial proliferation (Iyengar et al. 2017) and pathogenesis in the lens (Qin et al. 2017), the localisation and interactions of HSPGs with different growth factors and their signalling complexes, remain largely uncharacterised in the lens (see chapters 3 and 4). As different HSPGs appear to influence overlapping signalling pathways with diverse and cell type-specific outcomes (Rossi et al. 2003), characterising different HSPG-growth factor interactions, and developing a more comprehensive understanding of the signalling pathways they activate, is an important area of study. Investigating the biological consequences of functionally impairing HS sulfation modification of HSPGs would allow insights into how these modifications affect their role as regulators of signalling in the epithelium in normal cell biology and pathology. Moreover, a better understanding of these underlying regulatory mechanisms may serve as a basis for the identification of novel therapeutic targets and molecular strategies that could be implemented to slow or prevent the development and progression of diseases characterised by abnormal growth factor-mediated epithelial cell proliferation and HSPG sulfation profiles, such as cataract.

Evidence compiled from ours, and other preliminary studies in other tissues, suggest that growth factor-mediated processes in the epithelium can be regulated by modulating HS-GAG sulfation (Wang et al. 2017, Iyengar and Lovicu, 2017). Studies in mutant animals clearly show that HS-GAG sulfation is essential for normal lens development and maintenance. For example, conditional ablation of genes HS-GAG sulfation enzymes, *Ndst1* and *Ndst2*, following lens induction resulted in decreased lens epithelial cell proliferation, apoptosis, and defective fibre differentiation (Qu et al. 2011). Although such genetic ablation studies are valuable for understanding the differential roles of specific sulfation enzymes, chemical and small molecule inhibitors of HS-sulfation have also been widely employed to fine-tune the structure of HS-GAGs in living cells. These studies have proven to be exceptionally useful for studying the role of GAGs in biological systems, and for elucidating the biological consequences of altering HGAG sulfation both *in vitro* and *in vivo*. Of these compounds, sodium chlorate and xylosides are by far the best characterised and most widely utilised.

Sodium chlorate (NaClO_3^-) is a competitive inhibitor of the synthesis of 3'-phosphoadenosine-5'-phosphosulfate (PAPS), the key sulfate donor in HS-GAG sulfation reactions, has been widely used *in vitro* to study the effects of HS-GAG de-sulfation on cell behaviour (Safaiyan et al. 1989, Conrad et al. 2001, Iyengar et al. 2017). The proliferative response of lens epithelial cells induced by aqueous or exogenous growth factors (EGF, PDGF-AA, IGF-I, FGF-2) was blocked when cultured in sodium chlorate-supplemented medium (Iyengar et al. 2017). Moreover, sodium chlorate specifically blocked MAPK/ERK1/2-signalling induced by aqueous and FGF-2 but not the other mitogens. These findings suggest that HSPGs are likely an important regulator of this family of growth factors in the lens. However, little is known about the importance of

HSPGs in the regulation of other key growth factors in the ocular milieu and their associated downstream signalling pathways.

Another common class of molecules employed to alter HS-GAG function are synthetic xylosides, which compete with endogenous proteins for precursors and biosynthetic enzymes to assemble GAGs. This competition reduces endogenous proteoglycan-bound GAGs while increasing xyloside-bound free GAGs (Lugemwa and Esko 1991, Chua and Kuberan 2017, Persson and Mani 2018). Chick embryos cultured with β -D-Xyloside showed a severe, specific abnormalities in lens development attributed to co disruption of lens cell proliferation and blocked fibre differentiation paired with a dramatic decrease in FGF-2 and perlecan core protein expression (Martín et al. 2017).

Although studies involving sodium chlorate and xylosides have been a valuable starting point for investigating the biological consequences of altering HS-GAG sulfation, these molecules are not ideal for this purpose. Firstly, they do not selectively inhibit the sulfation of HS-GAG over other GAGs (i.e. chondroitin sulfate, dermatan sulfate, keratin sulfate). Second, they do not universally affect the different sulfation sites of HS-species. For instance, MDCK cells cultured with increasing concentrations of sodium chlorate were found to have HS-GAGs with proportionally decreasing O-sulfation, while HS-GAG N-sulfation was unaffected. Further investigation of O-desulfation revealed that low concentrations of sodium chlorate (5 or 20 mM) decreased 6-O but not 2-O HS sulfation, while high concentrations of sodium chlorate blocked all O-sulfation (Safaiyan et al. 1989). Thus, it is clear that a more selective, specific inhibitor would further validate and improve the results achieved with these early inhibitors.

One such candidate is surfen (bis-2-methyl-4-amino-quinolyl-6-carbamide, [Figure 2.2](#)), a small molecule antagonist of HS-GAG sulfation that was first described as an excipient to produce depot insulin (Schuksz et al. 2008). Later studies discovered that surfen, that selectively and specifically interferes with HS-protein interactions through a competitive electrostatic interaction, binds to the negatively charged sulfated HS-GAG chains and effectively impairs the functional binding of HS-GAGs to their wide array of protein binding partners (Schuksz et al. 2008). Preliminary studies in CHO cells found that treatment with surfen blocked FGF-2 binding and downstream ERK1/2-signalling activity, which are dependent on HS-GAG sulfation, in a dose-dependent manner (Schuksz et al. 2008). However, the effects of surfen treatment have not been studied in detail.

Here, we investigate the differential requirements for HSPG/HS-GAG sulfation in essential lens cellular processes – lens epithelial cell proliferation and fibre cell differentiation – induced by ocular humour and some of the major growth factors known to drive these signalling processes in the lens. By specifically modulating HS-GAG sulfation using surfen and further characterising which growth factors are modulated by HSPGs, we hope to better understand how key lens HSPGs regulate different growth factor signalling cellular processes.

5.3 Results

5.3.1 Surfen effectively blocks HSPG activity in lens cells in vitro

Previous studies in our laboratory had shown that rat lens epithelial cell proliferation induced by aqueous or exogenous growth factors (EGF, PDGF-AA, IGF-I, FGF-2) was blocked when cultured in sodium chlorate-supplemented medium (Iyengar et al. 2017). This study also reported that sodium chlorate specifically blocked ERK1/2 phosphorylation induced by aqueous and FGF-2 but not the other mitogens (Iyengar et al. 2017). Here, we demonstrate that sodium chlorate-supplemented media blocked LEC proliferation induced by FGF-2, but not aqueous (Figure 5.1).

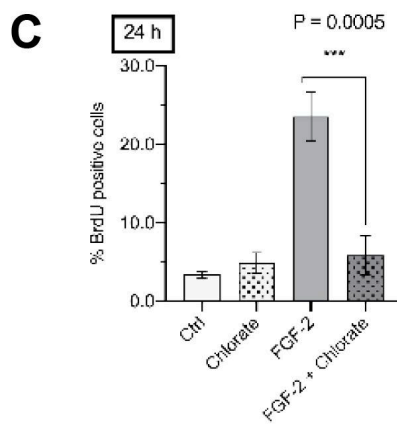
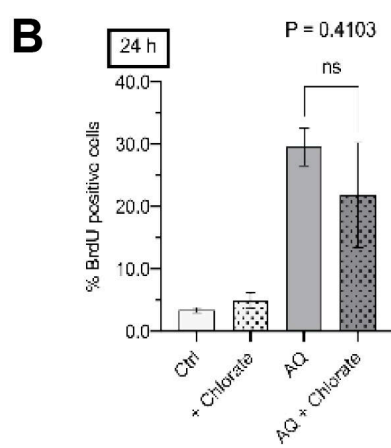
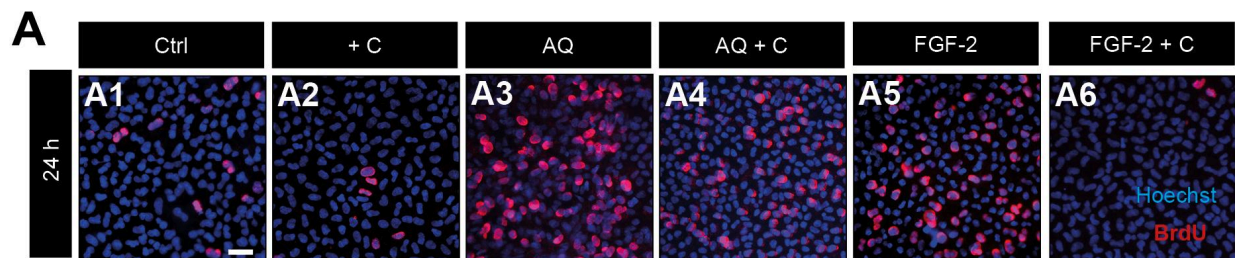


Figure 5.1. Sodium chlorate effectively blocks lens cell proliferation induced by FGF-2 but not aqueous. *A:* Representative images of 5'-2'-bromo-deoxyuridine (BrdU) incorporation. *B, C:* Quantification of lens cell proliferation (% BrdU incorporation). 10-day-old rat lens epithelial explants were treated with aqueous humour (AQ) or a proliferative dose of FGF-2 (1 ng/ml) with or without sodium chlorate (+C, 30 mM). Relative to control (Ctrl, A1) and sodium chlorate alone (A2), lens epithelial cell proliferation induced by aqueous was partially blocked with sodium chlorate (A3, B). FGF-2-induced proliferation was blocked completely in the presence of sodium chlorate (A3, C), but did not significantly decrease aqueous-induced proliferation. Data are the mean $\pm$ SEM from at least three independent experiments. Different from FGF-2 or aqueous alone (one-way ANOVA): ns: not statistically significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Nuclei counterstained with Hoechst dye (blue). Scale bar = 25 μ m.

A promising candidate inhibitor of HS-GAG sulfation is surfen, a selective electrostatic inhibitor of HS-GAG sulfation that targets all sulfation sites (Schuksz et al. 2008). FGF-2 is known to depend on HS-GAGs for signalling activation. Treatment of LECs with FGF-2 and increasing doses of surfen were used to determine the effective dose for blocking HS-GAG sulfation in lens epithelial explants. Explants treated with 1 or 5 μ M surfen showed no signs of decreased cell viability. 10 μ M surfen led to a significant decrease in cell number with a marked increase in apoptotic-like cells relative to control explants (Figure 5.2 C). Co-treatment with 5 or 10 μ M Surfen, but not 1 μ M, effectively blocked FGF-2-induced proliferation at 24 h (Figure 5.2 B). 5 μ M surfen was therefore determined to be the optimal concentration for HS-GAG inhibition in lens epithelial explants and was used for all following experiments in this study.

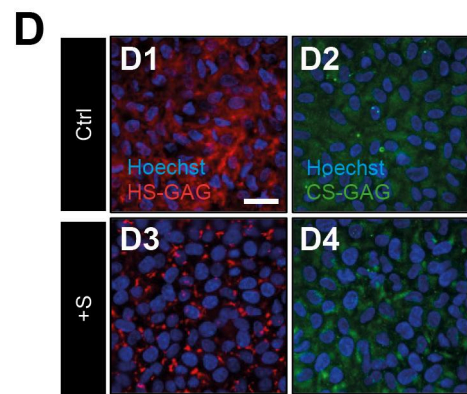
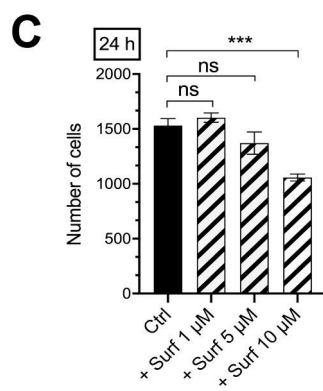
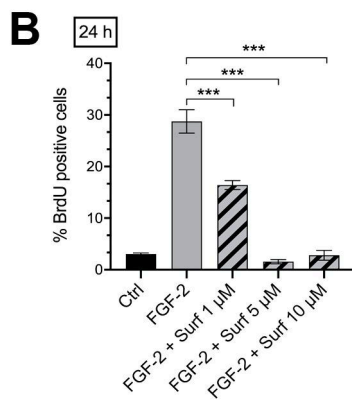
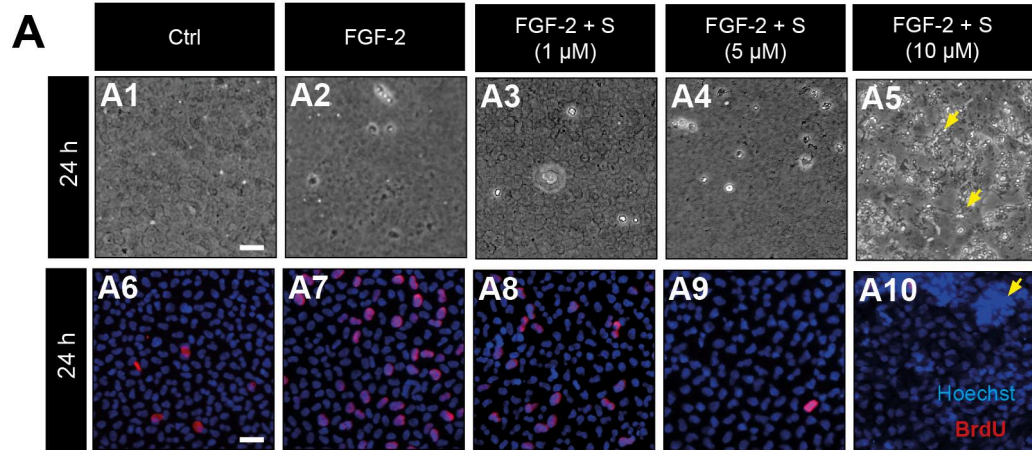


Figure 5.2. *Optimisation of Surfen hydrate, a selective inhibitor of HS-GAG activity. 10-day-old rat lens epithelial explants treated with a proliferative dose of FGF-2 (1 ng/ml) with or without Surfen. Relative to vehicle controls (Ctrl), explants treated for 24 h with increasing doses of surfen (Surf/+S, 1 μ M, 5 μ M, or 10 μ M) showed decreased cell viability (A1-5, C, number of cells) and FGF-2 (1 ng/ml)-induced cell proliferation (A6-10, B, % BrdU-incorporation) in a concentration-dependent manner. C) Explants treated with 1 or 5 μ M Surfen alone for 24 h showed no signs of decreased cell viability (number of cells) 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 μ M ($P=0.007$). Phase micrographs of these showed pyknotic cell nuclei characteristic of cell death (yellow arrows, A5 and A10). D) Treatment with 5 μ M Surfen alone for 1 h decreased immunolabelling of sulfated “active” GAG chains (HS-10E4, D2) relative to control explants (D1, D2), but not sulfated “active” CS-GAG chains (CS-56, D4). Data are the mean $\pm$ SEM from at least two independent experiments. Different from non-treated control (C) or FGF-2 alone (B) (one-way ANOVA): ns: not statistically significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Nuclei counterstained with Hoechst dye (blue). Scale bars = 25 μ m.s*

5.3.3 Differential requirements for HS-GAG sulfation of aqueous humour and key ocular mitogens.

In order to investigate the differential requirements for HSPG/HS-GAG sulfation in lens epithelial cell proliferation induced by different mitogens, we compared the effects of HS-GAG inhibition (with surfen) on downstream signalling activity (phospho-ERK1/2 immunolabelling at 4 h) and lens epithelial cell proliferation (% BrdU incorporation at 24 h) in 10-day-old rat lens epithelial explants treated with aqueous humour and each of the major ocular growth factors known to stimulate lens epithelial cell proliferation (For summary see [Table 5.1](#)).

Treatment with aqueous humour, and each of the mitogens led to activation of phospho-ERK1/2 at 4 h corresponding to lens cell proliferation at 24 h, although as previously shown in Figure 1, the levels of cell proliferation were different with different exogenous growth factors used. Co-treatment of cells with mitogens and surfen differentially impacted both activation of ERK1/2-signalling and lens cell proliferation for all mitogens (Figure 5.3-5.7) except for PDGF-AA (Figure 5.8), that was not significantly affected by HS-GAG inhibition. Surfen co-treatment partially blocked ERK1/2-signalling activity and lens cell proliferation induced by aqueous (Figure 5.3), EGF (Figure 5.6) and IGF-I (Figure 5.7), and completely blocked the downstream effects induced by FGF-2 (Figure 5.4) and HB-EGF (Figure 5.5).

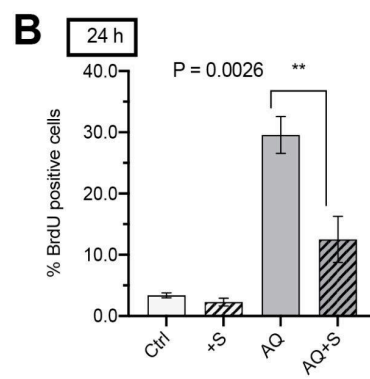
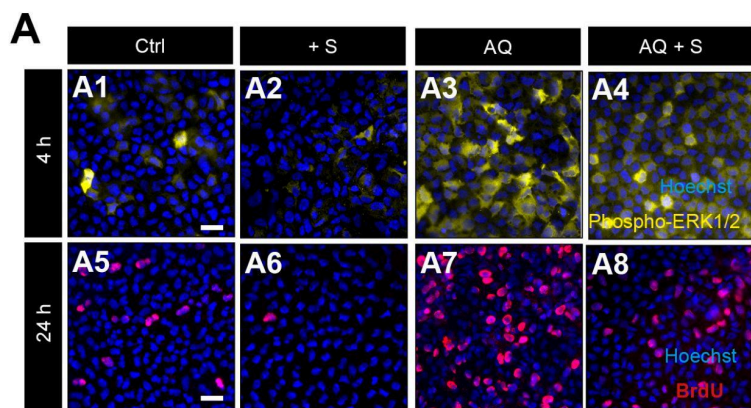


Figure 5.3. Surfen partially blocks aqueous-induced lens epithelial cell proliferation.

Downstream signalling activity (phospho-ERK1/2 immunolabelling at 4 h, A1-4) and cell proliferation (% BrdU incorporation at 24 h, A5-8, B) were investigated in 10-day-old rat lens epithelial explants treated with aqueous (AQ, 10% v/v in M199) with or without surfen (+S, 5 μ M). Relative to vehicle controls (Ctrl, A1, A5) and surfen (+S, 5 μ M) alone (A2, A6), explants treated with AQ (A3, A7) showed a significant increase in phospho-ERK1/2 immunolabelling (A3) and BrdU incorporation (A7, B: $P < 0.001$). Pre-treatment with surfen (1 h) moderately decreased AQ-induced phospho-ERK1/2-signalling (A4) and cell proliferation (A8, B: $P = 0.0026$) relative to AQ alone (A3, A7). A5-8: representative images of BrdU-labelling/proliferation. B) Quantification of cell proliferation data. Data are the mean $\pm$ SEM from at least three independent experiments. Different from AQ alone (Student t-test): ns: not statistically significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Cell nuclei counterstained with Hoechst dye (blue). Scale bars = 25 μ m.

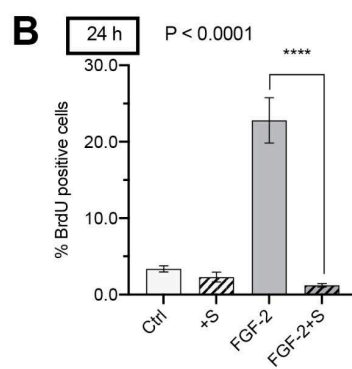
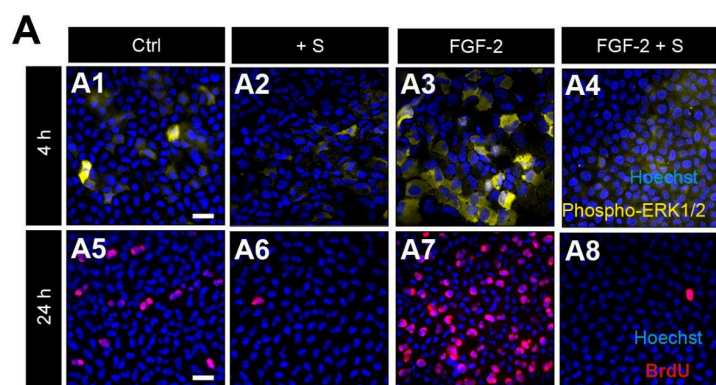


Figure 5.4. Surfen blocks FGF-2-induced lens epithelial cell proliferation. Downstream signalling activity (phospho-ERK1/2 immunolabelling at 4 h, A1-4) and cell proliferation (% BrdU incorporation at 24 h, A5-8, B) were investigated in 10-day-old rat lens epithelial explants treated with a proliferative dose of FGF-2 (1 ng/ml) with or without surfen (+S, 5 μ M). Relative to vehicle controls (Ctrl, A1, A5) and surfen (+S, 5 μ M) alone (A2, A6), explants treated with FGF-2 (A3, A7) showed a significant increase in phospho-ERK1/2 immunolabelling (A3) and BrdU incorporation (A7, B: $P < 0.001$). Pre-treatment with surfen (1 h) blocked FGF-2-induced phospho-ERK1/2 signalling (A4) and cell proliferation (A8, B: $P < 0.0001$) relative to FGF-2 alone (A3, A7). A5-8: representative images of BrdU proliferation. B) Quantification of BrdU proliferation data. Data are the mean $\pm$ SEM from at least three independent experiments. Different from FGF-2 alone (Student *t*-test): ns: not statistically significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Cell nuclei counterstained with Hoechst dye (blue). Scale bars = 25 μ m. Representative images (A) and data (B) of vehicle controls (A1, A5, B: “Ctrl”) and surfen alone (A2, A6, B: “+S”) adapted from Figure 4 for purpose of visual comparison.

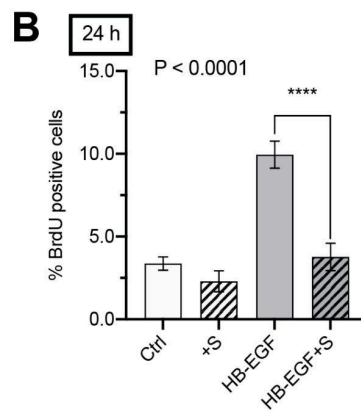
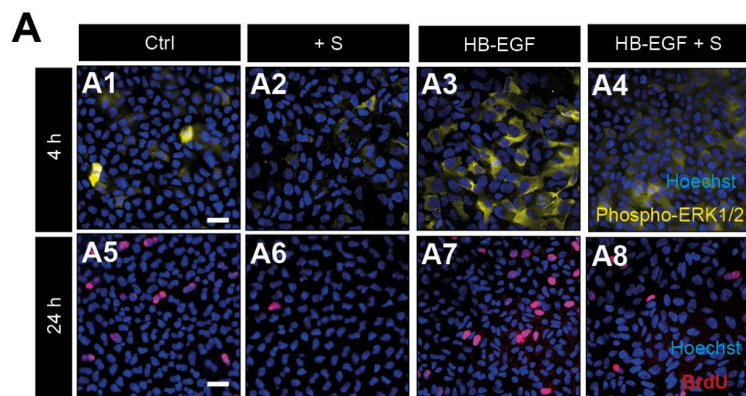


Figure 5.5. Surfen blocks HB-EGF-induced lens epithelial cell proliferation. Downstream signalling activity (phospho-ERK1/2 immunolabelling at 4 h, A1-4) and cell proliferation (% BrdU incorporation at 24 h, A5-8, B) were investigated in 10-day-old rat lens epithelial explants treated with HB-EGF (50 ng/ml) with or without surfen (+S, 5 μ M). Relative to vehicle controls (Ctrl, A1, A5) and surfen (+S, 5 μ M) alone (A2, A6), explants treated with HB-EGF (A3, A7) showed a significant increase in phospho-ERK1/2 immunolabelling (A3) and BrdU incorporation (A7, B: $P < 0.001$). Pre-treatment with Surfen (1 h) blocked HB-EGF-induced phospho-ERK1/2 signalling (A4) and cell proliferation (A8, B: $P < 0.0001$) relative to HB-EGF alone (A3, A7). A5-8: representative images of BrdU proliferation. B) Quantification of BrdU proliferation data. Data are the mean $\pm$ SEM from at least three independent experiments. Different from HB-EGF alone (Student *t*-test): ns: not statistically significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Cell nuclei counterstained with Hoechst dye (blue). Scale bars = 25 μ m. Representative images (A) and data (B) of vehicle controls (A1, A5, B: “Ctrl”) and surfen alone (A2, A6, B: “+S”) adapted from Figure 4 for the purpose of visual comparison.

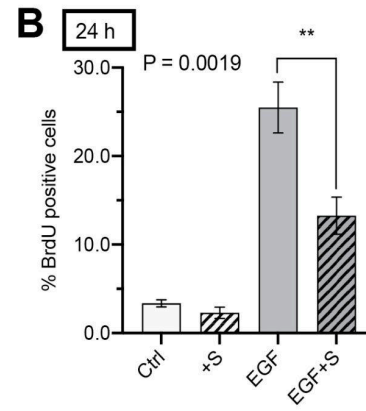
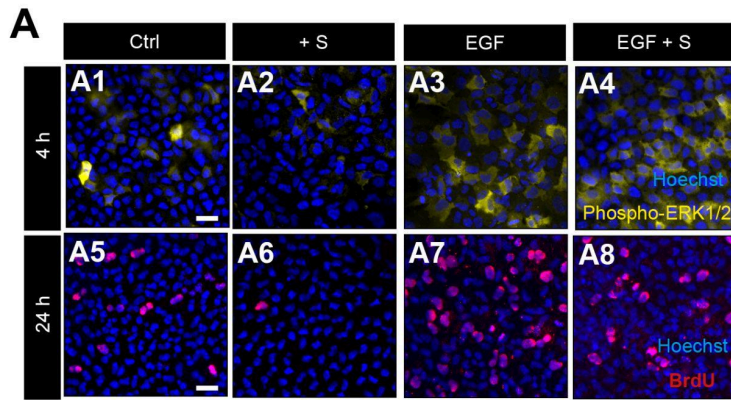


Figure 5.6. Surfen partially blocks EGF-induced lens epithelial cell proliferation. Downstream signalling activity (phospho-ERK1/2 immunolabelling at 4 h, A1-4) and cell proliferation (% BrdU incorporation at 24 h, A5-8, B) were investigated in 10-day-old rat lens epithelial explants treated with EGF (10 ng/ml) with or without surfen (+S, 5 μ M). Relative to vehicle controls (Ctrl, A1, A5) and surfen (+S, 5 μ M) alone (A2, A6), explants treated with EGF (A3, A7) showed a significant increase in phospho-ERK1/2 immunolabelling (A3) and BrdU incorporation (A7, B: $P < 0.001$). Pre-treatment with surfen (1 h) partially blocked EGF-induced phospho-ERK1/2 signalling (A4) and cell proliferation (A8, B: $P = 0.0019$) relative to EGF alone (A3, A7). A5-8: representative images of BrdU proliferation. B) Quantification of BrdU proliferation data. Data are the mean $\pm$ SEM from at least three independent experiments. Different from EGF alone (Student *t*-test): ns: not statistically significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Cell nuclei counterstained with Hoechst dye (blue). Scale bars = 25 μ m. Representative images (A) and data (B) of vehicle controls (A1, A5, B: “Ctrl”) and surfen alone (A2, A6, B: “+S”) adapted from Figure 4 for the purpose of visual comparison.

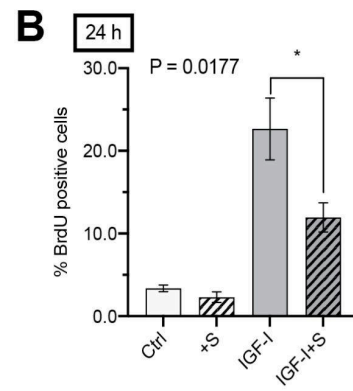
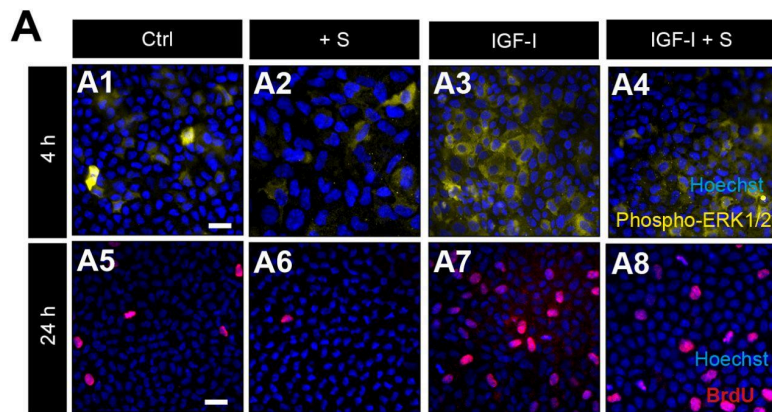


Figure 5.7. Surfen partially blocks IGF-I-induced lens epithelial cell proliferation. Downstream signalling activity (phospho-ERK1/2 immunolabelling at 4 h, A1-4) and cell proliferation (% BrdU incorporation at 24 h, A5-8, B) were investigated in 10-day-old rat lens epithelial explants treated with IGF-I (75 ng/ml) with or without surfen (+S, 5 μ M). Relative to vehicle controls (Ctrl, A1, A5) and surfen (+S, 5 μ M) alone (A2, A6), explants treated with IGF-I (A3, A7) showed a significant increase in phospho-ERK1/2 immunolabelling (A3) and BrdU incorporation (A7, B: $P < 0.001$). Pre-treatment with surfen (1 h) partially blocked IGF-I-induced phospho-ERK1/2 signalling (A4) and cell proliferation (A8, B: $P = 0.0177$) relative to IGF-I alone (A3, A7). A5-8: representative images of BrdU proliferation. B) Quantification of BrdU proliferation data. Data are the mean $\pm$ SEM from at least three independent experiments. Different from IGF-I alone (Student t-test): ns: not statistically significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Cell nuclei counterstained with Hoechst dye (blue). Scale bars = 25 μ m. Representative images (A) and data (B) of vehicle controls (A1, A5, B: “Ctrl”) and surfen alone (A2, A6, B: “+S”) adapted from Figure 4 for the purpose of visual comparison.

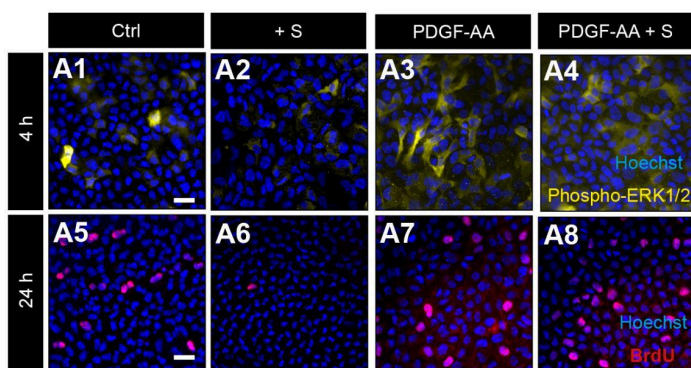
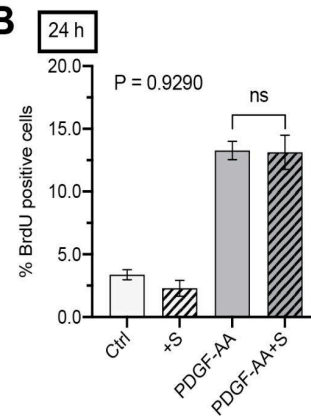
A**B**

Figure 5.8. Surfen does not block PDGF-AA-induced lens epithelial cell proliferation.

Downstream signalling activity (phospho-ERK1/2 immunolabelling at 4 h, A1-4) and cell proliferation (% BrdU incorporation at 24 h, A5-8, B) were investigated in 10-day-old rat lens epithelial explants treated with PDGF-AA (10 ng/ml) with or without surfen (+S, 5 μ M). Relative to vehicle controls (Ctrl, A1, A5) and surfen (+S, 5 μ M) alone (A2, A6), explants treated with PDGF-AA (A3, A7) showed a significant increase in phospho-ERK1/2 immunolabelling (A3) and BrdU incorporation (A7, B: $P < 0.001$). Pre-treatment with surfen (1 h) did not block PDGF-AA-induced phospho-ERK1/2 signalling (A4) or cell proliferation (A8, B: $P = 0.9290$) relative to PDGF-AA alone (A3, A7). A5-8: representative images of BrdU proliferation. B) Quantification of BrdU proliferation data. Data are the mean $\pm$ SEM from at least three independent experiments. Different from PDGF-AA alone (Student *t*-test): ns: not statistically significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Cell nuclei counterstained with Hoechst dye (blue). Scale bars = 25 μ m. Representative images (A) and data (B) of vehicle controls (A1, A5, B: “Ctrl”) and surfen alone (A2, A6, B: “+S”) adapted from Figure 4 for the purpose of visual comparison.

To validate phospho-ERK1/2 immunolabelling and further elucidate the ability of surfen to disrupt this signalling pathway in proliferating lens cells, Western blots were performed to analyse early ERK1/2 signalling activation at 1 h and 4 h. In FGF-2 treated explants, both surfen and sodium chlorate blocked ERK1/2 phosphorylation at 1 and 4 h, although this block was more significant in explants co-treated with surfen compared with sodium chlorate (Figure 5.10 A, B). Surfen blocked phospho-ERK1/2 activity at 1 h and 4 h induced by AQ. In HB-EGF-treated explants, surfen blocked HB-EGF-induced phospho-ERK1/2 activity at 1 h but not 4 h.

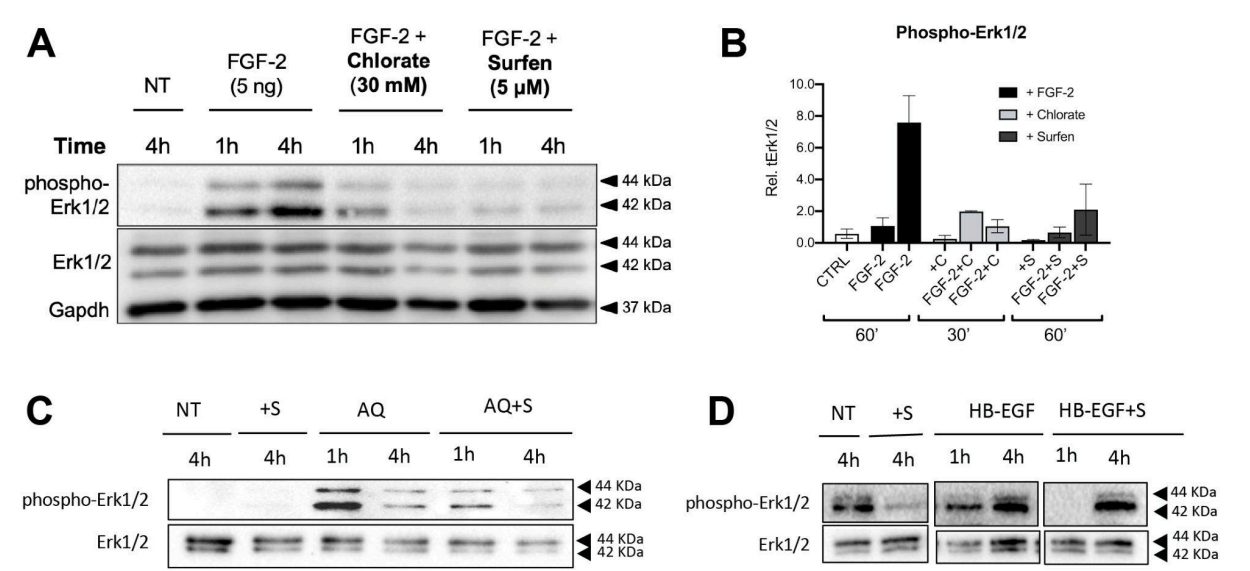


Figure 5.9. *Effects of sodium chlorate and surfen on downstream phosphorylation of ERK1/2 protein. Western blotting was used to validate phosphorylated-ERK1/2 (phospho-ERK1/2) and total-ERK1/2 (ERK1/2) protein at 1 h and 4 h in explants treated with a proliferative dose of FGF-2 (1 ng/ml), aqueous (AQ, 10%) or HB-EGF (50 ng/ml) with or without sodium chlorate (+C, 30 mM) and/or Surfen (+S, 5 μ M). A) Relative to vehicle controls (NT), FGF-2-induced phospho-ERK1/2 activity at 1h and 4h, was blocked by treatment with sodium chlorate and surfen. B) Quantification of phospho-ERK1/2 relative to total-ERK1/2 (t-ERK1/2) protein in A (n=2). Gapdh (housekeeping protein) labelling confirmed that t-ERK levels are not affected by mitogen treatment (consistent for all mitogens in this chapter, data not shown). Data are the mean $\pm$ SEM from at least three independent experiments. C) Increased phospho-ERK1/2 activity at 1 h and 4 h induced by AQ was blocked by treatment with surfen. D) Increased phospho-ERK1/2 activity at 1 h and 4 h induced by HB-EGF was blocked by treatment with surfen at 1 h but not 4 h. A, C and D are representative images of western blots from at least two independent experiments.*

5.3.4 *Surfen blocks vitreous and FGF-induced lens fibre differentiation*

After 5 days of culture with vitreous (Figure 5.11) or a fibre differentiating dose (150 ng/ml, Iyengar et al. 2007) of FGF-2 (Figure 5.12), cells in lens epithelial explants had an elongate morphology (Figure 5.11 A7, Figure 5.12 A7) and labelled strongly for β -crystallin and aquaporin-0 (Figure 5.11 B5, B8, C3, Figure 5.12 B5, B8), while cells in vehicle control explants remained cuboidal (Figure 5.11 A5, Figure 5.12 A5) and did not accumulate lens fibre protein markers (Figure 5.11 B4, B7, C1, Figure 5.12 B4, B7). Explants treated with surfen alone resembled control explants (Figure 5.11 A2, A6, C2, Figure 5.12 A2, A6), but when explants were co-treated with a differentiating dose of FGF-2 or vitreous and surfen, cell elongation (Figure 5.11 A8, Figure 5.12 A8), and the appearance of β -crystallin and AQP0 was significantly decreased in cells (Figure 5.11 B6, B9, C4, Figure 5.12 B6, B9), compared with cells treated with FGF-2 or vitreous alone. Surfen was also able to block phosphorylation of ERK1/2 after a 4 h exposure to vitreous (Figure 5.11 B12) or a high dose of FGF-2 (Figure 5.12 B12). Western blot analysis indicates that surfen blocks FGF-2-induced β -crystallin protein accumulation as early as 48 h after exposure to a high dose of FGF-2 (Figure 5.12 D, E).

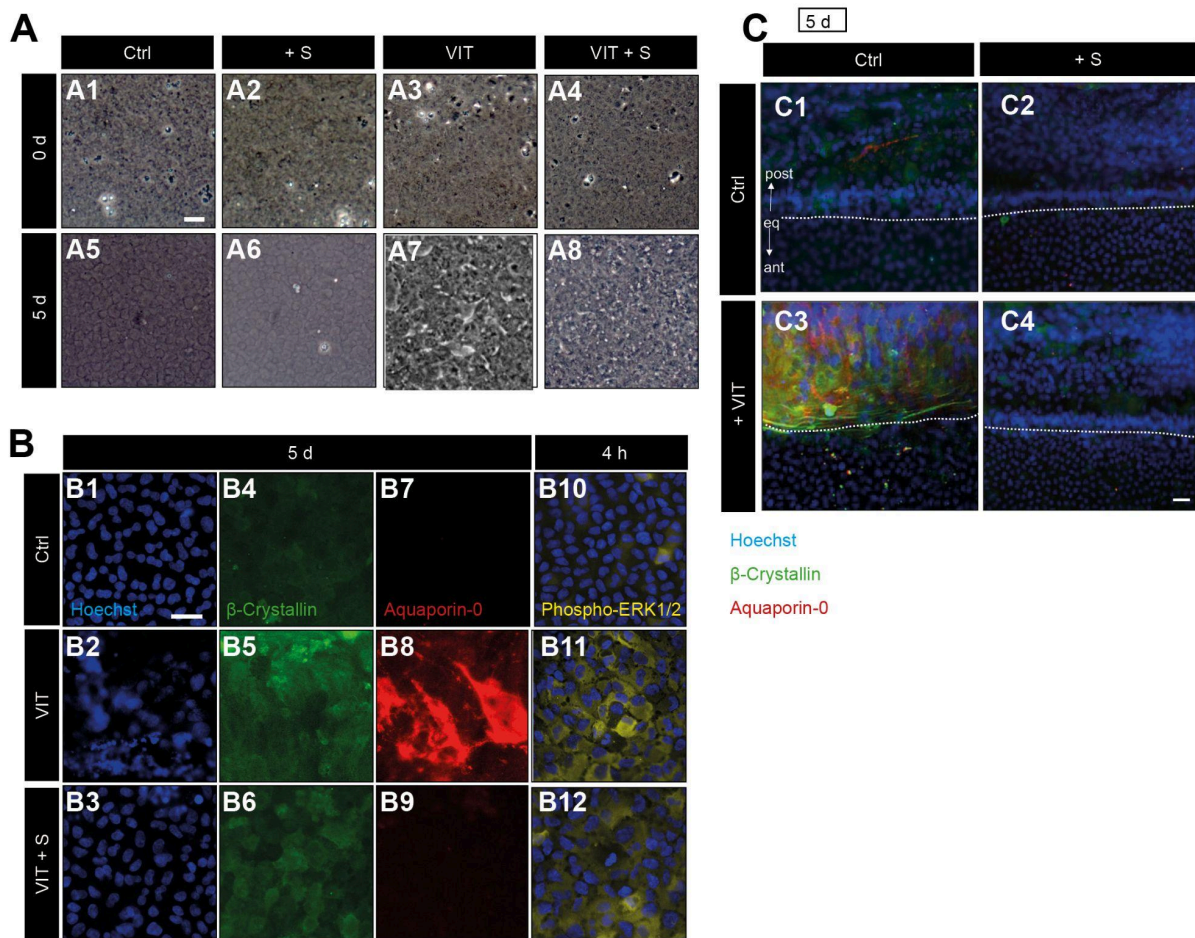


Figure 5.10. *HS-GAG sulfation is required for vitreous-induced lens fibre differentiation.*

10-day-old rat lens epithelial explants were treated up to 5 days with vitreous (VIT, 10% v/v in M199), with or without surfen (5 μ M). Relative to controls (A1, A5, B4, B7, C1) and surfen alone (A2, A6, C2), cells in explants treated with vitreous alone for 5 days underwent classical morphological changes consistent with secondary fibre differentiation (i.e. elongated, A7) and showed increased immunolabelling for fibre protein markers, (B8, C3). Vitreous-treated explants showed increased phosphorylated-ERK1/2 immunolabelling at 4 h (B11) relative to controls (B10). Co-treatment with surfen blocked vitreous-induced fibre elongation (A8), accumulation of fibre markers (B6, B9, C4), and ERK1/2 phosphorylation (B12). C) Low power image of explant treated for 5 days with vitreous (C3), with (C4) or without (C3) surfen, immunolabelled for fibre protein markers (merged). Dotted lines indicate the approximate lens equator in situ (C). Cell nuclei counterstained with Hoechst dye (blue). Scale bars = 25 μ m.

As immunolabelling of HS-GAGs in intact lens (see chapter 3) showed differential patterns in lens epithelial cells (cytoplasmic) and differentiating lens fibre cells (cytoplasmic, cell membrane and nuclear), we compared this to lens epithelial explants treated with either a low proliferative dose of FGF-2 for 24 h, or a high fibre differentiating dose of FGF-2 for 3 and 5 days and labelled with HS-GAG (Figure 5.12 C). While there was no change in HS-GAG labelling in explants treated with or without a low dose of FGF-2 (data not shown), when explants were treated with a fibre 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 (Figure 5.12 C1) to the cell nuclei of newly differentiating fibre cells (Figure 5.12 C2-3), comparable to that seen in the intact lens.

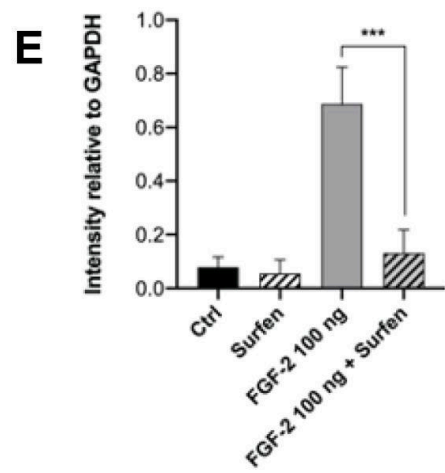
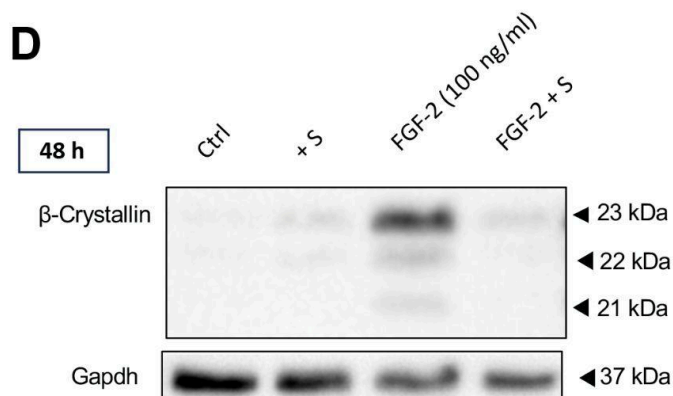
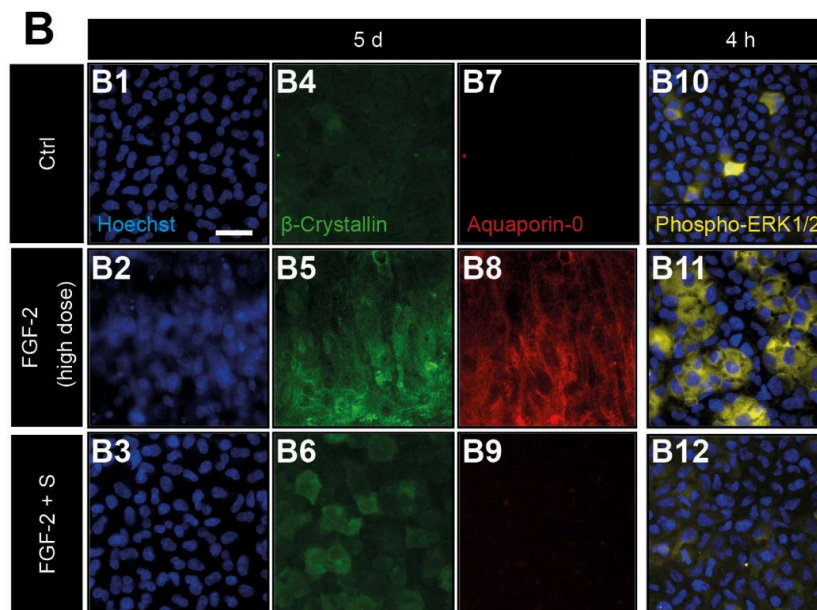
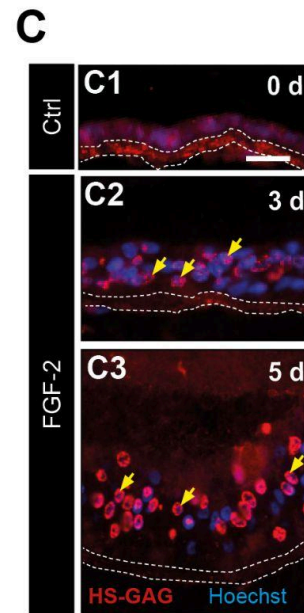
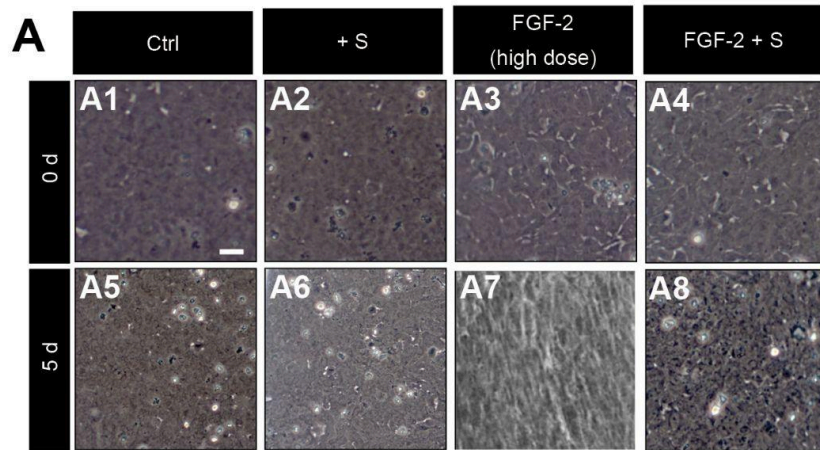


Figure 5.11. *HS-GAG sulfation is required for FGF-2-induced lens fibre differentiation.*

10-day-old rat lens epithelial explants were treated up to 5 days with a fibre differentiating dose of FGF-2 (150 ng/ml), with or without surfen (5 μ M). Relative to controls (A1, A5, B4, B7, C1) and surfen alone (A2, A6, C2), explants treated with FGF-2 alone for 5 days labelled for fibre protein markers, β -crystallin (B5) and aquaporin-0 (B8). FGF-2-treated explants showed increased phosphorylated-ERK1/2 labelling at 4 h (B11) relative to controls (B10). Co-treatment with Surfen blocked FGF-induced fibre cell elongation (A8), suppressed accumulation of fibre markers (B6, B9), and ERK1/2 phosphorylation (B12). Mid-sagittal sections through FGF-2-treated explants labelled for HS-GAG (HS-10E4, C) up to 5 days show elongated fibre cell morphology (C2, C3) and translocation of HS-GAG from the lens capsule at day 0 (C1) to the nuclei of elongating cells at 3- and 5-days post-treatment (yellow arrows, C2, C3). Dotted lines indicate the border of the lens capsule (C1-3). Representative western blot (D) shows surfen blocks FGF-2-induced β -crystallin protein accumulation at 48 h (E, $P < 0.0001$).

5.4 Discussion

This is the first study to conclusively demonstrate that HS-GAG sulfation is required to regulate lens epithelial cell proliferation and fibre differentiation in rat lens explants induced by ocular humour and their key component growth factors. Surfen, a novel small molecule inhibitor of HS-protein interactions, produced a more complete block of aqueous and FGF-2-induced LEC proliferation, relative to sodium chlorate, highlighting the putative higher selectivity and specificity of Surfen for HS-GAGs. Surfen co-treatment partially blocked early ERK1/2-signalling activity and lens cell proliferation induced by aqueous, EGF and IGF-I, and completely blocked the downstream effects induced by FGF-2 and HB-EGF. PDGF-AA-induced proliferation was independent of HS-GAG sulfation. HS-GAGs are localised to the nuclei of differentiating lens fibre cells, and surfen blocks FGF-2 and vitreous-induced fibre differentiation. Similarly to what was observed *in situ* (see chapters 3 and 4) here we show that HA-GAGs translocate to the nuclei of differentiating fibre cells *in vitro* in lens epithelial explants treated with vitreous humour or a differentiating dose of FGF-2. Moreover, we show that blocking HS-GAG sulfation results in a block of fibre cell differentiation. Taken together, these data reveal the differential dependence of different mitogens and morphogens to regulate these lens epithelial cell proliferation and fibre differentiation, and adds further evidence to the selectivity of HSPG interactions with different growth factors.

5.4.1 Surfen is an effective inhibitor of HS-GAG sulfation *in vitro*

To determine the requirement of HS-sulfation for LEC proliferation induced by different ocular mitogens comprising aqueous humour, we compared activation of early signalling activation and proliferation of LECs exposed to different growth factors with and without inhibitors of

HS-GAG sulfation. To investigate the biological consequences of altering HS-GAG sulfation, we first compared the effects of sodium chlorate, an established inhibitor of post-translational GAG sulfation (Safaiyan et al. 1999, Conrad et al. 2001, Iyengar et al. 2017), and surfen, a novel selective small molecule antagonist of HS-GAG-protein interactions (Schuksz et al. 2008). Here, we demonstrate that sodium chlorate-supplemented media completely blocks LEC proliferation and ERK1/2-signalling activity induced by FGF-2 but does not significantly block aqueous-induced proliferation. Previous studies in our laboratory had shown that rat lens epithelial cell proliferation induced by aqueous or exogenous growth factors (EGF, PDGF-AA, IGF-I, FGF-2) was blocked when cultured in sodium chlorate-supplemented medium, and that sodium chlorate specifically blocked ERK1/2 phosphorylation induced by aqueous and FGF-2 but not the other mitogens (Iyengar et al. 2017). On the other hand, interference with HS-GAG sulfation using surfen completely blocked LEC proliferation and ERK1/2 activity induced by FGF-2 and HB-EGF (proliferation only), and partially blocked the proliferative response to aqueous, EGF and IGF-1. Proliferation induced by PDGF-AA was unaffected by the presence of surfen. These results suggest that lens mitogens and their capacity to induce proliferation in LECs are differentially regulated by HS-GAG sulfation.

5.4.2 Differential requirements of HSPG sulfation for lens cell proliferation induced by different ocular mitogens.

To characterised the mitogenic competency of growth factors present in the ocular environment and their individual and collective contributions to lens cell proliferation, we compared LEC proliferation induced by aqueous humour, and different key mitogens found in the ocular media: FGF-2, HB-EGF, EGF, IGF-I and PDGF-AA. Consistent with the findings of previous studies

(Iyengar et al. 2006, Kok et al. 2009) here we report that cultured rat LECs undergo proliferation when exposed to aqueous humour, and its primary component mitogens. We found that all exogenous growth factors significantly increased cell proliferation, with aqueous humour producing the highest increase, followed by IGF-I, EGF, FGF-2, PDGF-AA and HB-EGF. The differential mitogenic capacity of lens growth factors to induce LEC proliferation, and that none alone are comparable to that of aqueous lends further evidence to the predominating theory that the proliferation and maintenance of the anterior lens epithelium is regulated by the activity and interplay of several native ocular growth factors in the aqueous humour (Ibaraki and Reddy 1995, Lovicu and McAvoy 2005, Igengar et al. 2006, Iyengar et al. 2009).

While HS-GAGs bind to and regulate a wide range of growth factors, not all require HSPGs for signalling activation. Many more are reported to be influenced directly by HSPGs, for instance, by binding to HS-chains in the pericellular matrix, or indirectly through HS-binding and regulation of other proteins directly related to growth factor signalling and bioavailability. For instance, of the mitogens tested, only two (FGF-2 and HB-EGF, Chu et al. 2005) are known to bind directly to sulfated HS-GAG chains, and of these two only FGF-2 is believed to require HS-sulfation for the formation of its ternary signalling complex with FGF receptors, which require HS-GAGs as co-receptors for signalling activation. Although HB-EGF can bind directly to HS chains, which can influence its bioavailability at the cell surface, its receptor binding activation is independent of HS-GAGs. EGF, IGF-I and PDGF-AA represent non-HS-binding growth factors which also do not require HS-GAGs for their receptors binding and signalling activation. For this reason, it would be expected that lens factors would be differentially affected by loss of HS-GAG activity.

Given the dependence of FGF-2 signalling on HS-GAG sulfation (Forsten-Williams et al. 2005), it is not surprising that FGF-2-induced proliferation was blocked by both sodium chlorate and surfen. The lesser extent of this block with sodium chlorate relative to surfen may be related to the fact that sodium chlorate impacts only a subset of HS-GAG sulfation sites (Safaiyan et al. 1999). HS-GAG chain interactions with HS-binding proteins are differentially influenced by GAG chain fine structure, specifically, chain length and the extent and patterning of post-translational sulfation sites (reviewed in chapter 1). For instance, *Hs6st*^{-/-} mutant animals (with HS-GAGs deficient for sulfation at the 6-O-position) exhibited lacrimal gland hypoplasia and disrupted FGF signalling, while *Hs2st*^{-/-} animals (with HS-GAGs deficient for sulfation at the 2-O-position) had a less severe phenotype (Qu et al. 2011), suggesting that FGF-HS interaction are promoted by the presence of specific O-sulfation sites on HS chains. As an electrostatic inhibitor, surfen binds to all sulfated sites on HS-GAGs and is predominantly influenced by the extent of GAG-chain sulfation. On the other hand, sodium chlorate selectively impairs O-sulfation in a dose dependent manner with low concentrations of sodium chlorate (5 or 20 mM) decreasing 6-O but not 2-O HS sulfation, and high doses of sodium chlorate (50 mM) reduced overall o-sulfation by 70% in MDCK cells (Safaiyan et al. 1989). Thus the relatively low dose of sodium chlorate (30 mM) used in this study and previous work from our laboratory likely did not impair FGF-promoting HS 2-O sulfation in lens epithelial cells to the same extent as surfen, which may explain why surfen was more effective at inhibiting FGF-2-induced proliferation relative to sodium chlorate.

Both surfen and sodium chlorate did not fully block the mitogenic effects of aqueous humour, which supports the accepted notion that the proliferative capacity of aqueous is the result of the cumulative effects of its component mitogens, several of which are not directly regulated by HS-GAG interactions. The more significant block of aqueous by surfen relative to sodium chlorate may also relate to the site-specific de-sulfation of HS-GAGs by sodium chlorate. Additionally, it is possible that sodium chlorate did not equally affect the binding of all HS-binding growth factors due to the heterogeneous nature of its desulfating effects. In addition to selective and dose-dependent de-sulfation of different HS O-sulfation site, N-sulfation of HS chains remains unchanged even when exposed to high concentrations of sodium chlorate (Safaiyan et al. 1989). As LEC proliferation is impaired in mice specifically lacking N-sulfation, it would be expected that the inhibition of HS-GAG sulfation at both O- and N-positions by surfen would produce a more complete block of any potential binding sites on HS-GAGs. This is important, because when binding to HS-GAG chains, FGF-2 and HB-EGF share low affinity binding sites, but also bind selectively and with higher affinity to smaller subsets of binding sites on HS-chains, which are exclusive to each growth factor (Chu et al. 2005). Additional research into the specific binding requirements of HS-binding GAGs in the lens, and how they are differentially affected by surfen vs. sodium chlorate is beyond the scope of this study but would be valuable to elucidate the fine structural requirements of HS-GAGs in the binding and regulation of ocular growth factors.

Surfen produced a partial block of HS proliferation induced by two non-HS-binding growth factors, EGF and IGF. Here for the first time, we compared the mitogenic activity of two members of the EGF family, one HS-binding (HB-EGF) (Chu et al. 2005) and the other

HS-independent (EGF). LEC proliferation induced by HB-EGF was abrogated in the presence of surfen, while EGF-induced proliferation was only partially blocked. The dependence of HB-EGF on HS-sulfation is in agreement with several studies in other systems where HS-binding is essential to HB-EGF receptor binding (Raab et al.1997) and function (Higashiyama et al. 1993, Iwamoto et al. 2010). Unlike FGF receptors, EGF receptors do not require HS-binding for activation, suggesting that the block of HB-EGF mitogenic activity in the presence of surfen is upstream of receptor binding interactions. One possibility relates to competitive inhibition of HB-EGF and surfen to HS-chains in the pericellular matrix. HS-GAG sequestration of growth factors is important for the regulation of the bioavailability of these growth factors at the cell surface, which may be compromised by competitive inhibition of HB-EGF/HS-GAG binding by surfen. The partial block of HS-independent EGF by surfen was more unexpected. Although EGF and HB-EGF share certain receptors (HER1 and HER4) only HB-EGF requires HS-binding (Raab et al.1997). One possible explanation for this is the indirect effects of HS-binding growth factors (i.e. FGF-2 and HB-EGF) on EGF activity. Simulations of HS-growth factor-receptor interactions reveal that HB-GF can regulate receptor binding EGF even when the two proteins share no binding sites (Forsten-Williams et al. 2008). It is plausible that surfen treatment of explants could compete for HSPGs in the pericellular matrix, releasing native HB-EGF which may disrupt EGF binding to its shared receptors, resulting in decreased EGF-induced proliferation.

Unlike EGF, the partial block of IGF-I activity by surfen was not entirely unexpected. HS-GAGs do not bind IGF-I or its cognate receptors (IGFRs), and thus have no direct effect on the bioavailability or receptor binding activity of IGF-I, however, the ability of HSPGs to influence

IGF-I signalling indirectly is well-established due to their binding and regulation of IGF-binding proteins (IGF-BPs) (Clemmons 1998, Allard and Duan 2018). IGFBPs are found in the pericellular matrix and are important molecules for regulating the bioavailability of IGFs to their receptors. Their primary role is as negative regulators of IGF-I signalling by sequestering IGFs in the ECM, inhibiting their binding to receptors at the cell surface. HSPG binding to IGF-BPs has been shown to increase IGF-BP concentrations in the matrix by protecting them from degradation (Smith et al. 1994).

The only growth factor tested which did not appear to be influenced by the presence of surfen was PDGF-AA. As PDGF-A has been reported to bind to HS-GAGs, this finding was somewhat unexpected (García-Olivas et al. 2003). One key difference between the other HS-binding growth factors in this study (FGF-2 and HB-EGF) and PDGF-A is the selectivity of HS-GAG binding. Most HS-protein interactions are attributable to low-affinity electrostatic interactions between the highly negatively charged HS-GAGs and positively charged regions of the proteins. These interactions are sufficient to categorise PDGF-A, FGF-2 and HB-EGF as HS-binding growth factors, however, unlike PDGF-A, in addition to these low-affinity binding interactions, FGF-2 and HB-EGF bind selectively and with higher affinity to smaller subsets of binding sites on HS-chains, which are exclusive to each growth factor (Chu et al. 2005). The same is not true for PDGF-A, which may explain its lower affinity for HS-binding relative to FGF-2 and HB-EGF (Raines et al. 1992). The non-specific and low-affinity electrostatic nature of the interaction of HS-GAGs and PDGF-A also means it can also readily bind to other sulfated GAGs, such as CS-GAG (Hamerman et al. 1987, Klagsbran et al. 1990). As surfen is a selective antagonist of HS-GAGs, it is reasonable to speculate that in the absence of HS-GAGs caused by

the competitive binding of surfen, PDGF-A would bind to other available GAGs, such as CS-GAG which are also present in the lens epithelium (see chapter 3). The observation that treatment with sodium chlorate, a non-specific inhibitor of all sulfated GAGs, but not surfen blocks PDGF-A-induced proliferation in LECs, lends further evidence to suggest that HS-binding may not be essential for the mitogenic effects of PDGF in lens epithelia (Iyengar et al. 2017).

Taken together, these findings highlight the diverse capacity of HSPGs to directly and indirectly regulate both HS-binding non-HS-binding growth factors and their downstream biological effects.

Table 5.1. Summary of effects of surfen on lens mitogen-induced signalling activity and proliferation.

Mitogen	Effect of surfen on mitogen-induced downstream signalling activity (phospho-ERK1/2 immunolabelling at 4 h)	Effect of surfen on mitogen-induced lens cell proliferation (% BrdU incorporation over 24 h)
AQ	Partially blocked	Partially blocked (**)
FGF-2 (1 ng/ml)	Blocked	Blocked (****)
HB-EGF	Blocked	Blocked (****)
EGF	Partially blocked	Partially blocked (**)
IGF-I	Partially blocked	Partially blocked (**)
PDGF-AA	Not blocked	Not blocked (ns)

Summary of figures 5.3-5.9. Surfen: 1 h pre-treatment prior to addition of mitogen. Different from mitogen alone (Student *t*-test): ns: not statistically significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Partially blocked = ns $> P > 0.0001$.

5.4.3 HSPG sulfation is required for lens fibre differentiation

Fibre differentiation is characterised by distinct molecular and morphologic changes, such as exit from the cell cycle, cell elongation, loss of cytoplasmic organelles including nuclei, as well as the accumulation of fibre-specific crystallin proteins and aquaporin-0 (Wride, 2011). Unlike the diverse effects of surfen on the different mitogens contributing to LEC proliferation, here for the first time we report that surfen blocks fibre differentiation mediated by both a lens fibre differentiating dose of FGF-2 and vitreous and surfen. Unlike LEC proliferation, which can be induced both by aqueous and by each of its component mitogens in isolation, although several growth factors contribute to the regulation and potentiation of lens fibre differentiation, the activity of FGF-2 is required for terminal differentiation. As discussed previously, it was expected that surfen would abrogate FGF-2 signalling, and thus it is not surprising that surfen would block fibre differentiation induced both by FGF-2 and vitreous. One interesting finding from these studies, was that although surfen could completely block the morphological changes and expression of aquaporin-0 in LECs treated with vitreous or a differentiating dose of FGF-2, an increase in β -crystallin expression was still observed. The morphological and protein changes associated with fibre differentiation are under the control of different signalling pathways downstream of receptor activation. Broadly, these fall into two major categories: i) elements of fibre differentiation that require activation of MAPK/ERK-signalling, and ii) those that are independent of the activation of this pathway, instead relying on the activation of other pathways (e.g., PI3K/AKT). Both the morphological changes and expression of aquaporin-0 in LECs are dependent on ERK1/2 activation. Consistent with this, surfen blocked ERK1/2 activation by vitreous and FGF-2 at 4 h. On the other hand, β -crystallin accumulation is considered to be

independent of ERK1/2 signalling (Lovicu et al. 2001). This work lends further compelling evidence for the requirement of FGF-2 in lens fibre differentiation.

In explants treated with a fibre differentiating dose of FGF-2, HS-GAG was seen to translocate from the lens capsule (where HSPGs can be sequestered) and cytosol of lens epithelia to the cell nuclei of newly differentiating fibre cells. This is similar to what is observed in the transitional zone of intact lenses (see chapters 3 and 4). While HSPGs localise to the nuclear compartment of various cell-types, the function of nuclear HS/HSPGs is yet to be understood (Kovalszky et al. 2014, Stewart and Sanderson, 2014), however, their association with HS-binding proteins, including growth factors and their high affinity receptors, may implicate them in the regulation of gene expression and the control of cell behaviour (reviewed in Stewart and Sanderson, 2014). For example, FGF-2 and even its receptor, FGFR-1, have been detected in the nuclei of different cell types (Maher, 1996), including differentiating lens fibre cells (de Iongh et al. 1996), where they can co-localise with HSPGs, influencing cell proliferation and differentiation in a cell type-specific manner (Amalric et al. 1994).

This study provides evidence that surfen can block the effects of vitreous and FGF-2 on lens epithelial cell differentiation and proliferation, suggesting that HSPGs may be a major regulatory factor in the control of lens fibre differentiation.

5.4.4 Conclusions

Taken together with previous work from our laboratory, this data supports a requirement for HSPG/GAG sulfation in growth factor-induced-signalling in the lens. This work highlights the

divergent and complex roles of HSPGs in the signalling regulation of different lens growth factors, and positions HSPGs as major regulators of lens cell proliferation and differentiation. By further characterising and specifically functionally modulating these different sulfated proteins, we hope to better understand how key lens HSPGs regulate different growth factor signalling associated with cellular processes. Moreover, gaining a better understanding of specific HSPG activity may allow us to develop novel ways of regulating cell behaviour, that will not only be relevant to lens biology and disease, such as cataract, but may extend to other systemic growth factor-mediated pathologies.

CHAPTER 6: RESULTS IV

HEPARAN SULFATE PROTEOGLYCANS IN FIBROTIC CATARACT

6.1 Preface

Part of the experimental work in this chapter involved the technical assistance of students under my co-supervision, Mr. James Heaton, Mr. Markus Smith and Mr. Gerard Wehbe.

6.2 Introduction

Cataract, characterised by the opacification of the lens resulting in partial or total vision loss, stands as the primary etiological factor for global blindness (Foster and Resnikoff, 2005, Lam et al. 2015, Pesudovs et al. 2021). Its significant influence on individual health and productivity results in substantial economic implications for healthcare systems (Brown et al. 2013,). Currently, surgical intervention remains the sole therapeutic strategy for cataract management (Allen and Vasavada 2006, Lam et al. 2015). This involves the surgical excision of the cataractous tissue from the surrounding lens capsule, and its replacement with an intraocular prosthetic implant ([Figure 1.9](#)). Despite the high efficiency and efficacy of cataract surgery (Chen et al. 2021), a considerable subset of patients can develop a secondary fibrotic cataract within a period of five years (Wang et al. 2017, Konopińska et al. 2021, Gu et al. 2022). This complication, known as posterior capsular opacification (PCO) is a fibrotic form of cataract with a similar pathogenic onset to anterior subcapsular cataract or capsular opacification (ASC/ACO), typically characterised by well-defined fibrotic events thought to be driven by TGF- β 2 (reviewed in Wormstone 2009). In PCO ([Figure 6.1](#)), lens epithelial cells left behind after surgical phacoemulsification of most lens cells respond to aberrant, elevated levels of active TGF- β 2 in the aqueous humour (Wallentin et al. 1998), which stimulates them to undergo EMT and

transdifferentiate into motile myofibroblastic cells (Awasthi et al. 2009, Shu and Lovicu, 2017, Jiang et al. 2018). Similar to fibrosis in other tissues (Leask and Abraham 2004), PCO (Reviewed in Wormstone et al. 2009, Lovicu et al. 2016) is characterised primarily by the transformation of cuboidal lens epithelial cells to spindle-shaped motile myofibroblastic cells. These cells deposit an aberrant matrix substrate rich in fibronectin and collagens-I and -III (rather than collagen IV) to which they readily adhere. Influenced by FGF-2 and integrin signalling, these myofibroblasts undergo migration and hyperproliferation, forming Soemmering's ring. LECs may also undergo aberrant differentiation into fibre cells, known as Elschnig's pearls. Subcapsular plaques lead to secondary opacification, compromising the functionality of the prosthetic IOL (Neumayer et al. 2017). The only existing treatment for PCO is Nd:YAG laser capsulotomy (Karahan et al. 2014), which is both expensive (Ursell et al. 2018, Raulinajtys-Grzybek et al. 2020) and runs the risk of serious complications (Javitt et al. 1992, Gu et al. 2022).

TGF- β -induced EMT is a complex stepwise phenomenon that plays a crucial role in fibrogenic disease, that is defined by a common set of intertwined cellular events, which are each differentially regulated by signalling molecules and pathways. First, induction of an injury response to lens epithelial cells causes the up-regulation of TGF- β production by lens epithelial cells, and its increased release from reservoirs in the lens capsule, along with other growth factors including FGF-2, EGFs and PDGFs by matrix metalloproteinases (MMPs) (Tholozan et al. 2007, Zelenka and Arpitha 2008). TGF- β binding to receptors on lens epithelial cells leads to the initiation of canonical TGF- β SMAD signalling, where SMAD2/3 forms a complex with SMAD4 and translocates to the nucleus, where it activates EMT-response genes and transcription factors (Figure 1.11).

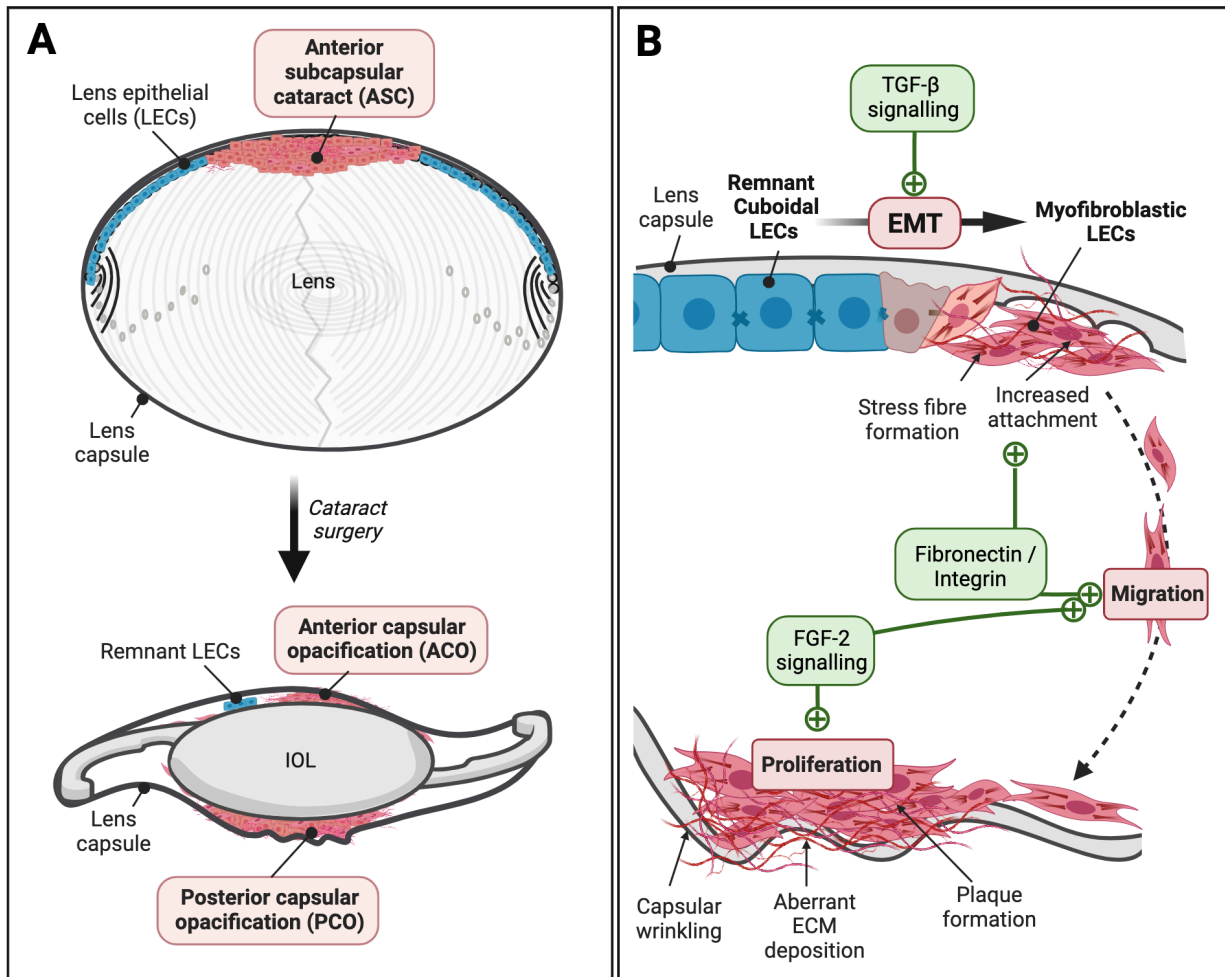


Figure 6.1. Pathogenesis of fibrotic cataract and lens TGF- β -induced epithelial-to-mesenchymal transition. (A) forms of fibrotic cataract. (B) molecular mechanisms underpinning the pathogenesis of posterior capsular opacification. Abbreviations: lens epithelial cells (LECs) anterior subcapsular cataract (ASC), anterior capsular opacification (ACO), posterior capsular opacification (PCO), transforming growth factor- β (TGF- β), epithelial-to-mesenchymal transition (EMT), fibroblast growth factor-2 (FGF-2), extracellular matrix (ECM).

TGF- β binds to its high affinity receptor subsequently leading to the downstream activation of its canonical signalling pathway, involving the phosphorylation of Small Mothers Against Decapentaplegic (SMAD) proteins, as well as non-canonical pathways (see [Figure 1.11](#)). For canonical signalling, TGF- β -signalling activation leads to the phosphorylation of SMAD2/3 in the cytoplasm, which then binds to SMAD4, and subsequently this ternary complex migrates to the cell nucleus to regulate the expression of TGF- β -responsive genes (Shirai et al. 2006, Meng et al. 2018). Various experimental models of TGF- β -induced EMT in the lens have also highlighted the involvement of non-canonical TGF- β signalling pathways, including MAPK, p38, β -catenin and Rho/ROCK mediated pathways (Taiyab, & West-Mays., 2022). The TGF- β -mediated wound healing response is also modulated and regulated by other growth factors (e.g. FGF-2, EGF) and ECM components (i.e. $\alpha 5\beta 1$ -integrin/fibronectin) (reviewed by Wormstone, 2002 and Liu and Kao, 2015).

TGF- β -induced EMT is a complex stepwise phenomenon that plays a crucial role in fibrogenic disease, that is defined by a common set of intertwined cellular events, which are each differentially regulated by signalling molecules and pathways. First, induction of an injury response to lens epithelial cells causes the up-regulation of TGF- β production by lens epithelial cells, and its increased release from reservoirs in the lens capsule, along with other growth factors including FGF-2, EGFs and PDGFs by MMPs. TGF- β binding to receptors on lens epithelial cells leads to the initiation of canonical TGF- β SMAD signalling, where SMAD2/3 forms a complex with SMAD4 and translocates to the nucleus, where it activates EMT-response genes and transcription factors. The result is the trans differentiation of cuboidal lens epithelial cells to myofibroblastic cells. The TGF- β -induced trans differentiation of lens epithelial cells to myofibroblastic cells is characterised by i) cytoskeletal reorganisation, including the formation of

actin stress fibres and increased expression of pro-contractile molecules (e.g. α -SMA, vimentin, tropomyosins), which manifest in spindle-shaped cell morphology, increased cell stiffness, reoriented microtubule polarity, and elevated cell motility (Korol 2007, Kubo et al. 2013), and ii) focal adhesion turnover and destabilisation of adherens junctions, indicated by the loss of epithelial markers (i.e. loss of junctional E-cadherin localisation) and the gain of mesenchymal markers (i.e. N-Cadherin, *Sail*) (Taiyab et al. 2022). Alterations to the actin cytoskeleton, focal adhesions, and stability of adherens junctions in TGF- β -induced EMT are regulated via a RhoA/ROCK-dependent pathway (Bhowmick et al. 2001). Having completed EMT, the myofibroblastic cells secrete abundant modified ECM, rich in fibrillar collagen I and fibronectin (Johar et al. 2007, Saika et al. 1998, Sappino et al. 1990). The motile myofibroblastic cells adhere to this modified matrix through interactions between matrix proteins (i.e. fibronectin) which induce focal adhesion formation at the cell–ECM interface through focal adhesion kinase (FAK)/integrin signalling-dependent mechanism. This alteration in both the epithelial cells and ECM results in a corresponding change to the lens capsule, which due to the increased adhesion of the contractile myofibroblastic cells, becomes wrinkled. At the same time, other factors in the ocular milieu, notably FGF-2, induce increased proliferation and migration of lens epithelial and myofibroblastic cells. The migratory and proliferative effects of FGF-2 have distinct pathways (Boilly et al. 2000). While FGF-2-induced proliferation is primarily mediated via ERK1/2 (Boilly et al. 2000, Lovicu and McAvoy 2001) and PI3K/AKT signalling (Boilly et al. 2000), FGF-2-induced migration rely on the signalling activity of Src kinase, p38 MAPK (Boilly et al. 2000) and integrins. Alongside FGF-2, other factors present in ocular milieu (i.e. PDGF, HB-EGF, IGF-Is) synergize with TGF- β to augment fibrosis through crosstalk, transactivation

and feed-forward mechanisms that include regulation of TGF- β levels, potentiation of pro-fibrotic pathways and suppression of TGF- β regulatory signalling.

The lens is an excellent model for the study of fibrotic disease (Eldred et al. 2011), and a range of *in vitro* and *in vivo* experimental models have been established to study the events underpinning fibrotic cataractogenesis (Wormstone and Eldred 2016). A growing body of work over the past decades in the lens field has made significant advances in our understanding of the underpinnings of fibrotic cataract, but there are still many unanswered questions. What is clear is that understanding the molecular basis of PCO is essential to elucidate potential strategies for prevention and treatment. The specific molecules and mechanisms that regulate PCO and its associated molecular mafia, remain largely unidentified and unexplored. Similarly, though the pathogenesis of fibrotic cataract has been extensively characterised both *in vitro* and *in vivo*, the field has yet to fully elucidate the role of external factors influencing the activation and progression of TGF- β -mediated EMT in the lens. Identifying the molecules that regulate cell behaviour and maintain the integrity of the lens is fundamental to discerning the underlying mechanisms involved in cataract formation. One such class of potential regulatory candidates are the HSPGs, which are known to impart a multifactorial influence over several key players and pathways involved in fibrosis.

Progressive advances in the field of glycobiology have revealed a critical role for proteoglycans in the activation and regulation of growth factor signalling and processes associated with mitogen-driven pathogenesis. As different HSPGs appear to influence overlapping signalling pathways with diverse and cell type-specific outcomes (Rossi 2003), characterising different HSPG-growth factor interactions to develop a more comprehensive understanding of the functional significance of HSPG-signalling changes with the onset of pathology, is an important

area of study. Studies from many tissues lend strong evidence to suggest an important role for HSPGs in the regulation of fibrotic disease (Solic et al. 2005, Westergren-Thorsson et al. 2017, Lord et al. 2018), however, the functional roles of HSPGs in fibrotic cataract remain largely uncharacterised.

HSPGs directly bind to and regulate the distribution and signalling activity of both FGF-2 and TGF- β in fibrosis (Chen et al., 2004, Qin et al. 2017), and have also been shown to be involved in the activity of key regulators of myofibroblast migration, including fibronectin (Kusano et al. 2000, Stepp et al. 2010), integrins (Couchman and Woods, 1999, Filla et al. 2006, Morgan et al. 2007, Qin et al. 2017), and focal adhesion kinase (Wilcox-Adelman et al. 2002, Shiha et al. 2020). HS/HSPGs have been shown to bind to TGF- β 2 (Lyon et al. 1997, Rider and Mulloy 2017) and it is important for TGF- β 2-signalling in some systems. For instance, disruption of HS synthesis (Ext1 knockdown) in mouse neural crest cells resulted in disrupted TGF- β 2 signalling (Iwao et al. 2009). HS-GAG also binds to protein antagonists of TGF- β 2 including gremlin, which has recently been reported to drive the development of PCO by promoting LEC proliferation and activation of TGF- β /SMAD signalling pathways (Ma et al. 2019). The effects of specifically disrupting HS-GAG in the lens on TGF- β 2 signalling has yet to be investigated.

For instance, a recent body of work has extensively characterised the role of Syndecan-4 in the pathogenesis of fibrotic cataract and demonstrated its potential as a therapeutic target for fibrotic lens pathologies such as fibrotic forms of cataract (i.e. ACO/PCO) (Qin et al. 2017). In this landmark study, syndecan-4 was reported to regulate FGF-2 and α 5 β 1-integrin signalling, with both FGF-driven proliferation and integrin-mediated adhesion and migration of LECs involved in the development of ACO/PCO requiring HSPG co-receptor activity. Moreover, mice lacking syndecan-4 have significantly reduced occurrence of lens opacification in an injury-induced

model of fibrotic wound healing (Qin et al. 2017). RNA interference knockdown of syndecan-4 in cultured human LECs also blocked FGF-induced proliferation and associated ERK1/2 and PI3/AKT/mTOR signalling pathway activation (Qin et al. 2017), with the same study reporting increased syndecan-4 expression in the LECs of age-related cataract patients (Qin et al. 2017).

Taken together, this data highlights HSPGs as potentially critical regulators of the aberrant cell behaviour underlying pathogenic processes, including TGF- β -induced EMT. A better understanding of these underlying regulatory mechanisms may serve as a basis for the identification of novel therapeutic targets and molecular strategies that could be implemented to slow or prevent the development and progression of diseases characterised by abnormal growth factor-mediated epithelial cell proliferation and HSPG sulfation profiles, such as fibrotic cataract. Here, we investigate the spatiotemporal immunolabelling of HSPGs in the pathogenesis of fibrotic cataract and also establish the first functional requirement for HSPGs in driving TGF- β -induced EMT in the lens. Here, we investigate the spatiotemporal immunolabelling and functional requirement of HSPGs in the pathogenesis of fibrotic cataract.

6.3 Results

6.3.1 Heparan sulfate is the predominant HSPG-GAG associated with lens fibrotic plaques.

Relative to wild-type lenses (Figure 6.2 A1), Alcian blue (AB) staining (indicating regions rich in sulfated GAGs) (Chute et al. 2019) is diminished from the lens capsule as it is increased in certain regions of lens fibrotic plaques (Figure 6.2 A2)., taken from transgenic mouse lines overexpressing TGF- β , indicating some heterogeneity in plaque sulfation patterns. A similar pattern was seen when labelling for HS-GAG chains, with increased staining in discrete cell

populations within the plaques, and a marked loss of staining from the lens capsule (Figure 6.2 B2). In contrast, relative to the lens of wild-type mice (Figure 6.2 C1), CS-GAG staining was uniformly lost in lens cells of transgenic mice that comprise the fibrotic plaques (Figure 6.2 C2).

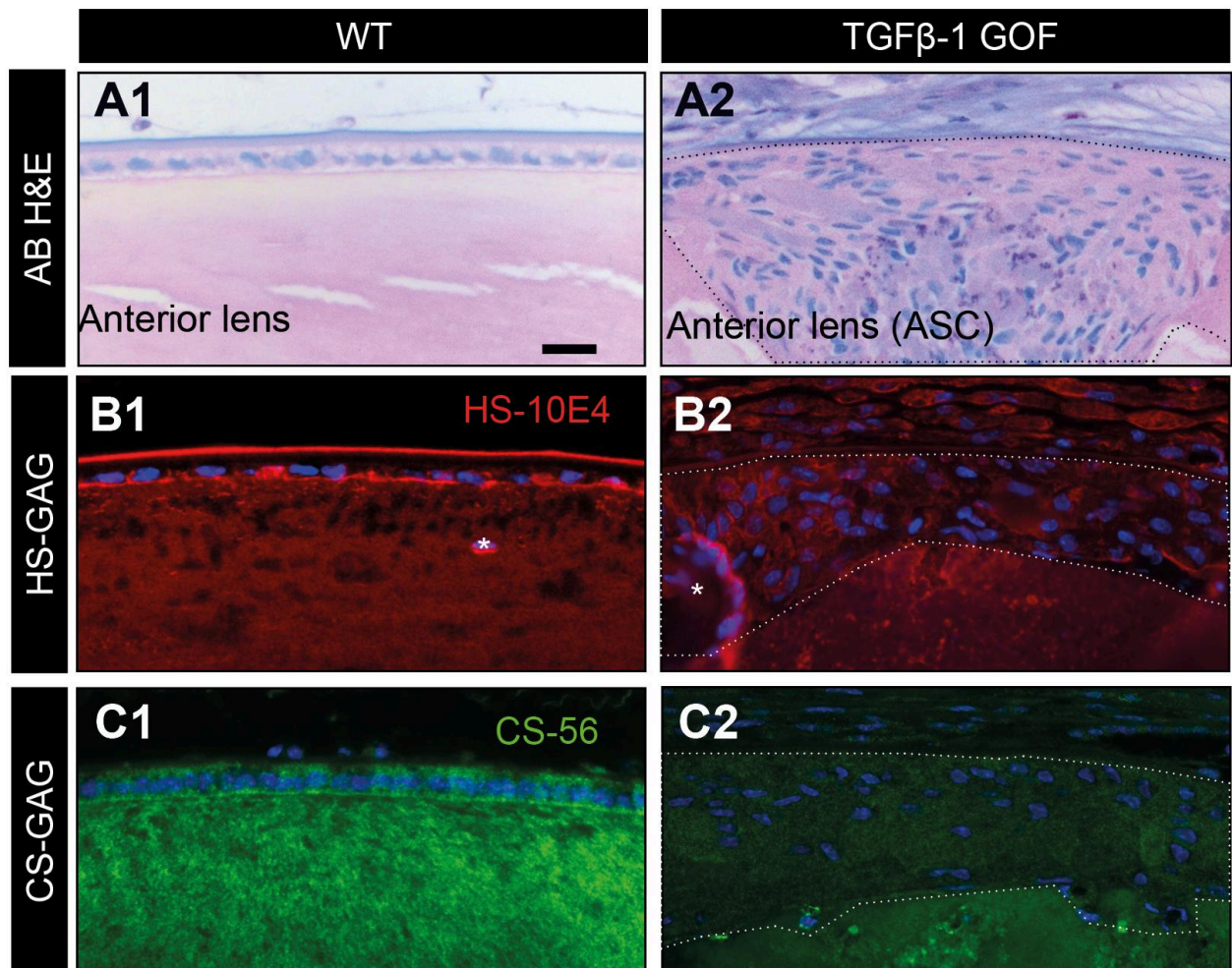


Figure 6.2. *Distribution of sulfated glycosaminoglycans in normal anterior lens vs fibrotic anterior subcapsular plaques of 21-day-old transgenic mice overexpressing TGF- β 1.*

Comparison between the normal anterior lenses of 21-day-old wild-type mice (WT, A1, B1, C1) relative to mice overexpressing TGF- β 1 in the lens (TGF- β 1 GOF, A2, B2, C2) presenting with anterior subcapsular plaques (ASC, outlined by white dotted lines). Mid-sagittal anterior lens sections stained with Alcian Blue and Haematoxylin & Eosin (AB H&E, A1-2) show distribution of sulfated GAGs, and morphology respectively. Staining shows comparative immunolabelling of the highly sulfated domains on heparan sulfate (HS-10E4, red, B1-2) or chondroitin sulfate (CS-56, green, C1-2) glycosaminoglycan chains. Cell nuclei are counterstained with Hoechst dye (blue). Scale bar = 25 μ m.

6.3.2 Immunolocalisation of HSPGs is differentially altered in the pathogenesis of fibrotic cataract.

When examining for HSPG labelling patterns in 21-day-old wildtype mouse lenses, these resembled those reported earlier for 10-day-old Wistar rats (see Chapter 3), except for glypicans 1, 5 and -6, that labelled strongly in the anterior epithelia of 10-day-old rats, but only stained weakly in 21-day-old mice lens (Figure 6.3 A1, E1, F1). Also, of note, syndecan-2 was not detected in the lens capsule of 21-day-old wildtype mouse lenses, although we found it to be present in 10-day-old Wistar rats (See chapter 3).

Compared with normal WT lenses, HSPG core proteins were markedly modified in cataractous plaques of transgenic lenses. These pathology-induced modifications could be grouped into different categories: **i)** changes in levels of protein and **ii)** changes in spatial and cellular patterns of labelling.

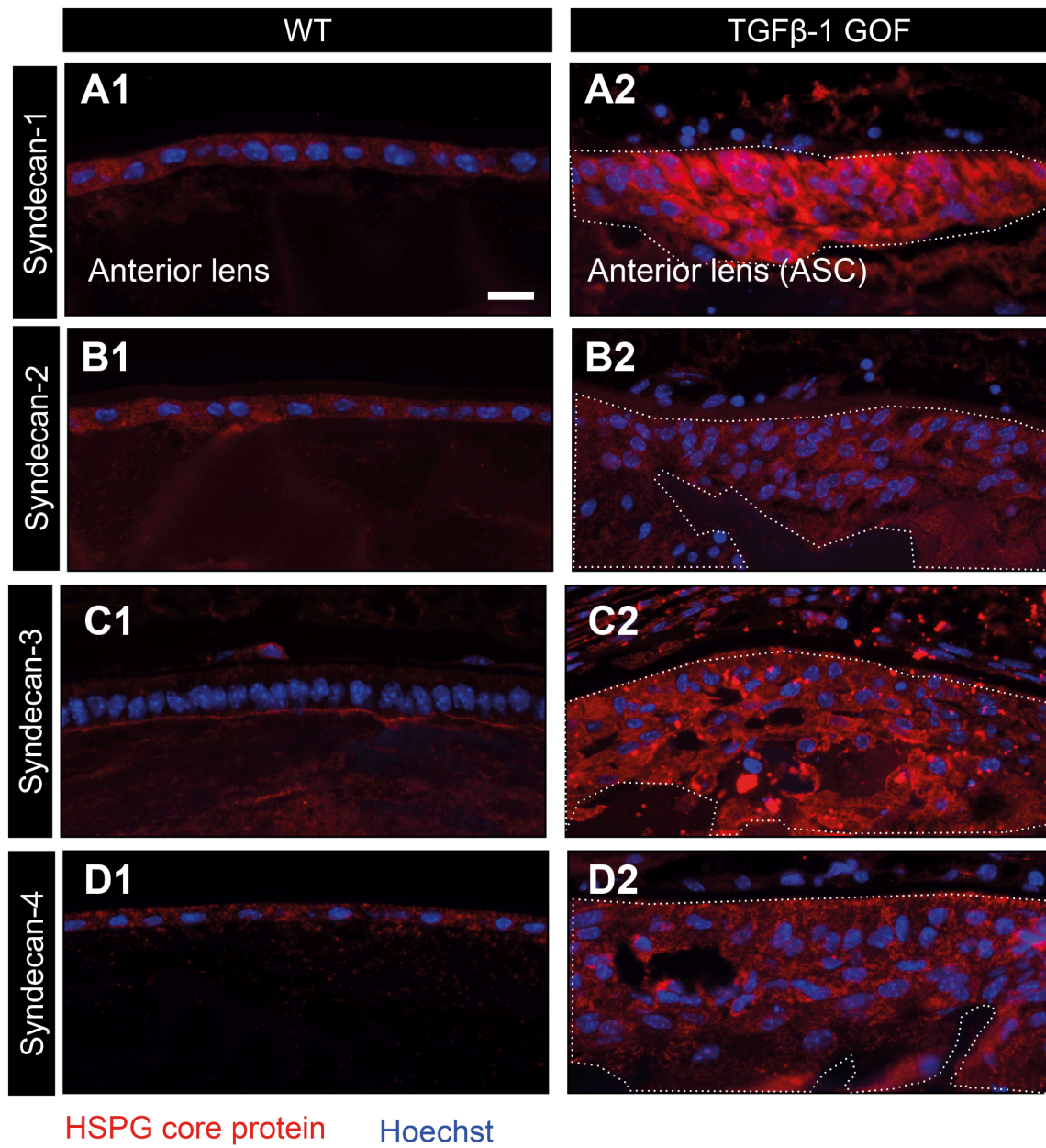
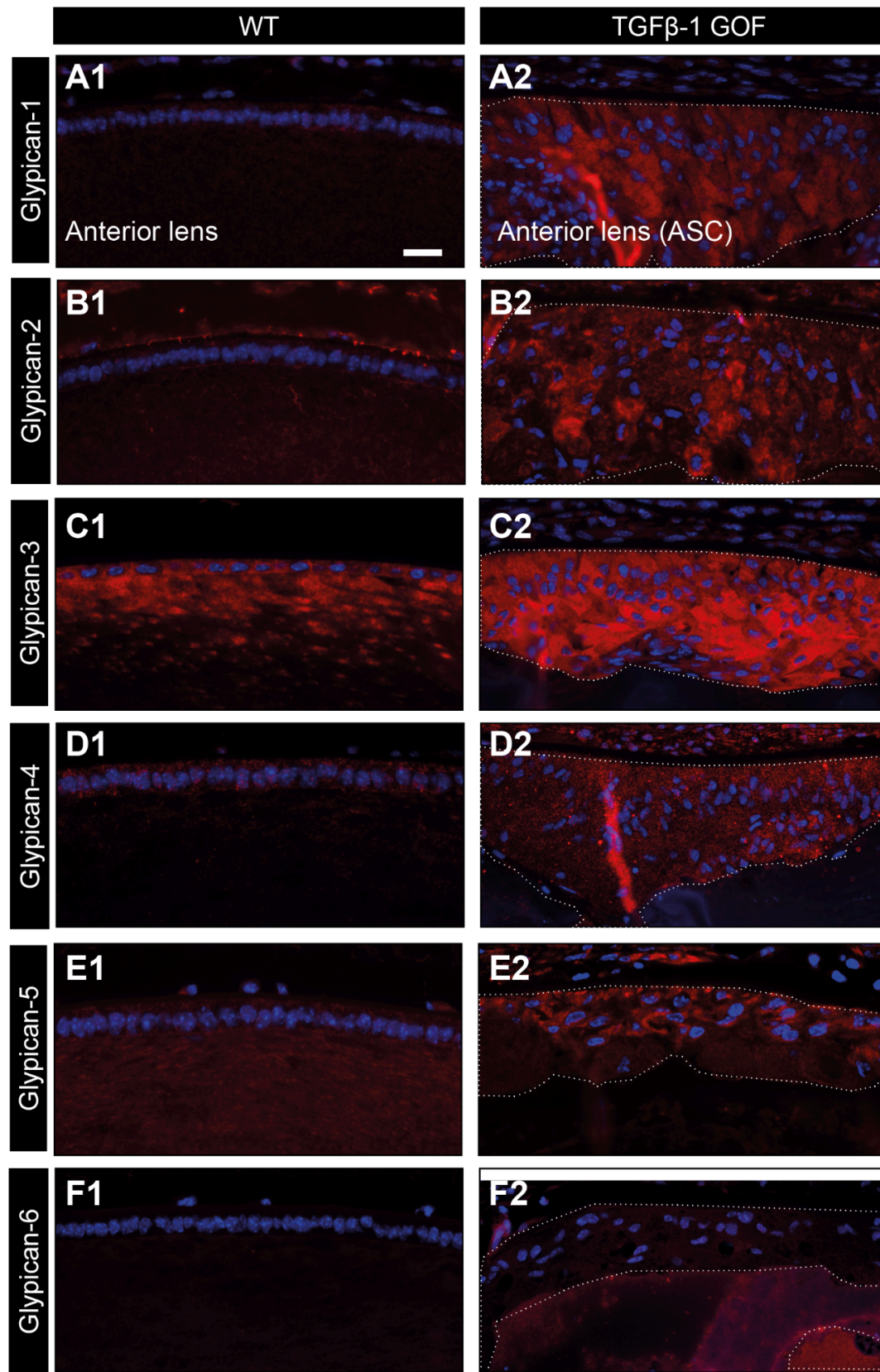


Figure 6.3. Immunolocalisation of syndecan core proteins in the normal anterior lens vs fibrotic anterior subcapsular plaques of 21-day-old mice. Mid-sagittal sections from 21-day-old wild-type mice (WT, A1, B1, C1, D1) and mice overexpressing TGF- β 1 in the lens (TGF- β 1 GOF, A2, B2, C2, D2) presenting with anterior subcapsular plaques (ASC) were immunolabelled for Syndecan (1-4) core proteins (red). Dotted white lines indicate the outline of fibrotic plaques. Cell nuclei counterstained with Hoechst (blue). Scale bar = 25 μ m.

Relative to wild-type, levels of different HSPGs were observed to increase or decrease in fibrotic plaques in a core protein-dependent manner. For instance, levels of labelling for syndecan-1, syndecan-3 (Figure 6.3 A, C), glypican-1, glypican-2, glypican-5 (Figure 6.4 A, B, E), collagen XVIII and agrin (Figure 6.5 A, B) were observed to be markedly increased in lens plaques of transgenic mice relative to wild-type mice. Only CS-GAG immunolabelling was observed to be decreased in lens fibrotic plaques of transgenic mice, relative to wild-type mice (Figure 6.2 C). The levels of syndecan-2, syndecan-4 (Figure 6.3 B, D), glypican-3, glypican-4 and glypican-6 (Figure 6.4 C, D, F) did not appear to increase or decrease.



HSPG core protein Hoechst

Figure 6.4. Immunolocalisation of glypican core proteins in the normal anterior lens vs fibrotic anterior subcapsular plaques of 21-day-old mice. Mid-sagittal sections from 21-day-old wild-type mice (WT, A1, B1, C1, D1, E1, F1) and transgenic mice overexpressing TGF- β 1 in the lens (TGF- β 1 GOF, A2, B2, C2, D2, E2, F2) presenting with anterior subcapsular plaques (ASC) were immunolabelled for glypican (1-6) core proteins (red). Dotted white lines indicate the border of fibrotic plaques. Cell nuclei counterstained with Hoechst (blue). Scale bar = 25 μ m.

Of the core proteins that were still present in plaques, two different localisation patterns were observed. Syndecan-1, syndecan-3 (Figure 6.3 A2, C2) and glypican-3 (Figure 6.4 C2) localised to fibrotic plaques relatively homogeneous, labelling most cells within the plaque. On the other hand, syndecan-2, syndecan-4 (Figure 6.3 B2, D2), glypican-1, glypican-2, glypican-3, glypican-5 (Figure 6.4 A2, B2, C2, E2), collagen XVIII and agrin (Figure 6.5 A2, B2) appeared to label only a specific subpopulation of cells within the plaque, giving a more heterogeneously pattern. This distinction can be further dissected into different core proteins displaying heterogeneous staining within or around cells throughout the whole plaque, extracellularly (i.e. collagen XVII, figure 6.5 A2, glypican-1; Figure 6.4 A2). On the other hand, several core proteins appear to label only a specific subpopulation of cells within the plaque, forming clear regions of division (i.e. Agrin, Figure 6.5 B2; glypican-2, Figure 6.4 B2).

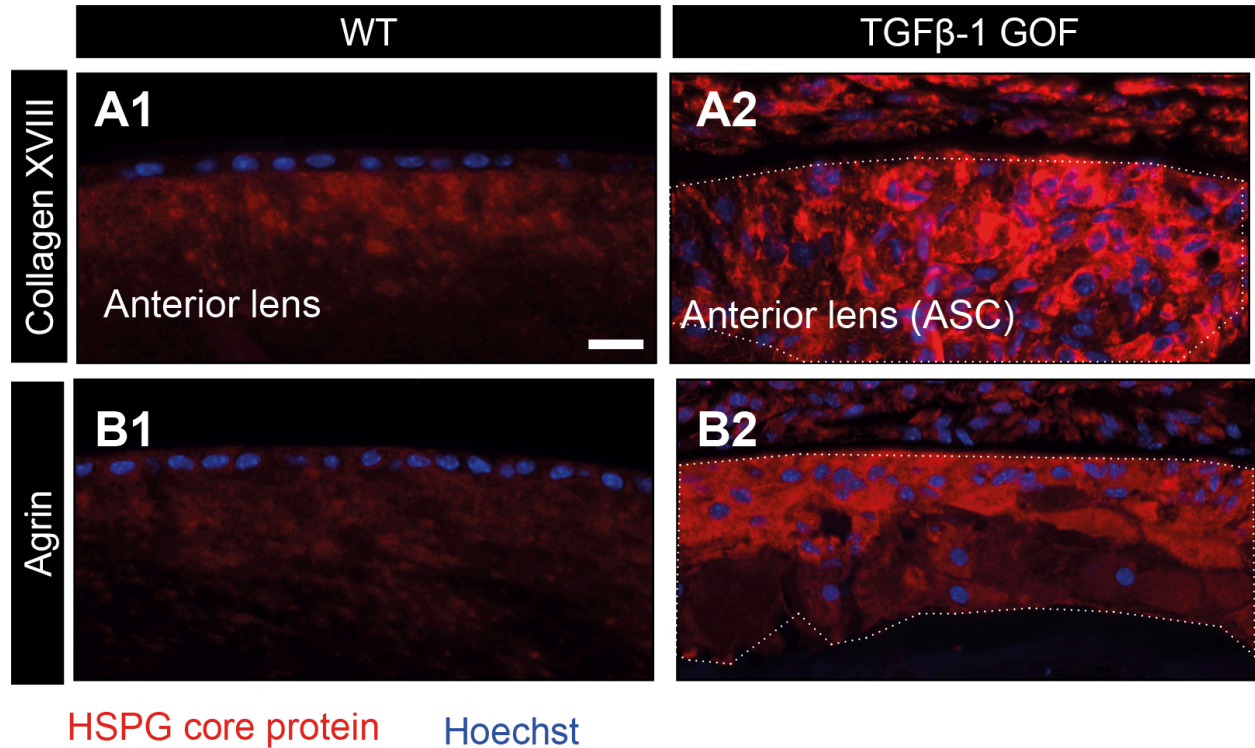


Figure 6.5. Immunolocalisation of collagen XVIII and agrin core proteins in the normal anterior lens vs fibrotic anterior subcapsular plaques of 21-day-old mice. Mid-sagittal sections from 21-day-old wild-type mice (WT, A1, B1) and transgenic mice overexpressing TGF- β 1 in the lens (TGF- β 1 GOF, A2, B2) presenting with anterior subcapsular plaques (ASC) were immunolabelled for collagen XVIII and agrin core proteins (red). Dotted white lines indicate the border of fibrotic plaques. Cell nuclei counterstained with Hoechst (blue). Scale bar = 25 μ m.

Table 6.1. Summary of changes to HSPG core protein immunolocalisation in fibrotic cataracts.

Change in level of labelling	<i>↑ Increase in TGF-β1 GOF relative to WT</i>	syndecan-1, syndecan-3, glypican-1, glypican-2, glypican-5, collagen XVIII, agrin
	<i>↓ in TGF-β1 GOF relative to WT</i>	HS-GAG, CS-GAG
	<i>No change in TGF-β1 GOF relative to WT</i>	Syndecan-2, syndecan-4, glypican-3, glypican-4, glypican-6
Core protein labelling of cells comprising fibrotic plaques	<i>Staining not observed in any cells within plaques</i>	glypican-6
	<i>Homogenous staining of cells within plaque</i>	Syndecan-1, syndecan-3, glypican-3,
	<i>Heterogeneous staining or staining of a specific subpopulation of cells within plaque</i>	HS-GAG, CS-GAG, syndecan-2, syndecan-4, glypican-1, glypican-2, glypican-3, glypican-5, collagen XVIII, agrin

Abbreviations: Heparan sulfate proteoglycan (HSPG), Increase (↑), Decrease (↓), transforming growth factor- β 1 (TGF- β 1), gain-of-function (GOF), wild-type (WT), heparan sulfate glycosaminoglycan (HS-GAG), chondroitin sulfate glycosaminoglycan (CS-GAG)

6.3.3 The HS-GAG antagonist surfen blocks TGF- β 2- EMT in the lens.

Pharmacological inhibition of HS-GAG activity *in vitro* blocked TGF- β -induced morphological changes in lens epithelial cells (i.e., elongation, migration) and downstream signalling (e.g. nuclear translocation of tSMAD2/3) associated with fibrotic epithelial-to-mesenchymal transition. Relative to cells in control explants (Figure 6.6 A1, B1) and those treated with surfen alone (Figure 6.6 B2, B2), cells treated with TGF- β 2 alone for 3 days underwent morphological changes consistent with epithelial-to-mesenchymal transition (i.e. elongation, apoptosis, capsular wrinkling, Figure 5 A3) and showed increased immunolabelling for the myofibroblastic stress-fibre marker, alpha-smooth muscle actin (α -SMA, Figure 6.6 B3) and altered labelling for the epithelial membrane marker beta-catenin (β -catenin, Figure 6.6 B3). Explants treated with TGF- β 2 for 3 days (Figure 6.6 C2) also showed decreased cellular staining of sulfated HS-GAG relative to untreated control explants (Figure 6.6 C1). Pre-treatment of lens epithelial explants with surfen blocked the TGF- β -induced myofibroblastic phenotype (Figure 6.6 A4), including the accumulation of α -SMA (Figure 6.6 B4).

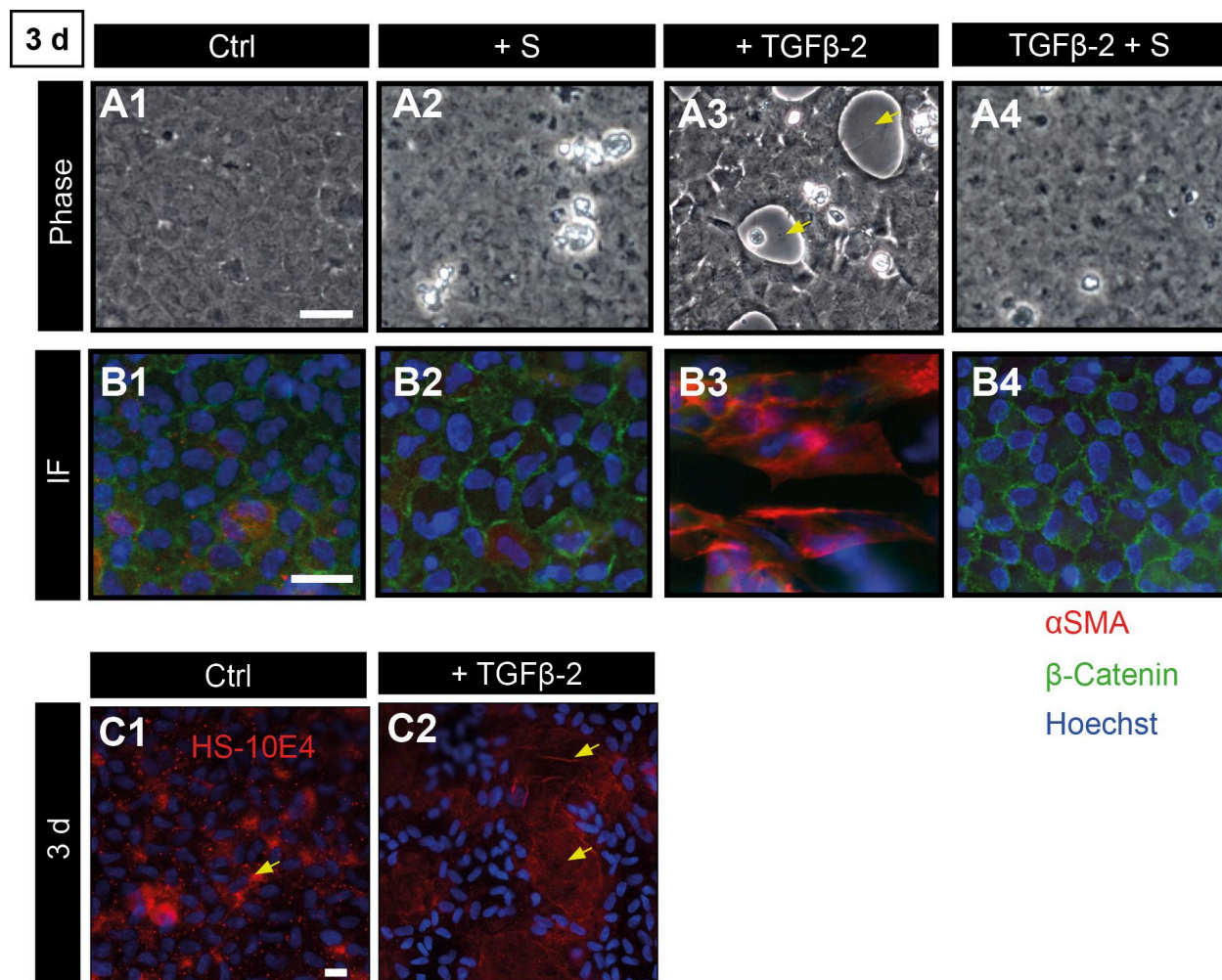


Figure 6.6. Surfen blocks TGF- β -induced epithelial-to-mesenchymal transition. 21-day-old rat lens epithelial explants were treated up to 3 days with TGF- β 2 (TGF- β 2, 300 pg/ml), with or without surfen (+S, 5 μ M), and labelled for myofibroblastic stress-fibre marker alpha-smooth muscle actin (α -SMA, red) and epithelial membrane marker beta-catenin (β -catenin, green). Capsular wrinkling indicated by yellow arrows (A3). C) Explants treated with TGF- β 2 for 3 days (C2) were compared with control explants and labelled for sulfated HS-GAG (HS-10E4, red). Dotted lines indicate approximate boundaries of regions of exposed capsule (C2). Cell nuclei counterstained with Hoechst dye (blue). Scale bars = 25 μ m.

Relative to controls explants (Figure 6.7 A1') and explants treated with surfen alone (Figure 6.7 B2'), cells in explants treated with TGF- β 2 alone (Figure 6.7 A3') for 2 h showed increased nuclear co-localisation of total SMAD2/3, that was blocked with pre-treatment with surfen (Figure 6.7 A4'). TGF- β 2-induced increased levels of vimentin, snai1, and phospho-ERK1/2 activity at 2 h (Figure 6.7 B), when compared to control explants,. TGF- β 2-induced increases in vimentin and phospho-ERK1/2 were completely blocked with surfen pre-treatment of cells, with Snai1 only partially blocked (Figure 6.7 B).

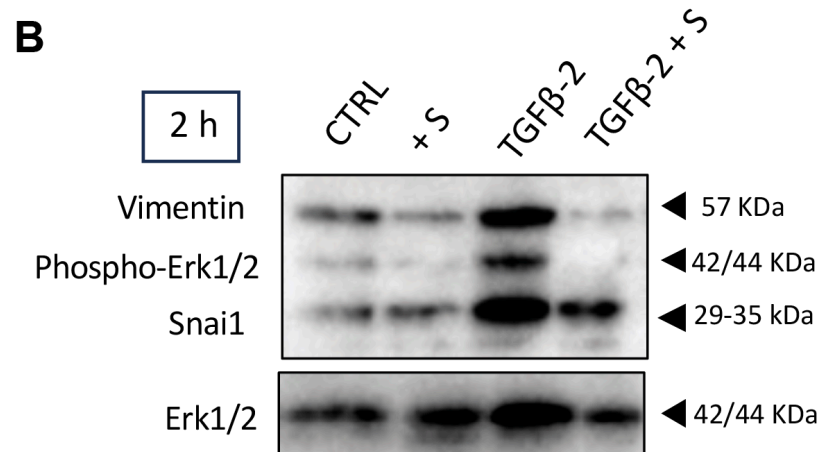
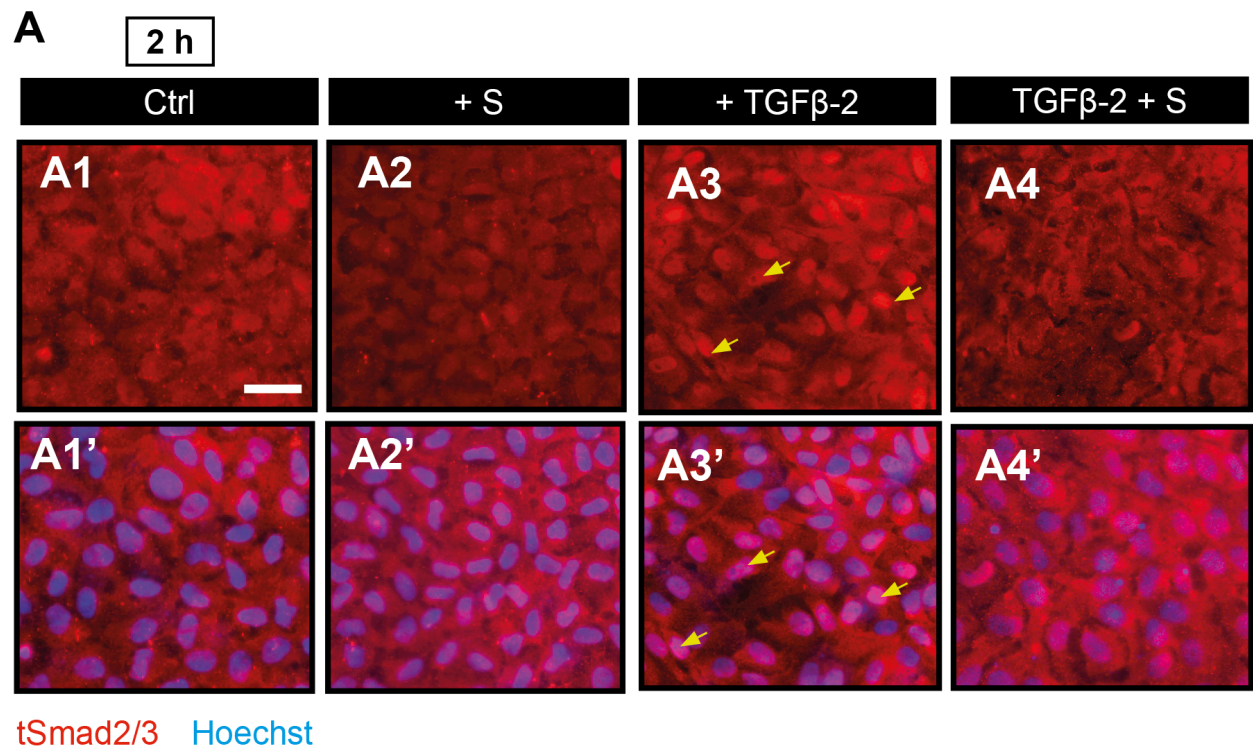


Figure 6.7. *Effects of surfen on early TGF- β -induced signalling. 21-day-old rat lens epithelial explants were treated for 2 h with TGF- β 2 (TGF- β 2, 300 pg/ml), with or without surfen (+S, 5 μ M). Nuclear translocation is indicated by yellow arrows. Western blotting was used to investigate early signalling markers associated with TGF- β 2-induced EMT (vimentin, Snai1, phosphorylated-ERK1/2 (phospho-ERK1/2) and total-ERK1/2 (ERK1/2)) at 2 h (B). Cell nuclei counterstained with Hoechst dye (blue). Scale bar = 25 μ m.*

Relative to controls (Figure 6.8 A1, B1, C1, D1) and surfen alone (Figure 6.8 A2, B2, C2, D2), All treatment timepoints of surfen blocked the induction of a TGF- β -induced myofibroblastic phenotype (Figure 6.8 A4, B4, C4, D4). In order to compare the ability of surfen to block TGF- β signalling after the induction of the critical initial phase of TGF- β -lens EMT, LECs were co- and post-treated with surfen (relative to TGF- β treatment). Explants in the co-treated and post-treated Surfen conditions showed a moderate increase in α -SMA staining, but cells remained cuboidal and did not form stress fibres (Figure 6.8 , B4, C4, D4).

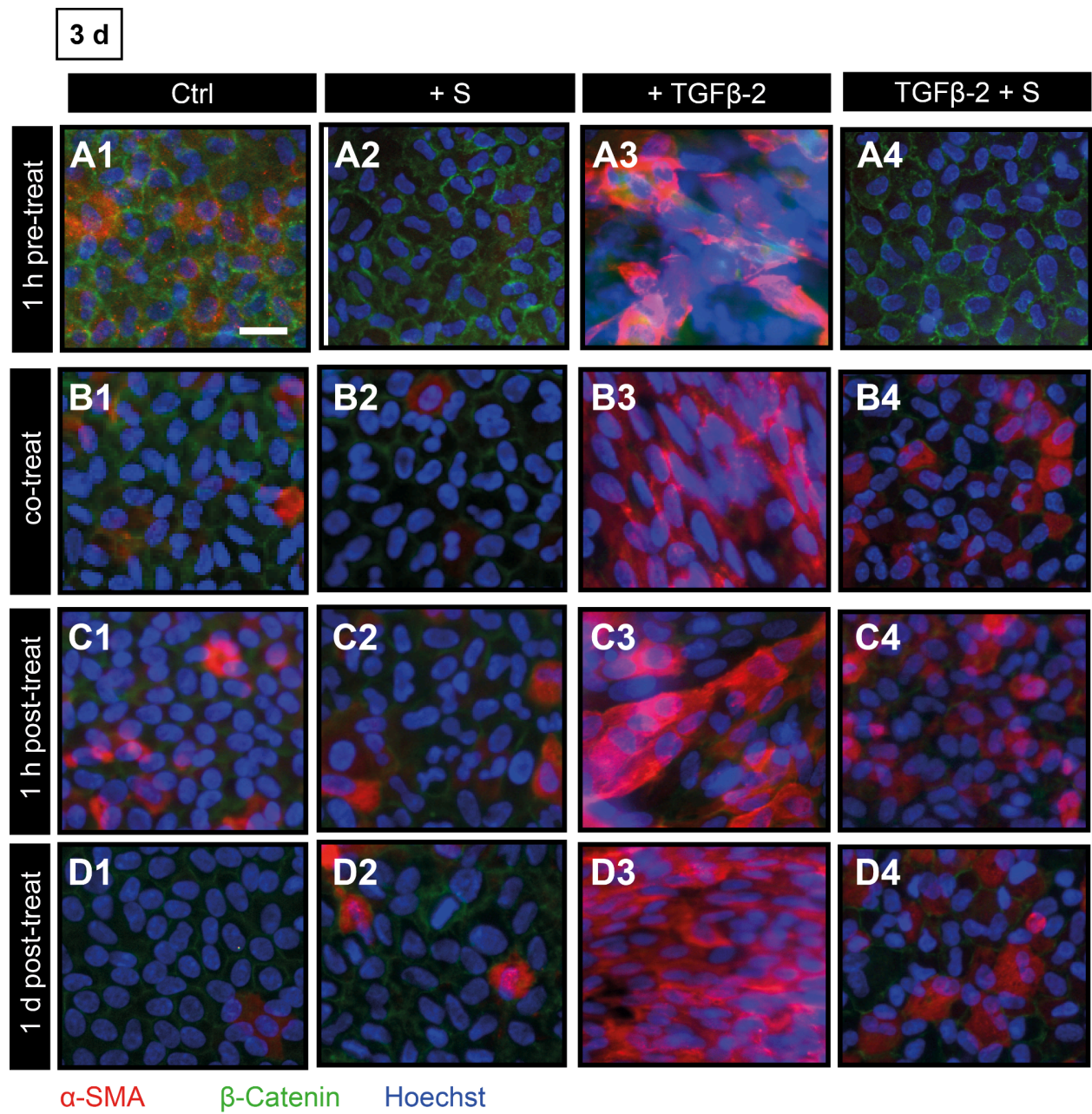


Figure 6.8. *Timing of Surfen treatment does not impact morphological changes associated with TGF- β -induced epithelial-to-mesenchymal transition. 21-day-old rat lens epithelial explants were treated up to 3 days with TGF- β 2 (TGF- β 2, 300 pg/ml), with or without surfen (+S, 5 μ M). Surfen was added to explant culture medium 1 h before TGF- β 2 (1 h pre-treat, A2, A4), together with TGF- β 2 (co-treat, B2, B4), 1 h after TGF- β 2 (1 h post-treat, C2, C4), or 1 d after TGF- β 2 (1 d post-treat, D2, D4). Cells were probed for myofibroblastic stress-fibre marker alpha-smooth muscle actin (α -SMA, red) and epithelial membrane marker beta-catenin (β -catenin, green). Cell nuclei counterstained with Hoechst dye (blue). Scale bar = 25 μ m.*

6.4 Discussion

Disruption of cellular processes associated with pathology have often been linked to significant changes in specific HSPGs (Heimann et al. 2001, Ibrahim et al. 2017) or HS-GAG sulfation enzymes (Saravanan et al. 2019), that can either drive these pathogenic processes, as seen in cataract (the opacification of the lens) (Qin et al. 2017), or can promote healing (reviewed in Park and Shukla, 2013). These changes can affect both specific core proteins (i.e. cleavage of specific cell surface HSPGs, up-/down-regulation of specific core proteins, changes in tissue expression patterns) and/or HS chain structure (i.e. cleavage of HS chains, up-/down-regulation of specific HS biosynthesis/sulfation enzymes). Moreover, this can highlight roles for specific HSPGs in the pathogenesis of disease.

6.4.1 *Distribution of sulfated glycosaminoglycans associated with lens fibrotic plaques.*

Proteoglycans make up a significant fraction of the matrix associated with fibrotic lesions, and accumulation of GAGs in the fibrotic areas can be visualised by Alcian Blue staining, indicating regions rich in sulfated GAGs (Chute et al. 2019), or by immunolabelling of sulfated GAG-chains. Relative to wild-type lenses we observed Alcian blue staining and HS-GAG immunofluorescent staining to be diminished from the lens capsule and increased in certain regions the subcapsular plaques from lenses of our transgenic mice, consistent with a decrease in sulfated HS-GAG staining observed in rat lens epithelial explants treated with TGF- β 2 for 3 days (see Figure 6.6 C). CS-GAG immunostaining showed decreased overall labelling in transgenic mouse lenses relative to wild-type. The loss of HS-GAG from the matrix associated with tissue fibrosis or injury is frequently reported owing to HS-GAG cleavage by increased activity of MMPs. MMPs, in particular MMP-2 and -9, are increased in the lens capsules of fibrotic

cataracts *in vivo* in humans and rats (Seomun et al. 2001, Wong et al. 2004) and have been shown to be up-regulated by TGF- β *in vitro* in rat lenses and lens epithelial cell lines preceding to the onset of EMT (Seomun et al. 2001, Dwivedi et al. 2006, Nathu et al. 2009, Liu et al. 2021). The cleavage of HSPGs from the capsule serves a regulatory function, as a mechanism of releasing pools of HS-bound ligands sequestered in the pericellular matrix to increase their bioavailability to cells. HSPGs are known to bind to several heparin-binding growth factors known to contribute to or potentiate lens fibrosis, including TGF- β (McCaffrey et al. 1992, Lyon et al. 1997), FGF-2, HB-EGF, PDGFs, BMPs and Wnts, several of which have been shown to increase in bioavailability with the onset of lens fibrosis. Moreover, Mice deficient in MMP-9 showed increased resistance to TGF- β 1-induced ASC plaque formation (Korol et al. 2014), and MMP inhibitors decrease fibrotic cataract formation *in vitro* (Dwivedi et al. 2006, Morarescu et al. 2010). Moreover, MMP inhibition prevents human lens epithelial cell migration and contraction of the lens capsule (Wong et al. 2004), cellular events associated with the signalling activity of these growth factors in lens epithelial cells, and MMP-9 deficiency HSPG-associated proteins involved in actin polymerization and cell migration (Liu et al. 2021) suggesting a possible link between HSPG and MMP-9 activity, and that the release of these factors from HS-GAG sequestration may be important for their biological activity.

HSPGs and their associated GAGs have also been shown to play a more direct role in the potentiation and regulation of several key events in fibrosis. For this reason, they are also commonly up-regulated in fibrotic lesions, where they are synthesised and deposited into the newly formed aberrant matrix, or targeted to the surface of fibrotic cells where they regulate growth factor signalling, focal adhesion and migration. For this reason, it was not surprising to

observe HS-GAG staining associated with the cells and new aberrant matrix synthesised and deposited within the subcapsular plaques. Although in other systems CS-GAGs have been shown to be up-regulated in fibrosis where they have the capacity to serve similar functions to HS-GAGs, here we show that the predominant GAGs associated with ASCs in these transgenic mice are HS. Our findings agree with earlier work that have identified increased HS- but not CS-GAGs localised to the basal lamina of lens epithelial cells in PCO plaques (Ishibashi et al. 1995). As different HSPGs can act as both positive and negative modulators of different factors driving fibrosis, depending on their associated core proteins, the presence or absence of GAG staining is generally insufficient to conclude a regulatory role, or lack thereof, for different sulfated GAGs in a tissue. As earlier chapters of this thesis (See chapters 3 and 4) showed that the lens expresses a unique profile of spatially and temporally regulated HSPGs, and as tissue HSPG profiles have been reported to change to support pathogenesis in a disease- and tissue-specific manner, we next characterised the specific HSPG core protein labelling profile in lenses of wild-type and our TGF- β 1 gain of function transgenic mice.

6.4.2 Immunolocalisation of HSPGs is differentially altered in the pathogenesis of fibrotic cataract.

Given the unique lens HSPG core protein profile we established earlier, we postulated that the significant molecular and phenotypic alterations to the lens with fibrotic cataract may be associated with changes in the expression and/or localisation of lens HSPGs. Compared with wild-type mice lenses, HSPG core proteins were markedly modified in cataractous plaques of transgenic lenses. These pathology-induced modifications could be grouped into different

categories: **i)** changes in levels of protein and **ii)** changes in spatial and cellular patterns of labelling. In lens fibrotic plaques of transgenic mice, we observed increased levels of labelling for syndecan-1, syndecan-3, syndecan-4, glypican-1, glypican-3, glypican-2, glypican-5, collagen XVIII and agrin relative to lenses of wild-type mice. The levels of syndecan-2, glypican-4 and glypican-6 did not appear to change. Pathogenic cell changes are often characterised by significant changes to the pathways and factors influencing cell signalling and behaviour.

Of the core proteins that were still present in plaques, labelling for syndecan-2, syndecan-4, glypican-1, glypican-2, glypican-3, glypican-5, collagen XVIII and agrin appeared more heterogeneous, potentially highlighting a specific subpopulation of cells within the plaque. A key feature common to fibrotic plaques observed in our transgenic TGF- β 1 GOF mice and in both human anterior subcapsular cataracts is their heterogeneous composition of spindle-shaped myofibroblasts (expressing myofibroblastic markers, e.g. α -SMA, Hales et al. 1994) and aberrant fibre cells (expressing fibre markers, e.g. beta-crystallin) surrounded by depositions of fibrillar ECM.

Our findings are consistent with several studies in the lens (Qin et al. 2017) and other tissues (Herum et al. 2013, Lunde et al. 2016, Lord et al. 2018, Udomsinprasert et al. 2020, Wilson et al. 2022) indicating that specific HSPGs are up-regulated with the onset of TGF- β -induced EMT and fibrosis.

Here we report that syndecan-4 is up-regulated in the localised to fibrotic plaques, and show increased immunostaining relative to the epithelium of wild-type lenses. This finding is in agreement with a recent body of work which extensively characterised the role of Syndecan-4 in the pathogenesis of TGF- β -induced lens EMT and demonstrated its potential as a therapeutic target for fibrotic lens pathologies such as fibrotic forms of cataract (i.e. ACO/PCO) (Qin et al. 2017). In this landmark study, syndecan-4 was reported to regulate FGF-2 and $\alpha 5 \beta 1$ -integrin signalling, with both FGF-driven proliferation and integrin-mediated adhesion and migration of LECs involved in the development of ACO/PCO requiring HSPG co-receptor activity. Moreover, mice lacking syndecan-4 have significantly reduced occurrence of lens opacification in an injury-induced model of fibrotic wound healing (Qin et al. 2017). RNA interference knockdown of syndecan-4 in cultured human LECs also blocked FGF-induced proliferation and associated ERK1/2 and PI3K/AKT/mTOR signalling pathway activation (Qin et al. 2017), with the same study reporting increased syndecan-4 expression in the LECs of age-related cataract patients (Qin et al. 2017). Our finding of syndecan-4 expression in the anterior plaques of transgenic mice overexpressing TGF- $\beta 1$ in the lens adds further evidence to the prospect of syndecan-4 as a key regulator of fibrotic disease in the lens.

In addition to the established roles in the regulation of FGF-2 and integrin signalling, one of the characteristic features of the syndecan family of HSPGs is their association with and regulation of the cellular cytoskeleton. All syndecans have been shown to interact with actin-associated proteins and evidence has been presented for signalling to the cytoskeleton (Morgan et al. 2007, Yoneda and Couchman, 2003). Syndecan-4 signals to the cytoskeleton primarily through PKC α that binds directly to the syndecan-4 core protein (Oh et al. 1997, Horowitz and Simons, 1998)

and this phosphorylation of the syndecan-4 core protein is sufficient for the modulation of focal adhesions and actin stress fibres (Echtermeyer et al. 1999), linked to lens fibre cell differentiation (Weber and Menko, 2006). A role for syndecan-3 may be in its association with Src kinases and cortactin (Filla et al. 2004, Kinnunen et al. 1998) which are essential regulators of the morphological changes underlying EMT (Walker et al. 2007, Li et al. 2019, Liu et al. 2021). Ephrin signalling, which is required for the phosphorylation of the syndecan-3 core protein (Asundi and Carey, 1997), has been implicated as a potential contributor to aberrant proliferation and loss of epithelial integrity and EMT in anterior lens epithelial cells (Murugan and Cheng 2022). Syndecan-3 deficiency impairs neuronal migration in the brain (Hienola et al. 2006), and explanted satellite cells from syndecan-3 null mice display aberrant differentiation, with a general increase in overall tyrosine phosphorylation (Cornelison et al. 2004).

Of all the syndecans, only syndecan-2 was observed to be decreased in plaques relative to wild-type. Unlike transgenic animals deficient in *sdcl*, *sdcl3* and *sdcl4* which have not been reported to have any dysgenic ocular phenotypes (Echtermeyer et al. 2001, Dickinson et al. 2016, Gougoula et al. 2019), *Sdc2*^{-/-} (*Sdc2*^{tm1b(KOMP)Wtsi}) mice have a high incidence of lens anomalies, including cataract (Dickinson et al. 2016), but these remain poorly characterised. It is not clear if this represents a lens-intrinsic- or secondary-phenotype, as syndecan-2 deficiency in ocular endothelial cells delays retinal vascular development and inhibits VEGF-signalling and angiogenesis (Corti et al. 2019). It is possible that syndecan-2 is driving cell proliferation, migration, invasion and EMT as it has been shown to do in colorectal cancer via MAPK signalling (Jang et al. 2016, Hua et al. 2020). Syndecan-2 is co-localised with fibronectin – a key protein found in modified ECM associated with fibrotic lens plaques (Shihan et al. 2020) – in the

anterior segment of the eye (Filla et al. 2004) and can regulate its assembly into the ECM (Klass et al. 2000).

Thus, despite their seemingly high structural and functional similarities, our findings provide additional evidence for distinct and separable roles for different syndecan family members in pathogenesis.

Due to the broad-reaching nature of HSPGs and the vast ray of proteins they bind, it can be difficult to dissect the roles of individual HSPG core proteins based on changes in core protein expression and localisation alone. However, given that the roles of specific HSPGs in fibrosis show similarities across different tissue types (Lord et al. 2018), taken together with previous detailed studies in the lens and other fibrotic diseases characterising the molecular interactions and regulatory roles of specific HSPG core proteins and families, the spatiotemporal changes in the overall HSPG profile observed in this study may provide indications of their potential roles in TGF- β -induced EMT and contribution to the pathogenesis of fibrotic cataracts.

We also show that two other members of the syndecan family of HSPGs, syndecans-1 and -3, four glypican family members (glypican-1, glypican-3, glypican-2, glypican-5), and the high molecular weight HSPGs collagen XVIII and agrin are localised to fibrotic plaques and show increased immunostaining relative to the epithelium of wild-type lenses. To our knowledge, this is the first reported association of these HSPGs with fibrotic cataract, however, their reported roles in the pathogenesis of other fibrotic diseases, and regulation of key pathways known to drive TGF- β -EMT may shed light on their potential roles in lens. In addition to their role as regulators of FGF-2 and integrin signalling, syndecan HSPGs are important regulators of cytoskeletal organisation and focal adhesion formation.

Glypicans-1 (Kleeff et al. 1998) and -3 (Midorikawa et al. 2003) are overexpressed in several cancers characterised by an up-regulation of TGF- β signalling (Friess et al. 1993).

TGF- β 1-induced SMAD2 phosphorylation pattern was attenuated in glypican-1 antisense transfected cells (Li et al. 2004). Glypican-1 and -3 control the responses of primary human suture mesenchymal cells to BMPs by inhibiting BMP signalling through SMAD-dependent and SMAD-independent pathways by sequestering BMPs away from the cells. Similarly, inhibition of endogenous GPC1 and GPC3 potentiates BMP2 responsiveness of human suture mesenchymal cells (Dwivedi et al. 2013). Glypican-3 is overexpressed in hepatocellular carcinoma, plays a key pro-oncogenic role in the progression of this cancer through its negative regulation of BMP-7 signalling through the SMAD signalling pathway (Midorikawa et al. 2003), and promotes of TGF- β -induced EMT through MAPK/ERK signalling (Wu et al. 2015). This regulation is selective to these growth factors, as enhanced expression of glypican-3 did not impact the signalling activity of IGF or HB-EGF, indicating that specific HSPGs may regulate the signalling activity of specific growth factors (Midorikawa et al. 2003). BMP-7 counteracts the fibrogenic activity of TGF- β in lens cells (Shu et al. 2017) and is down-regulated in cataract. Thus, it is possible that the increased expression of glypican-3, and possibly also the related glypican-1, we have observed here in the fibrotic plaques is related to the suppression of BMP signalling characteristic of lens fibrosis (Shu et al. 2017).

Transgenic mice overexpressing endostatin (C-terminal domain of type collagen XVIII) develop nuclear cataracts, and progressive occurrence of vacuoles in the epithelial cell layer. In older mice, the epithelial cells became spindle-shaped and formed clusters on the anterior of the lens.

This process resembled the epithelial-mesenchymal transition in the lens caused by transforming growth factor- β . Endostatin is also known to cause disassembly of the actin cytoskeleton via down-regulation of RhoA activity, which drives alterations to the actin cytoskeleton, focal adhesions, and stability of adherens junctions involved in TGF- β -induced EMT (Bhowmick et al. 2001, Korol 2017, Taiyab and West-Mays 2022).

Beyond the overall levels of the proteoglycan observed here, it is also important to note that the localisation of specific HSPG core proteins is relevant to understanding their contribution to pathogenesis. For instance, loss of cell surface staining, but acquisition of cytoplasmic or nuclear staining can indicate significant changes in cell-HSPG interactions/activity which are relevant to disease progression. For instance, the Syndecan-1 protein plays a crucial role in cell proliferation, adhesion, and migration, however, it was somewhat unexpected to find that it was upregulated in fibrotic plaques, as syndecan-1 is typically known to be characteristically localised to epithelial cells, where it is co-regulated with E-cadherin, and in fact often plays a key role in maintaining the epithelial phenotype. For instance, loss of syndecan-1 from the cell surface is associated with down-regulation of E-cadherin and myofibroblastic transformation (Kato et al. 1995). On the other hand, increased levels of matrix-associated syndecan-1 are associated with poor prognosis in several cancers, thought to be related to its regulation of growth factor bioavailability from within the pericellular matrix (Handra-Luca 2020). Thus, it is possible that this may be the case for syndecan-1 in fibrotic plaques.

Although we were not able to investigate the comparative immunostaining of perlecan in fibrotic lenses due to lack of a commercially available antibody, previous studies report increased

perlecan staining in the corneal stroma of transgenic mice overexpressing TGF- β 1, suggesting that perlecan may represent a component of the matrix-associated GAGs regulating TGF- β -induced EMT in the lens. Moreover, the roles of perlecan in fibrosis are similar across different tissue types (Lord et al. 2018). Future studies would benefit from adding comparative immunostaining of the perlecan core protein to those investigated here, to gain a more complete picture of the different HSPG core protein profile associated with ASC.

6.4.3 HSPGs are required for TGF- β 2-induced TGF- β EMT in lens.

TGF- β -induced EMT is a complex stepwise phenomenon that plays a crucial role in fibrogenic disease, that is defined by a common set of intertwined cellular events, which are each differentially regulated by signalling molecules and pathways. HSPGs have been implicated in the regulation of several key pathways involved in TGF- β -induced EMT.

TGF- β binding to receptors on lens epithelial cells leads to the initiation of canonical TGF- β SMAD signalling, where SMAD2/3 forms a complex with SMAD4 and translocates to the nucleus, where it activates EMT-response genes and transcription factors. The result is the trans-differentiation of cuboidal lens epithelial cells to myofibroblastic cells (Shirai et al. 2006, Shirai et al. 2014, Shirai et al. 2018). We demonstrated that pharmacological inhibition of HS-GAG activity *in vitro* with surfen blocked TGF- β -induced trans differentiation of lens epithelial cells to myofibroblastic cells (i.e., elongation, migration).

HSPG activity is also required for TGF- β -mediated cytoskeletal re-organisation and destabilisation of adherens junctions indicated by the ability of surfen to block accumulation of the pro-contractile stress-fibre marker α -SMA. Surfen also prevented loss of epithelial membrane marker β -catenin and the gain of mesenchymal markers (i.e. Snail) (Taiyab et al. 2022), associated with fibrotic EMT. Surfen also blocked early indicators of TGF- β 2-mediated downstream signalling. TGF- β 2-induced nuclear translocation of SMAD2/3 and increases in vimentin and phospho-ERK1/2 were completely blocked with surfen pre-treatment of cells, with Snail, (an important transcriptional repressor of E-cadherin) only partially blocked.

The motile myofibroblastic cells adhere to this modified matrix rich in fibrillar collagen I and fibronectin (Johar et al. 2007, Saika et al. 1998, Sappino et al. 1990) through interactions between matrix proteins (i.e. fibronectin) which induce focal adhesion formation at the cell–ECM interface through focal adhesion kinase (FAK)/integrin signalling-dependent mechanism (Mamuya et al. 2014). This alteration in both the epithelial cells and ECM results in a corresponding change to the lens capsule, which due to the increased adhesion of the contractile myofibroblastic cells, becomes wrinkled. Surfen maintained the epithelial cobblestone phenotype of the LECs and maintained the integrity and structure of the native lens capsule. HSPGs bind to (Laurie et al. 1986, Tumova et al. 2000) and regulate the deposition of collagen and fibronectin into the ECM (Klass et al. 2000, Filla et al. 2004), and regulate focal adhesion, migration and enhance matrix contractility via FAK/integrin and MAPK/ERK signalling in fibrosis (Laterra et al. 1983, Midwood et al. 2004, Chen et al. 2005, Chen et al. 2008, Qin et al. 2017). Corneal fibroblasts cultured on fibronectin (imitating a fibrotic corneal stroma environment) showed significantly higher HS-GAG nuclear localisation compared to

controls cultured on collagen (imitating a normal corneal stroma) (Richardson et al. 2001), indicating that HSPG-fibronectin interactions may represent an additional pathway for HSPG nuclear localisation in fibrosis. Thus, blocking HSPG activity with surfen may prevent EMT by inhibiting HSPG-mediated fibrotic modifications to the lens capsule, and the interactions between HSPGs and fibrotic matrix proteins (i.e., fibronectin) required for HSPG-matrix interaction-induced adhesion, migration, and matrix contractility in lens EMT.

Other factors in the ocular milieu, notably FGF-2, induce increased proliferation and migration of lens epithelial and myofibroblastic cells. The migratory and proliferative effects of FGF-2 have distinct pathways (Boilly et al. 2000). While FGF-2-induced proliferation is primarily mediated via ERK1/2 (Boilly et al. 2000, Lovicu and McAvoy 2001) and PI3K/AKT signalling (Boilly et al. 2000), FGF-2-induced migration rely on the signalling activity of Src kinases, p38 MAPK (Boilly et al. 2000) and integrins. As shown in the previous chapter, HSPGs regulate FGF-2 signalling in the lens via the ERK pathway to induce LEC proliferation. FGF-2 is significantly up-regulated in the aqueous humour after cataract surgery (Iyengar et al. 2017, Wen et al. 2022), and has been shown to facilitates cytoskeleton reorganisation, proliferation, migration and fibrosis of lens cells via FAK/Integrin and MAPK/ERK signalling to promote fibrosis (Qin et al. 2017, Wen et al. 2022). Thus, inhibition of HSPG activity may block proliferation, EMT, cell adhesion and migration induced by FGF-2 signalling, which would otherwise be promoted by increased HSPG activity in fibrotic plaques.

Alongside FGF-2, other factors present in ocular milieu (i.e. PDGF, HB-EGF, IGFs) synergize with TGF- β to augment fibrosis through crosstalk, transactivation and feed-forward mechanisms

that include regulation of TGF- β levels, potentiation of pro-fibrotic pathways and suppression of TGF- β regulatory signalling. Since we have shown that LEC proliferation induced by FGF-2, HB-EGF and IGF-I is under the control of lens HSPGs (see chapter 5), it is reasonable to conclude that HSPGs in the cataractous lens may also be driving the signalling and bioavailability of these factors to promote LEC proliferation, migration and potentiation of TGF- β signalling.

Other growth factors may also play emerging synergistic roles in EMT. Immunohistochemical studies of fibrotic plaques of patients before and after cataract surgery show higher levels of IGF and PDGF relative to pre-operative lenses (Shigemitsu et al. 1998), and hyperactivation of PDGF receptor β induces fibrotic EMT in lens cells (Chandler et al. 2021). Although our earlier results indicate that PDGF-induced proliferation is not regulated by lens HSPGs, several studies have reported changes in growth factor regulation correlating with pathology-induced changes in the levels and localisation of HSPGs and their associated GAGs.

6.4.4 HSPGs are differentially required for both the induction and progression of TGF- β 2-induced EMT.

PCO is a condition that progresses over time and is likely to develop in two phases (Wormstone, 2002). Injury to the lens (by surgery, for instance) induces a transient but significant increase in levels of active TGF- β 2 in the aqueous humour, initiating the initial phase of PCO characterised by induction of early pro-fibrotic signalling in LECs (i.e. SMAD2/3 signalling) leading to changes to gene expression, together with elevation of protein levels (e.g. α -SMA, Hales et al. 1994). The impact of this period is significant as the resulting changes provide the platform from

which PCO continues to develop through a second slower phase driven largely by autocrine mechanisms of lens cells which gives rise to persistent, long-term signalling events (i.e. cytoskeletal reorganisation, destabilisation of junctional proteins, secretion of modified fibrillar matrix and migration) resulting matrix contraction and transdifferentiation (Wormstone et al. 1997, Wormstone et al. 2000, Wormstone et al. 2001, Korol 2017, Taiyab and West-Mays 2022). These two phases have also been observed in our rat epithelial explant model. TGF- β 2-treated LECs will commit to EMT fate following TGF- β 2 treatment, even when only briefly exposed (Wormstone, 2002). As our initial studies were intended to investigate the requirement of HSPGs in this critical initial phase of TGF- β -lens EMT, LECs were pre-treated with surfen to eliminate any potential regulatory functions of HSPGs. Here we have shown that blocking HS-GAGs prior to the introduction of exogenous TGF- β 2 prevents both stages of EMT. While this result indicates that HSPG activity is essential for the initial inductive phase of EMT, it was unclear if HSPG activity was also required for the regulation of more long-term signalling events resulting in matrix contraction and transdifferentiation. Co-treatment of surfen together with TGF- β 2, and post-treatment of cells with surfen 24 h after addition of TGF- β 2 blocked the migration and myofibroblastic transformation of LECs, but did not completely block expression of α -SMA. These findings suggest that HSPGs appear to regulate both the early phase (indicated by the ability of surfen pre-treatment to block TGF- β 2-induced SMAD2/3 signalling and downstream expression of α -SMA) and late phase of EMT (indicated by the ability of surfen co- and post-treatment to block the downstream phenotypic and behavioural changes of lens cells of EMT, via an α -SMA-independent pathway).

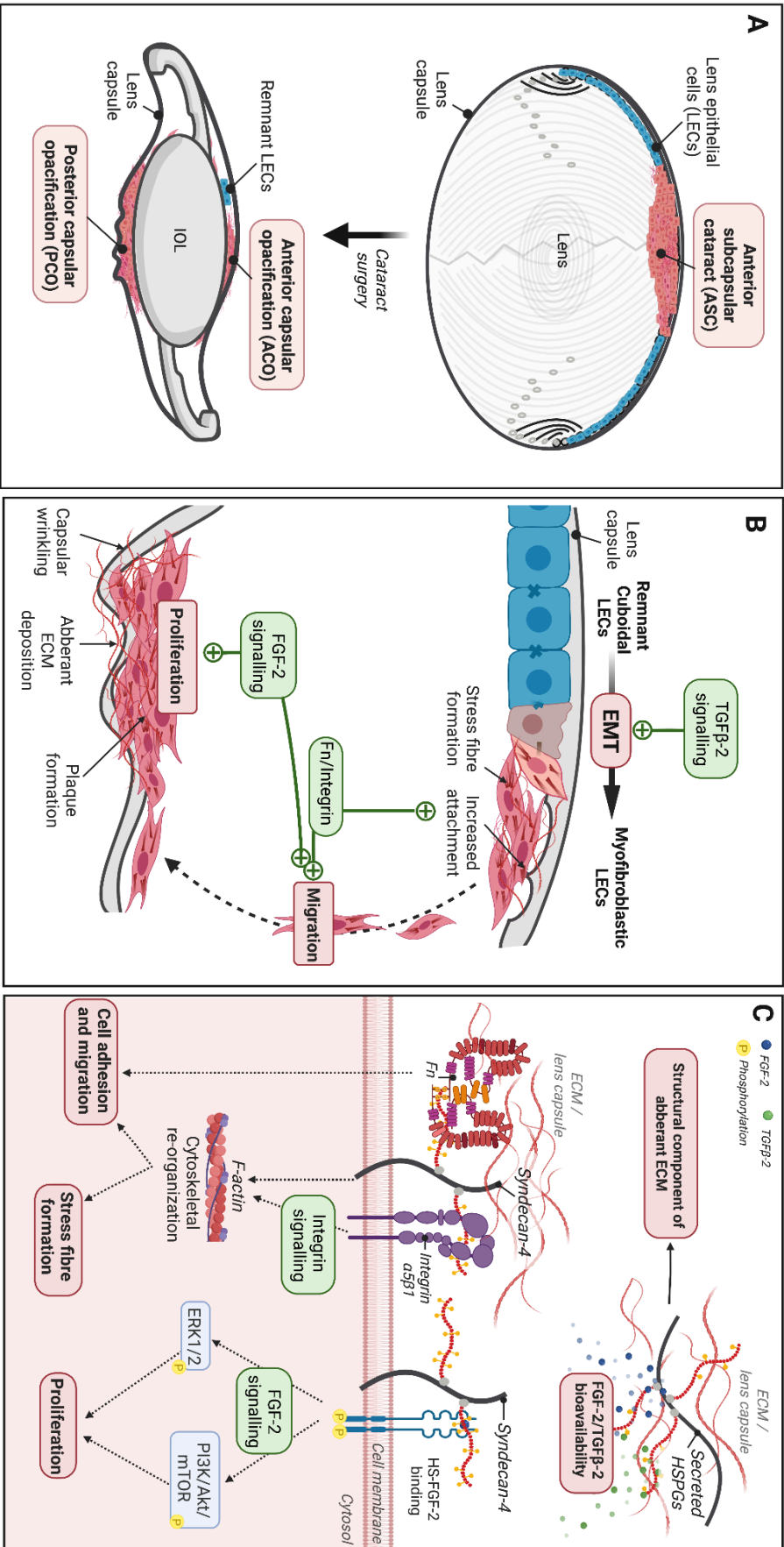


Figure 6.9. Schematic diagram of the aetiology and pathogenesis of fibrotic cataracts, and the role of HSPGs in the regulation of key cellular events. A) Fibrotic cataracts may form at the anterior or posterior pole of the lens, resulting in a plaque and opacification of the lens, such as in primary anterior subcapsular cataract (ASC), or secondary cataracts, such as anterior capsular opacification (ACO) or posterior capsular opacification (PCO). These secondary cataracts arise from LECs left behind during cataract surgery that undergo a wound healing response, causing them to transdifferentiate into myofibroblasts, where they form a plaque. B) The pathogenesis of PCO involves three main steps: i) LECs undergo an epithelial-to-mesenchymal transition (EMT) under the influence of TGF β -2 signalling to become motile myofibroblastic cells. These cells undergo cytoskeletal reorganisation (e.g. stress fibre formation) and secrete abundant aberrant extracellular matrix containing fibronectin (Fn), as they adhere to the lens capsule. ii) The myofibroblastic LECs migrate to the posterior capsule driven by FGF-2 and integrin signalling. iii) The myofibroblastic LECs undergo FGF-2 driven proliferation to form a plaque, adhering to and modulating the posterior lens capsule. C) Heparan sulfate proteoglycans (HSPGs) can contribute to the fibrotic events underpinning ASC/ACO/PCO pathogenesis in several ways: i) Secreted HSPGs form an integral part of the new secreted matrix, where they can sequester TGF β -2 and FGF-2, controlling their bioavailability to the myofibroblastic cells, ii) through interactions with matrix proteins (e.g. α 5 β 1-integrin/fibronectin, (Fn) HSPGs (e.g. syndecan-4) can regulate cell adhesion and migration, and stress-fibre formation, iii) HSPGs (e.g. syndecan-4) are essential co-receptors for FGF-2. FGF receptor (FGFR)-FGF-2-HS signalling complexing and activation results in ERK1/2 and PI3K/Akt/mTOR signalling activation required for FGF-2 driven proliferation of myofibroblastic LECs.

6.4.5 Conclusions

Here, we investigate the spatiotemporal immunolabelling of HSPGs in the pathogenesis of fibrotic cataract and also establish the first functional requirement for HSPGs in driving TGF- β -induced EMT in the lens. Taken together, this data highlights HSPGs as potentially critical regulators of the aberrant cell behaviour underlying pathogenic processes, including TGF- β -induced EMT. A better understanding of these underlying regulatory mechanisms may serve as a basis for the identification of novel therapeutic targets and molecular strategies that could be implemented to slow or prevent the development and progression of diseases characterised by abnormal growth factor-mediated epithelial cell proliferation and HSPG sulfation profiles, such as fibrotic cataract.

We propose that alterations to the expression and localisation of specific HSPG core proteins and their associated HS-GAG chains in fibrotic cataract may differentially orchestrate the cellular processes driving the pathogenesis of ASC and PCO. Moreover, the results of this *in vitro* work indicate that the modulation of these HSPGs may be effective in blocking TGFB-induced signalling leading to fibrotic cataract. As disruption to the lens epithelium leads to fibrotic cataract, establishing a greater knowledge of the molecules/mechanisms involved in maintaining these cells will provide a better understanding of this disease.

CHAPTER 7: GENERAL DISCUSSION

7.1 *Introduction*

HSPGs can be likened to the mysterious "dark matter" of the biological world: a ubiquitous and fundamental element that is yet to be fully integrated into the mainstream dogma of molecular biology. Present in all mammalian cells, HSPGs encompass dynamic and distinct roles in the development and maintenance of tissues, both on the cell surface and within the ECM, acting as a multifunctional bridge between cells and their environment. Progress in glycobiology has uncovered a significant function for HSPGs in the regulation of growth factors, matrix proteins, and signalling molecules. This positions HSPGs as key orchestrators of biological processes, including cell growth, differentiation, and survival. The eye lens is a relatively simple but prolifically studied tissue that presents an ideal stage on which to characterise and model the expression and unique roles of individual HSPGs in the ocular milieu. The well defined stages of development, refined architecture and well characterised biology of the vertebrate lens makes it an excellent model to study how basic cellular processes are coordinated, through the specific activity of native ocular growth factors triggering a complex interplay of intracellular signalling pathways (Lovicu and McAvoy 2005), potentially all regulated at some level and degree by HSPGs.

7.2 *Heparan sulfate is the predominant HSPG-associated GAG in the lens*

The specificity of HSPG-protein interactions does not only depend on the core protein type (e.g. syndecan, glypican), or their localisation (e.g. the cell surface or ECM), but also on the type (i.e. HS-GAG or CS-GAG) and fine structure (i.e. sulfation) of their GAG chains. In this body of work we show that although developing and postnatal lens cells produce proteoglycans carrying

both HS- and CS-GAGs, it is the HS that is the predominant sulfated GAG associated with the lens. We also show that this HS-GAG labelling is analogous in similar regions of lenses across different vertebrate species.

Despite differences in the levels of GAGs in the lens, we also show that regional labelling for HS-GAG and CS-GAG do not always overlap, suggesting that chondroitin sulfate proteoglycans (CSPGs) may play a distinct role in ocular biology to HSPGs (see Tao et al. 2022). The distinct functional roles of HS-GAG and CS-GAG (Karamanos et al. 2018) align with their ability to each interact selectively with different proteins in a structure-dependent manner (Vallet et al. 2021), possibly accounting for their different tissue localisation (Smetsers et al. 2003, Clark et al. 2011, Maeda et al. 2011). Importantly, only HS-GAG was present in the developing lens until E14.5, indicating that HS-GAGs are likely the major species regulating the earliest events of lens induction, invagination, and cell fate specification. The overlapping and divergent patterns of HS-GAG and CS-GAG in the lens (e.g. in newly differentiating secondary lens fibres) implies that these GAGs may both contribute to the regulation of some functional processes in the lens (e.g. fibre differentiation), while other processes are likely to be regulated predominantly by HS (e.g. morphogen gradient formation in the lens capsule).

The regional distribution of highly sulfated “active” HS-GAGs may provide an indication of their relative contributions to cell behaviour. These patterns were often associated with regions of lens cell activity in both the developing lens (i.e. lens placode, posterior aspect of lens pit/vesicle and lens transitional zone) and postnatal lens (i.e increased HS-GAG labelling in the transitional and germinative zones of the rat lens, specifically in actively dividing LECs). Of particular

interest was the nuclear localisation of HS observed in newly differentiating secondary lens fibres. This phenomenon has also been observed *in vitro* in lens epithelial cells undergoing FGF-induced lens fibre differentiation. The functions of HS/HSPGs in the nucleus are not fully understood, but they are known to transport HS-binding proteins, including growth factors and their receptors, to the nucleus to regulate gene expression and control cellular functions. We also observed an increase in the intensity of sulfated HS-labelling extending from the central epithelium to the equatorial germinative zone, suggesting a correlation between a change in the mitotic activity of lens epithelial cells in the germinative zone with a change in the specific HSPG profile of the epithelial cells.

7.3 The lens has a unique profile of HSPG core proteins that are spatiotemporally regulated in lens development

The localisation of HSPG core proteins can provide valuable information about their functional roles in a specific tissue. As increasing evidence suggests that HSPG core proteins play important roles in lens signalling and architecture, here, we present for the first time a comprehensive atlas of the unique HSPG profile of the murine ocular lens throughout key stages of life. Most HSPGs are expressed in the lens, that aligns with other postnatal lens HSPG transcript expression profiles (Hoang et al. 2014, Khan et al. 2015, Audette et al. 2016, Cavalleiro et al. 2017, Kakrana et al. 2018). Lens HSPGs are differentially localised to specific cell types (i.e. LECs, fibre cells) and functional regions of the lens (i.e. germinative zone, transitional zone, lens capsule). The differential localisation of HSPGs within anterior lens epithelia suggests they play distinct roles in the maintenance and polarity of the lens epithelium and may hold functional significance for the regulation of different HSPG-protein interactions

and signalling networks. Given their roles in other tissues, it can be speculated that these HSPGs likely play a role in the formation of local gradients by binding to native factors and regulating their bioavailability to adjacent cells.

An interesting finding of this work was the significantly divergent expression patterns of the closely related HSPGs (e.g. syndecans) that suggested that specific HSPG core proteins may be differentially regulating lens growth factors or specific biological events (e.g. lens proliferation, fibre differentiation, fibre elongation etc). The variety of labelling patterns and intensity vary in the different subcellular functional compartments (e. cytoplasm, nucleus) and regions (anterior vs posterior) of these cells. Despite their seemingly high structural and functional similarities, our findings provide additional evidence for distinct and separable roles for different HSPG family members.

The unique localisation and expression patterns of HSPG core proteins in the lens indicate that different HSPGs may have diverse roles in regulating lens cell processes like epithelial cell proliferation and fibre differentiation. The differential spatial and temporal labelling of HSPG core proteins suggests that lens cells acquire a distinctive repertoire of HSPGs as they differentiate, resulting in selective patterns of HS-protein interactions and activity. Taken together with the current literature, our findings support a requirement for HS sulfation in growth factor-induced lens cellular processes during embryogenesis. Moreover, these findings highlight the diverse roles of different HSPGs within a single system and indicate the lens as a candidate tissue for future studies seeking to elucidate the different roles of related proteoglycans.

7.4 HSPGs differentially regulate key lens growth factors and are required for lens epithelial cell proliferation and lens fibre differentiation

The interplay and tight control of lens growth factors and their downstream signalling pathways maintains the integrity of the vertebrate lens throughout life. For the first time, here we conclusively demonstrate that HS-GAG sulfation is required to regulate lens epithelial cell proliferation and fibre differentiation in rat lens cells induced by ocular humour and different ocular growth factors. The fine structure requirements of HS-ligand interactions varies with the ligand, conferred by variations in the sulfation patterns of HS chains, leading to differential regulation of biological functions. By treating lens epithelial cells in explants with a HS-specific antagonist Surfen, we were able to effectively block sulfation of only HS-GAG, leading to impaired lens epithelial cell proliferation and fibre differentiation, as well as a block to the corresponding activation of intracellular ERK1/2-signalling induced by these factors.

In addition to its essential role for lens cell survival (Chamberlain and McAvoy, 1989, Chow et al. 1995, Garcia et al. 2005), FGF-induced lens fibre differentiation promotes cell elongation (Lovicu and McAvoy, 1989) and the accumulation of fibre-specific β -crystallin (Chamberlain and McAvoy, 1989, Peek et al. 1992) and aquaporin-0 (AQP0/MIP) (Golestaneh et al. 2008). Interestingly, FGF-induced ERK1/2-signalling is required for the elongation of fibre cells (Lovicu and McAvoy, 2001, Li and Musil, 2001) and the associated expression of filensin and AQP0/MIP (Lovicu and McAvoy, 2001, Golestaneh et al. 2008), but not for β - and γ -crystallins (Lovicu and McAvoy, 2001, Wang et al. 2009), that may be under the control of alternate signalling pathways, such as PI3K/AKT (Wang et al. 2009). As we show that Surfen is able to block FGF-induced β -crystallin accumulation (Wishart and Lovicu, 2021), this suggests that

these alternative pathways regulating crystallin expression are also dependent on HS sulfation, consistent with HSPGs role as an upstream co-receptor for FGFR-signalling in lens. As FGFs are the only class of growth factors to induce lens fibre differentiation in mammals (Lovicu and McAvoy, 2005), Surfen blocking studies signify HSPGs as important regulators of lens cell fate (Wishart and Lovicu, 2021).

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 amongst the different HSPGs. We validate the previously reported, well-established effects of sodium chlorate (that targets all sulfated molecules) on lens and build on this by focussing only on the role of HS-sulfation, by using Surfen. Impaired HS blocks FGF-2-induced ERK1/2-phosphorylation and lens epithelial cell proliferation and fibre differentiation. Taken together with previous work from our laboratory, this data supports a requirement for HSPG/GAG sulfation in growth factor-induced-signalling in the lens. The Surfen blocking studies support that the functional roles of HSPGs in the lens are predominantly through HS sulfation and not via other sulfated GAGs (e.g. CS-GAG). These experimental blocking studies *in vitro* also focus attention on the different modalities of HSPG function, as co-receptors for matrix molecules and their ability to serve as independent receptors for heparin-binding growth factors. HSPGs also indirectly influence the intracellular signalling (e.g. MAPK/ERK, PI3K/AKT/mTOR, and the Rho family of GTPases of these molecules) by regulating receptor binding interactions, activation and trafficking (Bishop et al. 2007).

It is important to note that to date, limited research has been directed toward the study of HSPGs and their regulatory networks in the lens, and many key areas of interest have not been investigated to the extent that they could be covered in this thesis. Of particular interest to the field of lens biology would be further investigation of the relevance of HS-GAG/ HSPG-protein interactions in regulating lens water transport and antioxidant systems, HS-interactions with lens extracellular proteins (e.g. SPARC/GRIFN), interactions between HS and intrinsic metal ions in the lens, and further in depth characterisation of signalling networks (e.g. regulation of protein phosphatases) by lens HSPGs. Our future studies will seek to investigate the differential functions and protein interactions of specific HSPG members.

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

7.5 HSPGs are differentially localised in fibrotic cataract, and HS-sulfation is essential for lens epithelial-to-mesenchymal transition

Given HSPGs are master orchestrators, either directly or indirectly, of most pathways and protein interactions involved in cell communication, maintenance and transformation, changes in key regulatory pathways leading to the onset of pathogenesis are often accompanied by changes in the levels and/or localisation of specific HSPG core proteins, highlight roles for specific HSPGs

in the pathogenesis of many different diseases (Heimann et al. 2001, Ibrahim et al. 2017). Here, for the first time we report on pathology-induced alterations to the spatiotemporal labelling of HSPGs in fibrotic cataract, and also highlight the functional requirement for HSPGs in driving TGF- β -induced EMT in the lens. The requirement of HS for lens cell EMT, combined with the differential expression of HS-GAG and specific HSPG core proteins in cataractous lenses suggests that changes in the selective patterns and interactions and activity of specific HSPGs may point to candidate targets for the regulation of fibrotic changes underpinning the pathogenesis of fibrotic cataract. We propose that these alterations to the levels and localisation of specific HSPG core proteins, and their associated HS-GAG chains in fibrotic cataract may differentially orchestrate the cellular processes driving the pathogenesis of ASC and PCO. Moreover, the results of this *in vitro* work indicate that the modulation of these HSPGs may be effective in blocking TGF- β -induced signalling leading to fibrotic cataract. As disruption to the lens epithelium can lead to fibrotic cataract, establishing a greater knowledge of the molecules/mechanisms involved in maintaining these cells will provide a better understanding of this disease. Due to the broad-reaching nature of HSPGs and the vast array of proteins they bind, it can be difficult to dissect the specific roles of individual HSPG core proteins based on changes in core protein expression and localisation alone, however, given that the roles of specific HSPGs in fibrosis show similarities across different tissue types (Lord et al. 2018), taken together with previous detailed studies in the lens and other fibrotic diseases characterising the molecular interactions and regulatory roles of specific HSPG core proteins and families, the spatiotemporal changes in the overall HSPG profile observed in this study may provide indications of their potential roles in TGF- β -induced EMT and contribution to the pathogenesis of fibrotic cataracts.

Future research may also seek to investigate changes in the HSPG profile of other forms of cataract (e.g. age-related, congenital). For instance, HPSGs have been implicated in the pathology of congenital cataract with a study by Sulochana and colleagues (1997) reporting that 65.9% of children with congenital cataracts also presented with elevated levels of HS and CS-GAGs in the blood, urine and lens. The authors speculated that the additional anionic GAG chains may accumulate in the lens and induce the extracellular accumulation of Na⁺ ions and water, causing cataracts in a similar manner to the pathogenesis of adult cortical cataract (Sulochana et al., 1997). It is possible that this change in GAG chain profile, would also be associated with a change in core protein profile, similar to what we have shown here with fibrotic cataract.

7.6 *Conclusions*

Advances in the field of glycobiology have revealed a critical role for HSPGs in the activation and regulation of growth factors, matrix proteins and signalling molecules. This places HSPGs at the forefront of master regulators of homeostatic processes including cell proliferation, differentiation, and survival. The broad expression of HSPGs throughout the different ocular cells and tissues regulate many key signalling molecules to promote ocular development and homeostasis.

Here we have detailed the spatial expression profile of all known HSPG core proteins in the ocular lens. These findings are supported by many studies in animal models showing that loss or overexpression of specific HSPG core proteins results in unique phenotypic outcomes for the lens, as well as other ocular tissues. Much of this may be brought on by the loss or dysregulation

of their HS chain structure and function leading to ocular pathogenesis. Indeed, similar to HSPG core proteins, HS sulfation enzymes are expressed in a cell- and tissue-specific manner and the disruption of different sulfation patterns impacts growth factors, perturbing cellular function. The fine structure of HS-GAGs, characterised by the sulfation pattern, is an important determinant of the specificity of HS-protein interactions. Although HSPG sulfation clearly plays a critical role in the lens, the specific HS chain sulfation patterns required for HSPG interactions with ocular growth factors are unknown. Evaluation of interactions between lens mitogens and structurally-defined HS oligosaccharides, and phenotypic analyses of animal models with lens-specific knockdown of different HS-sulfotransferases would be valuable to further elucidate relationships between HS fine structure and HSPG interaction with different key lens growth factors and the contribution of different sulfation patterns to ligand binding kinetics and receptor dimerization, lens epithelial cell proliferation, fibre differentiation and EMT, along with the respective downstream signalling pathways. In many eye tissues, including the lens, significant changes in HSPG expression and function can either promote repair or drive pathogenesis. Often, this comes down to changes and regulatory activity of only a handful of specific HSPGs that are unique to different pathological processes. Given the importance of RTK-signalling in development and disease, and the known regulation of these RTKs by HSPGs, it is surprising that few studies have undertaken comprehensive developmental evaluation of tissues lacking different HSPG components. Further characterisation of the lenses in existing HSPG-mutant animals, and the generation of lens-specific knockdown animals, and comprehensive evaluation of how the established HSPG profile of the lens changes throughout lens development, or in disease (e.g. fibrotic cataract) would be a valuable step towards a better understanding of specific HSPG functional modulation pathways in the lens. Once identified, these HSPGs may serve as

valuable biomarkers and targets for the development of novel therapeutics, as has been done for other ocular and systemic diseases, including cancer.

REFERENCES

Afratis, N. A., Nikitovic, D., Multhaupt, H. A. B., Theocharis, A. D., Couchman, J. R., & Karamanos, N. K. (2017). Syndecans – key regulators of cell signalling and biological functions. *The FEBS Journal*, 284, 27–41.

Aikio, M., Hurskainen, M., Brideau, G., Hägg, P., Sormunen, R., Heljasvaara, R., Gould, D. B., & Pihlajaniemi, T. (2013). Collagen XVIII short isoform is critical for retinal vascularization, and overexpression of the Tsp-1 domain affects eye growth and cataract formation. *Investigative Ophthalmology & Visual Science*, 54, 7450–7462.

Akiyama, T., Kamimura, K., Firkus, C., Takeo, S., Shimmi, O., Nakato, H., 2008. Dally regulates Dpp morphogen gradient formation by stabilizing Dpp on the cell surface. *Developmental Biology* 313, 408–419.

Akutsu, N., Yamamoto, H., Sasaki, S., Taniguchi, H., Arimura, Y., Imai, K., & Shinomura, Y. (2010). Association of glypican-3 expression with growth signalling molecules in hepatocellular carcinoma. *World Journal of Gastroenterology*, 16, 3521–3528.

Allard, J. B., & Duan, C. (2018). IGF-binding proteins: why do they exist and why are there so many?. *Frontiers in endocrinology*, 9, 117.

Allen, D., & Vasavada, A. (2006). Cataract and surgery for cataract. *Bmj*, 333(7559), 128-132.

Amalric, F., Bouche, G., Bonnet, H., Brethenou, P., Roman, A. M., Truchet, I., & Quarto, N. (1994). Fibroblast growth factor-2 (FGF-2) in the nucleus: Translocation process and targets. *Biochemical Pharmacology*, 47, 111–115.

Amor, D. J., Stephenson, S. E. M., Mustapha, M., Mensah, M. A., Ockeloen, C. W., Lee, W. S., Tankard, R. M., Phelan, D. G., Shinawi, M., de Brouwer, A. P. M., Pfundt, R., Dowling, C.,

Arai, T., Parker, A., Busby Jr, W., Clemmons, D. R. (1994). Heparin, heparan sulfate, and dermatan sulfate regulate formation of the insulin-like growth factor-I and insulin-like growth factor-binding protein complexes. *Journal of Biological Chemistry*, 269, 20388-20393.

Arikawa-Hirasawa, E., Watanabe, H., Takami, H., Hassell, J. R., Yamada, Y. (1999). Perlecan is essential for cartilage and cephalic development. *Nature Genetics*, 23, 354–358.

Ashikari-Hada, S., Habuchi, H., Kariya, Y., Itoh, N., Reddi, A. H., & Kimata, K. (2004). Characterization of Growth Factor-binding Structures in Heparin/Heparan Sulfate Using an Octasaccharide Library. *Journal of Biological Chemistry*, 279, 12346–12354.

Ashikari-Hada, S., Habuchi, H., Kariya, Y., Kimata, K., 2005. Heparin regulates vascular endothelial growth factor165-dependent mitogenic activity, tube formation, and its receptor phosphorylation of human endothelial cells. Comparison of the effects of heparin and modified heparins. *Journal of Biological Chemistry* 280, 31508–31515.

Ashikari-Hada, S.; Habuchi, H.; Kariya, Y.; Itoh, N.; Reddi, A. H.; Kimata, K. Characterization of Growth Factor-binding Structures in Heparin/Heparan Sulfate Using an Octasaccharide Library. *Journal of Biological Chemistry* 2004, 279, 12346–12354.

Asundi, V. K., Carey, D. J. (1997). Phosphorylation of recombinant N-syndecan (syndecan 3) core protein. *Biochemical Biophysical Research Communications*, 240, 502–506.

Audette, D. S., Anand, D., So, T., Rubenstein, T. B., Lachke, S. A., Lovicu, F. J., & Duncan, M. K. (2016). Prox1 and fibroblast growth factor receptors form a novel regulatory loop controlling lens fibre differentiation and gene expression. *Development*, 143, 318–328

Avnur, Z. & Geiger, B., 1984. Immunocytochemical localisation of native chondroitin-sulfate in tissues and cultured cells using specific monoclonal antibody. *Cell*, 38(3), pp.811–822.

Awasthi, N., 2009. Posterior Capsular Opacification. *Archives of Ophthalmology*, 127, 555.

Ayers, K.L., Mteirek, R., Cervantes, A., Lavenant-Staccini, L., Thérond, P.P., & Gallet, A. (2012). Dally and notum regulate the switch between low and high level hedgehog pathway signalling. *Development*, 139, 3168–3179.

Barth, K. A., & Wilson, S. W. (1995). Expression of zebrafish nk2. 2 is influenced by sonic hedgehog/vertebrate hedgehog-1 and demarcates a zone of neuronal differentiation in the embryonic forebrain. *Development*, 121, 1755–1768.

Bass, M. D., Morgan, M. R., & Humphries, M. J. (2007). Integrins and syndecan-4 make distinct, but critical, contributions to adhesion contact formation. *Soft Matter*, 3, 372–376.

Bassnett, S., & Beebe, D. C. (1992). Coincident loss of mitochondria and nuclei during lens fibre cell differentiation. *Developmental Dynamics*, 194(2), 85-93.

Belecky-Adams, T.L., Adler, R. & Beebe, D.C., 2002. Bone morphogenetic protein signalling and the initiation of lens fibre cell differentiation. *Development*, 129(16), pp.3795–3802.

Bellin, R.M., Kubicek, J.D., Frigault, M.J., Kamien, A.J., Steward, R.L., Barnes, H.M., DiGiacomo, M.B., Duncan, L.J., Edgerly, C.K., Morse, E.M., Park, C.Y., Fredberg, J.J., Cheng, C.-M., & LeDuc, P.R. (2009). Defining the role of syndecan-4 in mechanotransduction using surface-modification approaches. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 22102–22107.

Berk, R.S., Dong, Z., Alousi, S., Kosir, M.A., Wang, Y., & Vlodavsky, I. (2004). Murine ocular heparanase expression before and during infection with *Pseudomonas aeruginosa*. *Investigative Ophthalmology & Visual Science*, 45, 1182-1187

Bernfield M. Biology of the Syndecans: A Family of Transmembrane Heparan Sulfate Proteoglycans. *Annual Review of Cell and Developmental Biology*. 1992;8(1):365-393. doi:10.1146/annurev.cellbio.8.1.365.

Bernfield, M., Hinkes, M.T., & Gallo, R.L. (1993). Developmental expression of the syndecans: possible function and regulation. *Development*, 119, 205–212.

Bezakova, G., & Lømo, T. (2001). Muscle Activity and Muscle Agrin Regulate the organisation of Cytoskeletal Proteins and Attached Acetylcholine Receptor (AChR) Aggregates in Skeletal Muscle fibres. *The Journal of Cell Biology*, 153, 1453–1463.

Bhat, S. P. (2001). The ocular lens epithelium. *Bioscience reports*, 21(4), 537-563.

Billings, P.C., & Pacifici, M. (2015). Interactions of signalling proteins, growth factors and other proteins with heparan sulfate: mechanisms and mysteries. *Connective Tissue Research*, 56, 272–280.

Bishop, J.R., Schuksz, M., Esko, J.D., 2007. Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature* 446, 1030–1037.

Boswell, B.A., Overbeek, P.A., Musil, L.S., 2008. Essential role of BMPs in FGF-induced secondary lens fibre differentiation. *Developmental Biology* 324, 202–212.

Brewitt, B. and Clark, J.I., 1988. Growth and transparency in the lens, an epithelial tissue, stimulated by pulses of PDGF. *Science*, 242, 777-779.

Brockstedt, U., Dobra, K., Nurminen, M., & Hjerpe, A. (2002). Immunoreactivity to cell surface syndecans in cytoplasm and nucleus: tubulin-dependent rearrangements. *Experimental Cell Research*, 274, 235–245.

Brown, G. C., Brown, M. M., Menezes, A., Busbee, B. G., Lieske, H. B., & Lieske, P. A. (2013). Cataract surgery cost utility revisited in 2012: a new economic paradigm. *Ophthalmology*, 120(12), 2367-2376.

Budak, G., Dash, S., Srivastava, R., Lachke, S.A., & Janga, S.C. (2018). Express: a database of transcriptome profiles encompassing known and novel transcripts across multiple development stages in eye tissues. *Experimental Eye Research*, 168, 57-68.

Bullock, S.L., Fletcher, J.M., Beddington, R.S., & Wilson, V.A. (1998). Renal agenesis in mice homozygous for a gene trap mutation in the gene encoding heparan sulfate 2-sulfotransferase. *Genes & Development*, 12, 1894–1906.

Busse, M., Feta, A., Presto, J., Wilén, M., Grønning, M., Kjellén, L., & Kusche-Gullberg, M. (2007). Contribution of EXT1, EXT2, and EXTL3 to heparan sulfate chain elongation. *Journal of Biological Chemistry*, 282, 32802–32810.

Bányai, L., Sonderegger, P., & Patthy, L. (2010). Agrin binds BMP2, BMP4 and TGF- β 1. *PLOS ONE*, 5, e10758.

Call, T.W., & Hollyfield, J.G. (1990). Sulfated proteoglycans in Bruch's membrane of the human eye: localisation and characterization using cupromeronic blue. *Experimental Eye Research*, 51, 451–462.

Campos-Xavier, A.B., Martinet, D., Bateman, J., Belluoccio, D., Rowley, L., Tan, T.Y., Baxová, A., Gustavson, K.H., Borochowitz, Z.U., Innes, A.M., Unger, S., & et al. (2009). Mutations in

the heparan-sulfate proteoglycan glypican 6 (GPC6) impair endochondral ossification and cause recessive omodysplasia. *The American Journal of Human Genetics*, 84, 760-770.

Cano-Gauci, D.F., Song, H.H., Yang, H., McKerlie, C., Choo, B., Shi, W., Pullano, R., Piscione, T.D., Grisaru, S., Soon, S., Sedlackova, L., Tanswell, A.K., Mak, T.W., Yeager, H., Lockwood, G.A., Rosenblum, N.D., & Filmus, J. (1999). Glypican-3-deficient mice exhibit developmental overgrowth and some of the abnormalities typical of Simpson-Golabi-Behmel syndrome. *Journal of Cell Biology*, 146, 255-164.

Capurro M, Martin T, Shi W, Filmus J. Glypican-3 binds to frizzled and plays a direct role in the stimulation of canonical Wnt signalling. *J Cell Sci*. 2014;127(7):jcs.140871-jcs.141575. doi:10.1242/jcs.140871.

Capurro, M. I., Xu, P., Shi, W., Li, F., Jia, A., & Filmus, J. (2008). Glypican-3 inhibits Hedgehog signalling during development by competing with patched for Hedgehog binding. *Developmental Cell*, 14, 700–711.

Capurro, M., Izumikawa, T., Suarez, P., Shi, W., Cydzik, M., Kaneiwa, T., Garipey, J., Bonafe, L., & Filmus, J. (2017). Glypican-6 promotes the growth of developing long bones by stimulating Hedgehog signalling. *Journal of Cell Biology*, 216, 2911–2926.

Capurro, M., Martin, T., Shi, W., & Filmus, J. (2014). Glypican-3 binds to frizzled and plays a direct role in the stimulation of canonical Wnt signalling. *Journal of Cell Science*, 127, 140871–1575.

Carrasco, H., Olivares, G. H., Faunes, F., Oliva, C., & Larraín, J. (2005). Heparan sulfate proteoglycans exert positive and negative effects in Shh activity. *Journal of Cell Biochemistry*, 96, 831–838.

Carulli, S., Beck, K., Dayan, G., Boulesteix, S., Lortat-Jacob, H., & Rousselle, P. (2012). Cell surface proteoglycans syndecan-1 and -4 bind overlapping but distinct sites in laminin $\alpha 3$ LG45 protein domain. *Journal of Biological Chemistry*, 287, 12204–12216.

Cavalheiro, G. R., Matos-Rodrigues, G. E., Zhao, Y., Gomes, A. L., Anand, D., Predes, D., ... Martins, R. A. P. (2017). N-myc regulates growth and fibre cell differentiation in lens development. *Developmental Biology*, 429, 105-117.

Chai, L., & Morris, J. E. (2009). Distribution of heparan sulfate proteoglycans in embryonic chicken neural retina and isolated inner limiting membrane. *Current Eye Research*, 13, 669-677.

Chamberlain CG, McAvoy JW. Fibre differentiation and polarity in the mammalian lens: A key role for FGF. *Progress in Retinal and Eye Research*. 1997;16(3):443-478.
doi:10.1016/S1350-9462(96)00034-1.

Chamberlain, C. G., & McAvoy, J. W. (1989). Induction of Lens fibre differentiation by acidic and basic fibroblast growth factor (FGF). *Growth Factors*, 1, 125-134.

Chamberlain, C. G., McAvoy, J. W., & Richardson, N. A. (1991). The effects of insulin and basic fibroblast growth factor on fibre differentiation in rat Lens epithelial explants. *Growth Factors*, 4, 183-188.

Chandler, H. L., Geisler, J. A., & Sizemore, G. M. (2021). Platelet-Derived Growth Factor Receptor β Promotes Cataract Formation. *Investigative Ophthalmology & Visual Science*, 62(8), 2065-2065.

Chang, T., Mazotta, J., Dumstrei, K., Dumitrescu, A., & Hartenstein, V. (2001). Dpp and Hh signalling in the *Drosophila* embryonic eye field. *Development*, 128, 4691-4704.

Chauhan, B. K., Lou, M., Zheng, Y., & Lang, R. A. (2011). Balanced Rac1 and RhoA activities regulate cell shape and drive invagination morphogenesis in epithelia. *Proceedings of the National Academy of Sciences U.S.A.*, 108, 18289–18294.

Chen Y, Stump RJW, Lovicu FJ, Shimono A, McAvoy JW, 2008. Wnt signalling is required for organisation of the lens fibre cell cytoskeleton and development of lens three-dimensional architecture. *Dev Biol.* 324(1):161-176. doi:10.1016/j.ydbio.2008.09.002.

Chen, L., Klass, C., & Woods, A. (2004). Syndecan-2 regulates transforming growth factor- β signalling. *Journal of Biological Chemistry*, 279, 15715-15718.

Chen, X., Xu, J., Chen, X., & Yao, K. (2021). Cataract: Advances in surgery and whether surgery remains the only treatment in future. *Advances in Ophthalmology Practice and Research*, 1(1), 100008.

Chen, Y. et al., 2008. Wnt signalling is required for organisation of the lens fibre cell cytoskeleton and development of lens three-dimensional architecture. *Developmental Biology*, 324(1), pp.161–176.

Chen, Y., Shi-Wen, X., van Beek, J., Kennedy, L., McLeod, M., Renzoni, E. A., ... & Leask, A. (2005). Matrix contraction by dermal fibroblasts requires transforming growth factor- β /activin-linked kinase 5, heparan sulfate-containing proteoglycans, and MEK/ERK: insights into pathological scarring in chronic fibrotic disease. *The American journal of pathology*, 167(6), 1699-1711.

Cheng C, Gong X. Diverse roles of Eph/ephrin signalling in the mouse lens. Buday L, ed. *PLOS ONE*. 2011;6(11):e28147. doi:10.1371/journal.pone.0028147.

Cheng C, Nowak RB, Fowler VM. The lens actin filament cytoskeleton: Diverse structures for complex functions. *Experimental Eye Research*. 2017;156:58-71. doi:10.1016/j.exer.2016.03.005.

Cheng, C. & Gong, X., 2011. Diverse roles of Eph/ephrin signalling in the mouse lens L. Buday, ed. *PLoS ONE*, 6(11), p.e28147.

Cheng, C., Ansari, M. M., Cooper, J. A., & Gong, X. (2013). EphA2 and Src regulate equatorial cell morphogenesis during lens development. *Development*, 140, 4237-4245.

Cheng, C., Nowak, R. B., & Fowler, V. M. (2017). The lens actin filament cytoskeleton: Diverse structures for complex functions. *Experimental Eye Research*, 156, 58-71.

Chiao, E., Fisher, P., Crisponi, L., Deiana, M., Dragatsis, I., Schlessinger, D., ... Efstratiadis, A. (2002). Overgrowth of a mouse model of the Simpson-Golabi-Behmel syndrome is independent of IGF signalling. *Developmental Biology*, 243, 185-206.

Cho, H. J., Baek, K. E., Saika, S., Jeong, M. J., & Yoo, J. (2007). Snail is required for transforming growth factor- β -induced epithelial–mesenchymal transition by activating PI3 kinase/AKT signal pathway. *Biochemical and biophysical research communications*, 353(2), 337-343.

Choi, J., Park, S. Y., & Joo, C. K. (2004). Hepatocyte growth factor induces proliferation of lens epithelial cells through activation of ERK1/2 and JNK/SAPK. *Investigative Ophthalmology & Visual Science*, 45, 2696-2704.

Chow, R. L., Roux, G. D., Roghani, M., Palmer, M. A., Rifkin, D. B., Moscatelli, D. A., & Lang, R. A. (1995). FGF suppresses apoptosis and induces differentiation of fibre cells in the mouse lens. *Development*, 121, 4383-4393.

Chow, R.L., Roux, G.D., Roghani, M., Palmer, M.A., Rifkin, D.B., Moscatelli, D.A., Lang, R.A., 1995. FGF suppresses apoptosis and induces differentiation of fibre cells in the mouse lens. *Development* 121, 4383–4393.

Christianson, H. C., Svensson, K. J., van Kuppevelt, T. H., Li, J.-P., & Belting, M. (2013). Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalisation and functional activity. *Proceedings of the National Academy of Sciences*, 110, 17380-17385.

Christianson, H.C., Svensson, K.J., van Kuppevelt, T.H., Li, J.-P., Belting, M., 2013. Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. *Proc. Natl. Acad. Sci. U.S.A.* 110, 17380–17385.

Chu, C. L., Goerges, A. L., & Nugent, M. A. (2005). Identification of common and specific growth factor binding sites in heparan sulfate proteoglycans. *Biochemistry*, 44(36), 12203-12213.

Chua, J. S., & Kuberan, B. (2017). Synthetic xylosides: probing the glycosaminoglycan biosynthetic machinery for biomedical applications. *Accounts of Chemical Research*, 50(11), 2693-2705.

Clark, S. J., Keenan, T. D. L., Fielder, H. L., Collinson, L. J., Holley, R. J., Merry, C. L. R., ... Bishop, P. N. (2011). Mapping the differential distribution of glycosaminoglycans in the adult human retina, choroid, and sclera. *Investigative Ophthalmology & Visual Science*, 52, 6511-6521.

Clemmons, D. R. (1998). Role of insulin-like growth factor binding proteins in controlling IGF actions. *Molecular and cellular endocrinology*, 140(1-2), 19-24.

Cohen, N. A., Kaufmann, W. E., Worley, P. F., & Rupp, F. (1997). Expression of agrin in the developing and adult rat brain. *Neuroscience*, 76(2), 581-596.

Conrad, H. E. (2001). Inhibition of Heparan Sulfate Synthesis by Chlorate. In *Proteoglycan Protocols* (pp. 325-328). Humana Press.

Cornelison, D. D. W., Wilcox-Adelman, S. A., Goetinck, P. F., Rauvala, H., Rapraeger, A. C., & Olwin, B. B. (2004). Essential and separable roles for Syndecan-3 and Syndecan-4 in skeletal muscle development and regeneration. *Genes & Development*, 18, 2231-2236.

Corti, F., Finetti, F., Ziche, M., & Simons, M. (2013). The syndecan-4/protein kinase C α pathway mediates prostaglandin E₂-induced extracellular regulated kinase (ERK) activation in endothelial cells and angiogenesis in vivo. *Journal of Biological Chemistry*, 288, 12712-12721.

Corti, F., Wang, Y., Rhodes, J. M., Atri, D., Archer-Hartmann, S., Zhang, J., ... Simons, M. (2019). N-terminal syndecan-2 domain selectively enhances 6-O heparan sulfate chains sulfation and promotes VEGFA165-dependent neovascularization. *Nature Communications*, 10, 1-14.

Couchman, J. R., & Pataki, C. A. (2012). An Introduction to Proteoglycans and Their localisation. *Journal of Histochemistry & Cytochemistry*, 60, 885-897.

Couchman, J. R., & Woods, A. (1999). Syndecan-4 and integrins: Combinatorial signalling in cell adhesion. *Journal of Cell Science*, 112, 3415-3420.

Couchman, J. R., Gopal, S., Lim, H. C., Nørgaard, S., & Multhaupt, H. A. B. (2015). Fell-Muir Lecture: Syndecans: From peripheral coreceptors to mainstream regulators of cell behaviour. *International Journal of Experimental Pathology*, 96, 1-10.

Couchman, J.R. & Pataki, C.A., 2012. An Introduction to Proteoglycans and Their localisation. *Journal of Histochemistry & Cytochemistry*, 60(12), pp.885–897.

Coulombre, J. L., & Coulombre, A. J. (1963). Lens development: fibre elongation and lens orientation. *Science*, 142(3598), 1489-1490.

Cvekl, A., & Ashery-Padan, R. (2014) and molecular mechanisms of vertebrate lens development. *Development*, 141, 4432–4447.

Cvekl, A., & Zhang, X. (2017). signalling and Gene Regulatory Networks in Mammalian Lens Development. *Trends in Genetics*, 33, 677–702.

Daniels, M.P. (2012). The role of agrin in synaptic development, plasticity and signalling in the central nervous system. *Neurochemistry International*, pp. 848–853.

Danysh, B., Patel, T.P., Edwards, D.A., Czymmek, K.J., & Duncan, M.K. (2008). Sulfate and carboxyl groups within the anterior lens capsule regulates diffusion charged proteins. *Investigative Ophthalmology & Visual Science*, 49, 1524-1524.

Danysh, B.P., & Duncan, M.K. (2009). The lens capsule. *Experimental Eye Research*, 88, 151-164.

Das, S., Majid, M., & Baker, A.B. (2016). Syndecan-4 enhances PDGF-BB activity in diabetic wound healing. *Acta Biomaterialia*, 42, 56–65.

David, G., Bai, X.M., Van der Schueren, B., Cassiman, J.-J., & van den Berghe, H. (1992). Developmental Changes in Heparan Sulfate Expression: In situ detection with mAbs. *The Journal of Cell Biology*, 119, 961–975.

Davies, Y., Lewis, D., Fullwood, N.J., Nieduszynski, I.A., Marcyniuk, B., Albon, J., & Tullo, A. (1999). Proteoglycans on normal and migrating human corneal endothelium. *Experimental Eye Research*, 68, 303–311.

Dawes, L. J., Shelley, E. J., McAvoy, J. W., & Lovicu, F. J. (2018). A role for Hippo/YAP-signalling in FGF-induced lens epithelial cell proliferation and fibre differentiation. *Experimental eye research*, 169, 122-133.

Dawes, L. J., Sugiyama, Y., Lovicu, F. J., Harris, C. G., Shelley, E. J., & McAvoy, J. W. (2014). Interactions between lens epithelial and fibre cells reveal an intrinsic self-assembly mechanism. *Developmental biology*, 385(2), 291-303.

Dawes, L. J., Sugiyama, Y., Tanedo, A. S., Lovicu, F. J., & McAvoy, J. W. (2013). Wnt-frizzled signalling is part of an FGF-induced cascade that promotes lens fibre differentiation. *Investigative Ophthalmology & Visual Science*, 54, 1582–1590.

Dawes, L.J. et al., 2013. Wnt-frizzled signalling is part of an FGF-induced cascade that promotes lens fibre differentiation. *Investigative Ophthalmology & Visual Science*, 54(3), pp.1582–1590.

Dawes, L.J., Sleeman, M.A., Anderson, I.K., Reddan, J.R., & Wormstone, I.M. (2009). TGF- β /SMAD4-Dependent and -Independent Regulation of Human Lens Epithelial Cells. *Investigative Ophthalmology & Visual Science*, 50, 5318–5327.

De Agostini, A.L., Lau, H.K., Leone, C., Youssoufian, H., & Rosenberg, R.D. (1990). Cell mutants defective in synthesizing a heparan sulfate proteoglycan with regions of defined monosaccharide sequence. *Proc. Natl. Acad. Sci. U.S.A.*, 87, 9784–9788.

De Cat, B. (2001). Developmental roles of the glypicans. *Seminars in Cell & Developmental Biology*, 12, 117–125.

De Iongh, R. U., Abud, H. E., & Hime, G. R. (2006). WNT/Frizzled signalling in eye development and disease. *Frontiers of Bioscience*, 11, 2442–2464.

de Iongh, R., Wederell, E., Lovicu, F. and McAvoy, J., 2005. Transforming Growth Factor- β -Induced Epithelial-Mesenchymal Transition in the Lens: A Model for Cataract Formation. *Cells Tissues Organs*, 179, 43-55.

de Iongh, R.U. et al., 1996. FGF receptor-1 (flg) expression is correlated with fibre differentiation during rat lens morphogenesis and growth. *Developmental Dynamics*, 206(4), pp.412–426.

de Iongh, R.U., Gordon-Thomson, C., Chamberlain, C.G., Hales, A.M., & McAvoy, J.W. (2001). TGF beta receptor expression in lens: Implications for differentiation and cataractogenesis. *Experimental Eye Research*, 72, 649–659.

de Iongh, R.U., Lovicu, F.J., Hanneken, A., Baird, A., & McAvoy, J.W. (1996). FGF receptor-1 (flg) expression is correlated with fibre differentiation during rat lens morphogenesis and growth. *Developmental Dynamics*, 206, 412–426.

De Rossi, G., Vähätupa, M., Cristante, E., Arokiasamy, S., Liyanage, S.E., May, U., Pellinen, L., Uusitalo-Järvinen, H., Bainbridge, J.W., Järvinen, T.A. and Whiteford, J.R., 2021. Pathological angiogenesis requires syndecan-4 for efficient VEGFA-induced VE-cadherin internalization. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 41, 1374-1389.

Dealy, C.N. et al., 1997. FGF-Stimulated Outgrowth and Proliferation of Limb Mesoderm Is Dependent on Syndecan-3. *Developmental Biology*, 184(2), pp.343–350.

Decarlo, A.A., Belousova, M., Ellis, A.L., Petersen, D., Grenett, H., Hardigan, P., O'Grady, R., Lord, M., & Whitelock, J.M. (2012). Perlecan domain 1 recombinant proteoglycan augments BMP-2 activity and osteogenesis. *BMC Biotechnology*, 12, 60–16.

DeDreu, J., Walker, J.L., & Menko, A.S. (2021). Dynamics of the lens basement membrane capsule and its interaction with connective tissue-like extracapsular matrix proteins. *Matrix Biology*, 96, 18-46.

Dickinson, M.E., Flenniken, A.M., Ji, X., Teboul, L., Wong, M.D., White, J.K., Meehan, T.F., Weninger, W.J., Westerberg, H., Adissu, H., Baker, C.N., Bower, L., Brown, J.M., Caddle, L.B., Chiani, F., Clary, D., Cleak, J., Daly, M.J., Denegre, J.M., Doe, B., Dolan, M.E., Edie, S.M., Fuchs, H., Gailus-Durner, V., Galli, A., Gambadoro, A., Gallegos, J., Guo, S., Horner, N.R., Hsu, C.-W., Johnson, S.J., Kalaga, S., Keith, L.C., Lanoue, L., Lawson, T.N., Lek, M., Mark, M., Marschall, S., Mason, J., McElwee, M.L., Newbigging, S., Nutter, L.M.J., Peterson, K.A., Ramirez-Solis, R., Rowland, D.J., Ryder, E., Samocha, K.E., Seavitt, J.R., Selloum, M., Szoke-Kovacs, Z., Tamura, M., Trainor, A.G., Tudose, I., Wakana, S., Warren, J., Wendling, O., West, D.B., Wong, L., Yoshiki, A., Wurst, W., MacArthur, D.G., Tocchini-Valentini, G.P., Gao, X., Flicek, P., Bradley, A., Skarnes, W.C., Justice, M.J., Parkinson, H.E., Moore, M., Wells, S., Braun, R.E., Svenson, K.L., de Angelis, M.H., Herault, Y., Mohun, T., Mallon, A.-M., Henkelman, R.M., Brown, S.D.M., Adams, D.J., Lloyd, K.C.K., McKerlie, C., Beaudet, A.L., Bućan, M., Murray, S.A., 2016. High-throughput discovery of novel developmental phenotypes. *Nature* 537, 508–514.

Dobra, K., Nurminen, M., & Hjerpe, A. (2003). Growth factors regulate the expression profile of their syndecan co-receptors and the differentiation of mesothelioma cells. *Anticancer Research*, 23, 2435–2444.

Dong S, Cole GJ, Halfter W. Expression of collagen XVIII and localisation of its glycosaminoglycan attachment sites. *Journal of Biological Chemistry*. 2003;278(3):1700-1707. doi:10.1074/jbc.M209276200.

Dong, S., Cole, G. J., & Halfter, W. (2003). Expression of collagen XVIII and localisation of its

Dowling, C., & Allen, N.J. (2018). Mice Lacking Glypican 4 Display Juvenile Hyperactivity and Adult Social Interaction Deficits. *Brain Plasticity*, 4, 197–209.

Dreyfuss, J. L., Regatieri, C. V., Jarrouge, T. R., Cavaleiro, R. P., Sampaio, L. O., & Nader, H. B. (2009). Heparan sulfate proteoglycans: Structure, protein interactions and cell signalling. *An Acad Bras Cienc*, 81, 409–429.

Dyksterhuis, L.D., White, J.F., Hickey, M., Kirby, N., Mudie, S., Hawley, A., Vashi, A., Nigro, J., Werkmeister, J.A., & Ramshaw, J.A.M. (2011). Impact of heparan sulfate chains and sulfur-mediated bonds on the mechanical properties of bovine lens capsule. *Biophysical Journal*, 100, 2077-2083.

Echtermeyer, F. et al., 1999. Syndecan-4 core protein is sufficient for the assembly of focal adhesions and actin stress fibres. *J Cell Sci*, 112(20), pp.3433–3441.

Echtermeyer, F., Streit, M., Wilcox-Adelman, S., Saoncella, S., Denhez, F., Detmar, M., Goetinck, P.F., 2001. Delayed wound repair and impaired angiogenesis in mice lacking syndecan-4. *J. Clin. Invest.* 107, R9–R14.

Elamaa, H., Sormunen, R., Rehn, M., Soininen, R., Pihlajaniemi, T., 2005. Endostatin Overexpression Specifically in the Lens and Skin Leads to Cataract and Ultrastructural Alterations in Basement Membranes. *Am. J. Pathol.* 166, 221–229.

Eldred, J. A., L. J. Dawes, and I. M. Wormstone. "The lens as a model for fibrotic disease." *Philosophical Transactions of the Royal Society B: Biological Sciences* 366.1568 (2011): 1301-1319.

Elenius, K. et al., 1990. Binding of human syndecan to extracellular matrix proteins. *The Journal of biological chemistry*, 265(29), pp.17837–17843.

Esko, J.D. & Selleck, S.B., 2002. Order out of chaos: Assembly of ligand binding sites in heparan sulfate, *Annual Reviews* 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA.

Esko, J.D., Prestegard, J.H. & Linhardt, R.J., 2017. Proteins That Bind Sulfated Glycosaminoglycans. In A. Varki et al., eds. *Essentials of Glycobiology*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, pp. 493–503.

Esko, J.D., Prestegard, J.H., Linhardt, R.J., 2017. Proteins That Bind Sulfated Glycosaminoglycans, in: Varki, A., Cummings, R.D., Esko, J.D., Stanley, P., Hart, G.W., Aebi, M., Darvill, A.G., Kinoshita, T., Packer, N.H., Prestegard, J.H., Schnaar, R.L., Seeberger, P.H. (Eds.), *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 493–503.

Ethell, I.M., Irie, F., Kalo, M.S., Couchman, J.R., Pasquale, E.B., Yamaguchi, Y., 2001. EphB/Syndecan-2 signalling in Dendritic Spine Morphogenesis. *Neuron* 31, 1001–1013.

Faber, S. C., Dimanlig, P., Makarenkova, H. P., Shirke, S., Ko, K., & Lang, R. A. (2001). Fgf receptor signalling plays a role in lens induction. *Development*, 128, 4425–4438.

Faber, S. C., Robinson, M. L., Makarenkova, H. P., & Lang, R. A. (2002). Bmp signalling is required for development of primary lens fibre cells.

Fan, G., Xiao, L., Cheng, L., Wang, X., Sun, B., Hu, G., 2000. Targeted disruption of NDST-1 gene leads to pulmonary hypoplasia and neonatal respiratory distress in mice. *FEBS Lett.* 467, 7–11.

Fannon, M., Nugent, M.A., 1996. Basic fibroblast growth factor binds its receptors, is internalized, and stimulates DNA synthesis in Balb/c3T3 cells in the absence of heparan sulfate. *Journal of Biological Chemistry* 271, 17949–17956.

Fedarko, N.S. & Conrad, H.E., 1986. A Unique Heparan Sulfate in the Nuclei of Hepatocytes: Structural Changes with the Growth State of the Cells. *The Journal of Cell Biology*, 102(2), pp.587–599.

Fedarko, N.S., Ishihara, M. & Conrad, H.E., 1989. Control of cell division in hepatoma cells by exogenous heparan sulfate proteoglycan. *Journal of Cellular Physiology*, 139(2), pp.287–294.

Fico, A., Maina, F. & Dono, R., 2011. Fine-tuning of cell signalling by glypicans. *Cellular and Molecular Life Sciences*, 68(6), pp.923–929.

Filla, M. S., David, G., Weinreb, R. N., Kaufman, P. L., & Peters, D. M. (2004). Distribution of syndecans 1-4 within the anterior segment of the human eye: Expression of a variant syndecan-3 and matrix-associated syndecan-2. *Experimental Eye Research*, 79, 61–74.

Filla, M.S., Woods, A., Kaufman, P.L., Peters, D.M., 2006. $\beta 1$ and $\beta 3$ integrins cooperate to induce syndecan-4-containing cross-linked actin networks in human trabecular meshwork cells. *Investigative Ophthalmology & Visual Science* 47, 1956–1967.

Filla MS, David G, Weinreb RN, Kaufman PL, Peters DM. Distribution of syndecans 1-4 within the anterior segment of the human eye: Expression of a variant syndecan-3 and matrix-associated syndecan-2. *Experimental Eye Research*. 2004;79(1):61-74. doi:10.1016/j.exer.2004.02.010.

Filmus, J. (2022). The function of Glypicans in the mammalian embryo. *American Journal Physiology and Cell Physiology*.

Filmus, J., Capurro, M., 2008. The role of glypican-3 in the regulation of body size and cancer. *Cell Cycle* 7, 2787–2790.

Fisher, M. C., Li, Y., Seghatoleslami, M. R., Dealy, C. N., & Kosher, R. A. (2006). Heparan sulfate proteoglycans including syndecan-3 modulate BMP activity during limb cartilage differentiation. *Matrix Biology*, 25, 27–39.

Flügel-Koch, C., Ohlmann, A., Piatigorsky, J., Tamm, E.R., 2002. Disruption of anterior segment development by TGF-beta1 overexpression in the eyes of transgenic mice. *Developmental Dynamics* 225, 111–125.

Ford-Perriss, M. et al., 2003. Localisation of specific heparan sulfate proteoglycans during the proliferative phase of brain development. *Developmental Dynamics*, 227(2), pp.170–184.

Forsberg, E., Kjellén, L., 2001. Heparan sulfate: lessons from knockout mice. *J. Clin. Invest.* 108, 175–180.

Forsten-Williams, K., Chu, C. L., Fannon, M., Buczek-Thomas, J. A., & Nugent, M. A. (2008). Control of growth factor networks by heparan sulfate proteoglycans. *Annals of biomedical engineering*, 36, 2134-2148.

Foster, A., Resnikoff, S., 2005. The impact of Vision 2020 on global blindness. *Eye*. 19, 1133-1135.

Fuerer, C., Habib, S. J., & Nusse, R. (2010). A study on the interactions between heparan sulfate proteoglycans and Wnt proteins. *Developmental Dynamics*, 239, 184–190.

Fuerst, P. G.; Rauch, S. M.; Burgess, R. W. Defects in eye development in transgenic mice overexpressing the heparan sulfate proteoglycan agrin. *Developmental Biology* 2007, 303, 165–180.

Fujimura, N., 2016. WNT/ β -Catenin signalling in Vertebrate Eye Development. *Front. Cell Developmental Biology* 4, 138.

Fukai, N., Eklund, L., Marneros, A.G., Oh, S.P., Keene, D.R., Tamarkin, L., Niemelä, M., Ilves, M., Li, E., Pihlajaniemi, T., Olsen, B.R., 2002. Lack of collagen XVIII/endostatin results in eye abnormalities. *The EMBO Journal* 21, 1535–1544.

Furuta, Y., Hogan, B.L., 1998. BMP4 is essential for lens induction in the mouse embryo. *Genes Dev.* 12, 3764–3775.

Galli, A. et al., 2003. Glypican 4 modulates FGF signalling and regulates dorsoventral forebrain patterning in *Xenopus* embryos. *Development*, 130(20), pp.4919–4929.

Garcia, C.M. et al., 2005. signalling through FGF receptor-2 is required for lens cell survival and for withdrawal from the cell cycle during lens fibre cell differentiation. *Developmental dynamics* : an official publication of the American Association of Anatomists, 233(2), pp.516–527.

Garcia, C.M., Yu, K., Zhao, H., Ashery-Padan, R., Ornitz, D.M., Robinson, M.L., Beebe, D.C., 2005. signalling through FGF receptor-2 is required for lens cell survival and for withdrawal from the cell cycle during lens fibre cell differentiation. *Developmental Dynamics* 233, 516–527.

García, B. et al., 2016. Differential Expression of Proteoglycans by Corneal Stromal Cells in Keratoconus. *Investigative Ophthalmology & Visual Science*, 57(6), pp.2618–2628.

Gautam, M., Noakes, P.G., Moscoso, L., Rupp, F., Scheller, R.H., Merlie, J.P., Sanes, J.R., 1996. Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* 85, 525–535.

Golestaneh, N. et al., 2004. Lens major intrinsic protein (MIP)/aquaporin 0 expression in rat lens epithelia explants requires fibroblast growth factor-induced ERK and JNK signalling. *Journal of Biological Chemistry*, 279(30), pp.31813–31822.

Golestaneh, N. et al., 2008. PKC putative phosphorylation site Ser235 is required for MIP/AQP0 translocation to the plasma membrane. *Molecular Vision*, 14, pp.1006–1014.

Golestaneh, N., Fan, J., Zelenka, P., Chepelinsky, A.B., 2008. PKC putative phosphorylation site Ser235 is required for MIP/AQP0 translocation to the plasma membrane. *Molecular Vision* 14, 1006–1014.

Gomes, B.F., Crema, A.S., Santhiago, M.R., Costa Filho, A.A., Moraes Jr, H.V., Paiva, D.S. and Miguel, N.C., 2021. Morphologic and molecular study on the lens anterior capsule in systemic sclerosis. *Arquivos Brasileiros de Oftalmologia*, 84, pp.271-274.

Gospodarowicz, D., Greenburg, G., Bialecki, H., Zetter, B.R., 1978. Factors Involved in the Modulation of Cell Proliferation In vivo and In vitro: The Role of Fibroblast and Epidermal Growth Factors in the Proliferative Response of Mammalian Cells. *In Vitro* 14, 85–118.

Gougoula, C., Bielfeld, A.P., Pour, S.J., Krüssel, J.-S., Götte, M., Benten, W.P.M., Baston-Büst, D.M., 2019. Physiological and anatomical aspects of the reproduction of mice with reduced Syndecan-1 expression. *Reprod. Biol. Endocrinol.* 17, 28–12.

Gould, S. E., Upholt, W. B., & Kosher, R. A. (1995). Characterization of Chicken Syndecan-3 as a Heparan Sulfate Proteoglycan and Its Expression during Embryogenesis. *Developmental Biology*, 168, 438–451.

Granés, F. et al., 2000. Ezrin links syndecan-2 to the cytoskeleton. *Journal of Cell Science*, 113(7), pp.1267–1276.

Grisaru, S. (2001). Glypican-3 modulates BMP- and FGF-mediated effects during renal branching morphogenesis. *Developmental Biology*, 231, 31–46.

Groffen, A.J. et al., 1998. Agrin is a major heparan sulfate proteoglycan in the human glomerular basement membrane. *Journal of Histochemistry & Cytochemistry*, 46(1), pp.19–27.

Gu, X., Chen, X., Jin, G., Wang, L., Zhang, E., Wang, W., ... & Luo, L. (2022). Early-onset posterior capsule opacification: incidence, severity, and risk factors. *Ophthalmology and Therapy*, 1-11.

Gubbiotti, M.A., Neill, T., Iozzo, R.V., 2017. A current view of perlecan in physiology and pathology: A mosaic of functions. *Matrix Biology* 57–58, 285–298.

Gunhaga, L. The lens: a classical model of embryonic induction providing new insights into cell determination in early development. *Philosophical Transactions: Biological Sciences* 2011, 366, 1193–1203.

Götte, M., Joussen, A.M., Klein, C., Andre, P., Wagner, D.D., Hinkes, M.T., Kirchhof, B., Adamis, A.P., Bernfield, M., 2002. Role of syndecan-1 in leukocyte-endothelial interactions in the ocular vasculature. *Investigative Ophthalmology & Visual Science* 43, 1135–1141.

HajMohammadi, S., Enjyoji, K., Princivalle, M., Christi, P., Lech, M., Beeler, D., Rayburn, H., Schwartz, J.J., Barzegar, S., de Agostini, A.I. and Post, M.J., 2003. Normal levels of anticoagulant heparan sulfate are not essential for normal hemostasis. *J clin invest*, 111, 989-999.

Hakim, S.A. & Abd Raboh, N.M., 2015. Immunohistochemical expression of glypican 3 in endometrial carcinoma and correlation with prognostic parameters. *International Journal of Clinical and Experimental Pathology*, 8(10), pp.13225–13232.

Hales AM, Schulz MW, Chamberlain CG, et al. TGF- β 1 induces lens cells to accumulate alpha-smooth muscle actin, a marker for subcapsular cataracts. *Curr Eye Res*. 1994, 13: 885–90.

Handler, M., Yurchenco, P.D., Iozzo, R.V., 1997. Developmental expression of perlecan during murine embryogenesis. *Developmental Dynamics* 210, 130–145.

Harvey, S.J., Jarad, G., Cunningham, J., Rops, A.L., Van Der Vlag, J., Berden, J.H., Moeller, M.J., Holzman, L.B., Burgess, R.W. and Miner, J.H., 2007. Disruption of glomerular basement membrane charge through podocyte-specific mutation of agrin does not alter glomerular permselectivity. *The American Journal of Pathology*, 171, 139-152.

Hay, E.D. & Meier, S., 1974. Glycosaminoglycan synthesis by embryonic inductors: neural tube, notochord, and lens. *The Journal of Cell Biology*, 62(3), pp.889–898.

Hayashida, A., Amano, S., Gallo, R.L., Linhardt, R.J., Liu, J., Park, P.W., 2015. 2-O-Sulfated Domains in Syndecan-1 Heparan Sulfate Inhibit Neutrophil Cathelicidin and Promote *Staphylococcus aureus* Corneal Infection. *Journal of Biological Chemistry* 290, 16157–16167.

Hayashida, A., Amano, S., Park, P.W., 2011. Syndecan-1 promotes *Staphylococcus aureus* corneal infection by counteracting neutrophil-mediated host defense. *Journal of Biological Chemistry* 286, 3288–3297.

Heathcote, J. G., & Orkin, R. W. (1984). Biosynthesis of Sulphated Macromolecules by Rabbit Lens Epithelium. I. Identification of the Major Macromolecules Synthesized by Lens Epithelial Cells in vitro. *The Journal of Cell Biology*, 99, 852–860.

Heimann, H., Coupland, S.E., Gochman, R., Hellmich, M., Foerster, M.H., 2001. Alterations in expression of mucin, tenascin-c and syndecan-1 in the conjunctiva following retinal surgery and plaque radiotherapy. *Graefe's Archive for Clinical and Experimental Ophthalmology* 239, 488–495.

Heljasvaara, R., Aikio, M., Ruotsalainen, H., Pihlajaniemi, T., 2017. Collagen XVIII in tissue homeostasis and dysregulation - Lessons learned from model organisms and human patients. *Matrix Biol.* 57-58, 55–75.

Hewitt, A.T., Nakazawa, K., Newsome, D.A., 1989. Analysis of newly synthesized Bruch's membrane proteoglycans. *Investigative Ophthalmology & Visual Science* 30, 478–486.

Hienola, A., Tumova, S., Kuleskiy, E., Rauvala, H., 2006. N-syndecan deficiency impairs neural migration in brain. *The Journal of Cell Biology* 174, 569–580.

Hoang, T. V., Kumar, P. K. R., Sutharzan, S., Tsonis, P. A., Liang, C., & Robinson, M. L. (2014). Comparative transcriptome analysis of epithelial and fibre cells in newborn mouse lenses with RNA sequencing. *Molecular Vision*, 20, 1491–1517.

Horowitz, A., Simons, M., 1998. Phosphorylation of the cytoplasmic tail of syndecan-4 regulates activation of protein kinase C alpha. *Journal of Biological Chemistry* 273, 25548–25551.

Hsia, E., Richardson, T.P., Nugent, M.A., 2003. Nuclear localisation of basic fibroblast growth factor is mediated by heparan sulfate proteoglycans through protein kinase C signalling. *J. Cell. Biochem.* 88, 1214–1225.

Hua, R., Yu, J., Yan, X., Ni, Q., Zhi, X., Li, X., ... & Zhu, J. (2020). Syndecan-2 in colorectal cancer plays oncogenic role via epithelial-mesenchymal transition and MAPK pathway. *Biomedicine & Pharmacotherapy*, 121, 109630.

Huang, J., Rajagopal, R., Liu, Y., Dattilo, L. K., Shaham, O., Ashery-Padan, R., & Beebe, D. C. (2011). The mechanism of lens placode formation: a case of matrix-mediated morphogenesis. *Developmental Biology*, 355, 32–42.

Hubka, K. M., Carson, D. D., Harrington, D. A., & Farach-Carson, M. C. (2019). Perlecan domain I gradients establish stable biomimetic heparin binding growth factor gradients for cell migration in hydrogels. *Acta Biomaterialia*, 97, 385–398.

Hufnagel, L., Kreuger, J., Cohen, S. M., & Shraiman, B. I. (2006). On the role of glypicans in the process of morphogen gradient formation. *Developmental Biology*, 300, 512–522.

Humphries, D.E., Silbert, J.E., 1988. Chlorate: A reversible inhibitor of proteoglycan sulfation. *Biochemical Biophysical Research Communications*. 154, 365–371.

Ibaraki, N., Lin, L. R., & Reddy, V. N. (1995). Effects of growth factors on proliferation and differentiation in human lens epithelial cells in early subculture. *Investigative ophthalmology & visual science*, 36(11), 2304-2312.

Ibrahim, S.A., Gadalla, R., El-Ghonaimy, E.A., Samir, O., Mohamed, H.T., Hassan, H., Greve, B., El-Shinawi, M., Mohamed, M.M., Götte, M., 2017. Syndecan-1 is a novel molecular marker for triple negative inflammatory breast cancer and modulates the cancer stem cell phenotype via the IL-6/STAT3, Notch and EGFR signalling pathways. *Molecular Cancer* 16, 57.

Inatani, M., Honjo, M., Oohira, A., Kido, N., Otori, Y., Tano, Y., Honda, Y., Tanihara, H., 2002. Spatiotemporal Expression Patterns of N-Syndecan, a Transmembrane Heparan Sulfate Proteoglycan, in Developing Retina. *Investigative Ophthalmology & Visual Science* 43, 1616–1621.

Inomata, T., Ebihara, N., Funaki, T., Matsuda, A., Watanabe, Y., Ning, L., Xu, Z., Murakami, A., Arikawa Hirasawa, E., 2012. Perlecan-deficient mutation impairs corneal epithelial structure. *Investigative Ophthalmology & Visual Science* 53, 1277–1284.

Iozzo, R.V., 2005. Basement membrane proteoglycans: from cellar to ceiling. *Nat Rev Mol Cell Biol* 6, 646–656.

Ishibashi, T., Araki, H., Sugai, S., Tawara, A. and Inomata, H., 1995. Detection of Proteoglycans in Human Posterior Capsule Opacification. *Ophthalmic Research*, 27, 208-213.

Ito, Y. et al., 2005. Structural characterization of the epitopes of the monoclonal antibodies 473HD, CS-56, and MO-225 specific for chondroitin sulfate D-type using the oligosaccharide library. *Glycobiology*, 15(6), pp.593–603.

Iwao, K., Inatani, M., Matsumoto, Y., Ogata-Iwao, M., Takihara, Y., Irie, F., Yamaguchi, Y., Okinami, S. and Tanihara, H., 2009. Heparan sulfate deficiency leads to Peters anomaly in mice by disturbing neural crest TGF- β 2 signalling. *J Clin Invest*, 119, 1997-2008.

Iwao, K., Inatani, M., Ogata-Iwao, M., Yamaguchi, Y., Okinami, S., Tanihara, H., 2010. Heparan sulfate deficiency in periocular mesenchyme causes microphthalmia and ciliary body dysgenesis. *Experimental Eye Research* 90, 81–88.

Iyengar L, Patkunanathan B, Lynch OT, McAvoy JW, Rasko JEJ, Lovicu FJ. Aqueous humour- and growth factor-induced lens cell proliferation is dependent on MAPK/ERK1/2 and Akt/PI3-K signalling. *Experimental Eye Research*. 2006;83(3):667-678. doi:10.1016/j.exer.2006.03.008.

Iyengar, L. et al., 2007. Duration of ERK1/2 phosphorylation induced by FGF or ocular media determines lens cell fate. *Differentiation*, 75(7), pp.662–668.

Iyengar, L., Lovicu, F.J., 2017. Aqueous humour-induced lens epithelial cell proliferation requires FGF-signalling. *Growth Factors* 35, 131–143.

Iyengar, L., Patkunanathan, B., Lynch, O.T., McAvoy, J.W., Rasko, J.E.J., Lovicu, F.J., 2006. Aqueous humour- and growth factor-induced lens cell proliferation is dependent on MAPK/ERK1/2 and Akt/PI3-K signalling. *Experimental Eye Research* 83, 667–678.

Iyengar, L., Patkunanathan, B., Mcavoy, J. W., & Lovicu, F. J. (2009). Growth factors involved in aqueous humour-induced lens cell proliferation. *Growth Factors*, 27(1), 50-62.

Iyengar, L., Wang, Q., Rasko, J.E.J., McAvoy, J.W., Lovicu, F.J., 2007. Duration of ERK1/2 phosphorylation induced by FGF or ocular media determines lens cell fate. *Differentiation* 75, 662–668.

Izvolosky, K.I., Lu, J., Martin, G., Albrecht, K.H., Cardoso, W.V., 2008. Systemic inactivation of Hs6st1 in mice is associated with late postnatal mortality without major defects in organogenesis. *Genesis* 46, 8–18.

J

Jackson, S.M., Nakato, H., Sugiura, M., Jannuzi, A., Oakes, R., Kaluza, V., Golden, C., Selleck, S.B., 1997. dally, a Drosophila glypican, controls cellular responses to the TGF-beta-related morphogen, Dpp. *Development* 124, 4113–4120.

Jang E, Albadawi H, Watkins MT, Edelman ER, Baker AB. Syndecan-4 proteoliposomes enhance fibroblast growth factor-2 (FGF-2)-induced proliferation, migration, and neovascularization of ischemic muscle. *Proc Natl Acad Sci USA*. 2012;109(5):1679-1684. doi:10.1073/pnas.1117885109.

Jang, B. et al., 2016. Syndecan-2 enhances E-cadherin shedding and fibroblast-like morphological changes by inducing MMP-7 expression in colon cancer cells. *Biochemical and Biophysical Research Communications*, 477(1), pp.47–53.

Jang, E. et al., 2012. Syndecan-4 proteoliposomes enhance fibroblast growth factor-2 (FGF-2)-induced proliferation, migration, and neovascularization of ischemic muscle. *Proceedings of the National Academy of Sciences of the United States of America*, 109(5), pp.1679–1684.

Javitt, J. C., Tielsch, J. M., Canner, J. K., Kolb, M. M., Sommer, A., Steinberg, E. P., ... & Wilkinson, C. P. (1992). National Outcomes of Cataract Extraction: Increased risk of retinal complications associated with Nd.-YAG laser capsulotomy. *Ophthalmology*, 99(10), 1487-1498.

Jemth, P., Smeds, E., Do, A.T., Habuchi, H., Kimata, K., Lindahl, U., Kusche-Gullberg, M., 2003. Oligosaccharide library-based assessment of heparan sulfate 6-O-sulfotransferase substrate specificity. *Journal of Biological Chemistry* 278, 24371–24376.

Jen, Y.H.L., Musacchio, M., Lander, A.D., 2009. Glypican-1 controls brain size through regulation of fibroblast growth factor signalling in early neurogenesis. *Neural Dev* 4, 33–19.

Jiang, J., Shiha, M.H., Wang, Y., Duncan, M.K., 2018. Lens epithelial cells initiate an inflammatory response following cataract surgery. *Investigative Ophthalmology & Visual Science* 59, 4986-4997.

Jidigam, V. K., Srinivasan, R. C., Patthey, C., & Gunhaga, L. (2015). Apical constriction and epithelial invagination are regulated by BMP activity. *Biology Open*, 4, 1782–1791.

Jin, H., Fisher, M., & Grainger, R. M. (2012). Defining progressive stages in the commitment process leading to embryonic lens formation. *Genesis*, 50, 728–740.

Jinno, A., Hayashida, A., Jenkinson, H.F., Park, P.W., 2020. Syndecan-1 Promotes *Streptococcus pneumoniae* Corneal Infection by Facilitating the Assembly of Adhesive Fibronectin Fibrils. *mBio* 11,e01907-20.

Joslyn, G., Wolf, F.W., Brush, G., Wu, L., Schuckit, M., White, R.L., 2011. Glypican Gene GPC5 Participates in the Behavioral Response to Ethanol: Evidence from Humans, Mice, and Fruit Flies. *G3 (Bethesda)* 1, 627–635.

Kakrana, A., Yang, A., Anand, D., Djordjevic, D., Ramachandrani, D., Singh, A., Huang, H., Ho, J.W.K., Lachke, S.A., 2018. ISyTE 2.0: A database for expression-based gene discovery in the eye. *Nucleic Acids Res.* 46, D875–D885.

Kaksonen, M., Pavlov, I., Vöikar, V., Lauri, S.E., Hienola, A., Riekk, R., Lakso, M., Taira, T., Rauvala, H., 2002. Syndecan-3-deficient mice exhibit enhanced LTP and impaired hippocampus-dependent memory. *Mol. Cell. Neurosci.* 21, 158–172.

Karahan, E., Er, D., & Kaynak, S. (2014). An overview of Nd: YAG laser capsulotomy. *Medical hypothesis, discovery and innovation in ophthalmology*, 3(2), 45.

Karamanos NK, Piperigkou Z, Theocharis AD, et al. Proteoglycan chemical diversity drives multifunctional cell regulation and therapeutics. *Chem Rev.* 2018;118(18):9152-9232. doi:10.1021/acs.chemrev.8b00354.

Karamanos, N. K., Piperigkou, Z., Theocharis, A. D., Watanabe, H., Franchi, M., Baud, S., Brézillon, S., Götte, M., Passi, A., Vigetti, D., Ricard-Blum, S., Sanderson, R. D., Neill, T., & Iozzo, R. V. (2018). Proteoglycan chemical diversity drives multifunctional cell regulation and therapeutics. *Chemical Reviews*, 118, 9152–9232.

Kato, M., Wang, H., Bernfield, M., Gallagher, J.T., Turnbull, J.E., 1994. Cell surface syndecan-1 on distinct cell types differs in fine structure and ligand binding of its heparan sulfate chains. *Journal of Biological Chemistry* 269, 18881–18890.

Kerever, A.; Mercier, F.; Nonaka, R.; de Vega, S.; Oda, Y.; Zalc, B.; Okada, Y.; Hattori, N.; Yamada, Y.; Arikawa Hiras.

Kerr, C.L., Huang, J., Williams, T., West-Mays, J.A., 2012. Activation of the hedgehog signalling pathway in the developing lens stimulates ectopic FoxE3 expression and disruption in fibre cell differentiation. *Investigative Ophthalmology & Visual Science* 53, 3316–3330.

Khan, A.O., Aldahmesh, M.A., Mohamed, J.Y., Al-Mesfer, S., Alkuraya, F.S., 2012. The distinct ophthalmic phenotype of Knobloch syndrome in children. *Br J Ophthalmol* 96, 890–895.

Khan, S. Y., Hackett, S. F., Lee, M.-C. W., Pourmand, N., Talbot, C. C., & Riazuddin, S. A. (2015). Transcriptome Profiling of Developing Murine Lens Through RNA Sequencing. *Investigative Ophthalmology & Visual Science*, 56, 4919–4926.

Khare, N., Baumgartner, S., 2000. Dally-like protein, a new *Drosophila* glypican with expression overlapping with wingless. *Mech. Dev.* 99, 199–202.

Khurana, R.N. et al., 2002. Inhibition of Rho-kinase induces α B-crystallin expression in lens epithelial cells. *Biochemical and Biophysical Research Communications*, 294(5), pp.981–987.

Kim, M.-S., Saunders, A. M., Hamaoka, B. Y., Beachy, P. A., & Leahy, D. J. (2011). Structure of the protein core of the glypican Dally-like and localisation of a region important for hedgehog signalling. *Proceedings of the National Academy of Sciences U.S.A.*, 108, 13112–13117.

Kim, M.J., Cotman, S.L., Halfter, W., Cole, G.J., 2003. The heparan sulfate proteoglycan agrin modulates neurite outgrowth mediated by FGF-2. *J. Neurobiol.* 55, 261–277.

Kim, M.J., Liu, I.H., Song, Y., Lee, J.-A., Halfter, W., Balice-Gordon, R.J., Linney, E., Cole, G.J., 2007. Agrin is required for posterior development and motor axon outgrowth and branching in embryonic zebrafish. *Glycobiology* 17, 231–247.

Kinnunen, T., Kaksonen, M., Saarinen, J., Kalkkinen, N., Peng, H.B., Rauvala, H., 1998. Cortactin-Src kinase signalling pathway is involved in N-syndecan-dependent neurite outgrowth. *Journal of Biological Chemistry* 273, 10702–10708.

Klass, C.M., Couchman, J.R. and Woods, A., 2000. Control of extracellular matrix assembly by syndecan-2 proteoglycan. *Journal of Cell Science*, 113, 493-506.

Knox, S., Merry, C., Stringer, S., Melrose, J., Whitelock, J., 2002. Not all perlecanins are created equal: interactions with fibroblast growth factor (FGF) 2 and FGF receptors. *Journal of Biological Chemistry* 277, 14657–14665.

Kok, A., Lovicu, F.J., Chamberlain, C.G., McAvoy, J.W., 2002. Influence of platelet-derived growth factor on lens epithelial cell proliferation and differentiation. *Growth Factors* 20, 27–34.

Kokenyesi, R., & Bernfield, M. (1994). Core protein structure and sequence determine the site and presence of heparan sulfate and chondroitin sulfate on syndecan-1. *Journal of Biological Chemistry*, 269, 12304–12309.

Kokkinos, M.I., Brown, H.J., de Iongh, R.U., 2007. Focal adhesion kinase (FAK) expression and activation during lens development. *Molecular Vision* 13, 418–430.

Kolluri, A., & Ho, M. (2019). The Role of Glypican-3 in Regulating Wnt, YAP, and Hedgehog in Liver Cancer. *Frontiers in Oncology*, 9, 708.

Kolset, S.O., Prydz, K., Pejler, G., 2004. Intracellular proteoglycans. *Biochem. J.* 379, 217–227.

Konopińska, J., Młynarczyk, M., Dmuchowska, D. A., & Obuchowska, I. (2021). Posterior capsule opacification: a review of experimental studies. *Journal of clinical medicine*, 10(13), 2847.

Korol, A. (2017). The Role of Cytoskeletal signalling in Lens EMT (Doctoral dissertation).

Korol, A., Pino, G., Dwivedi, D., Robertson, J. V., Deschamps, P. A., & West-Mays, J. A. (2014). Matrix Metalloproteinase-9–Null Mice Are Resistant to TGF- β –Induced Anterior Subcapsular Cataract Formation. *The American Journal of Pathology*, 184(7), 2001–2012.

Koso, H., Iida, A., Tabata, Y., Baba, Y., Satoh, S., Taketo, M.M., Watanabe, S., 2008. CD138/syndecan-1 and SSEA-1 mark distinct populations of developing ciliary epithelium that are regulated differentially by Wnt signal. *Stem Cells* 26, 3162–3171.

Kovalszky, I., Hjerpe, A. & Dobra, K., 2014. Nuclear translocation of heparan sulfate proteoglycans and their functional significance. *Biochimica Et Biophysica Acta*, 1840(8), pp.2491–2497.

Koyama, Y. et al., 2008. Matrix metalloproteinase-9 associated with heparan sulphate chains of GPI-anchored cell surface proteoglycans mediates motility of murine colon adenocarcinoma cells. *Journal of Biochemistry*, 143(5), pp.581–592.

Kröger, S., Pfister, H., 2009. Agrin in the nervous system: Synaptogenesis and beyond. *Future Neurology* 4, 67–86.

Kubo, E., Hasanova, N., Fatma, N., Sasaki, H., & Singh, D. P. (2013). Elevated tropomyosin expression is associated with epithelial–mesenchymal transition of lens epithelial cells. *Journal of cellular and molecular medicine*, 17(1), 212–221.

Kuriyama, S., Mayor, R., 2009. A role for Syndecan-4 in neural induction involving ERK- and PKC-dependent pathways. *Development* 136, 575–584.

Kusano, Y., Oguri, K., Nagayasu, Y., Munesue, S., Ishihara, M., Saiki, I., Yonekura, H., Yamamoto, H., Okayama, M., 2000. Participation of Syndecan 2 in the Induction of Stress fibre Formation in Cooperation with Integrin $\alpha 5 \beta 1$: Structural Characteristics of Heparan Sulfate Chains with Avidity to COOH-Terminal Heparin-Binding Domain of Fibronectin. *Experimental Cell Research* 256, 434–444.

Lam, D., Rao, S. K., Ratra, V., Liu, Y., Mitchell, P., King, J., ... & Chang, D. F. (2015). Cataract. *Nature reviews Disease primers*, 1(1), 1-15.

Lamanna, W.C., Kalus, I., Padva, M., Baldwin, R.J., Merry, C.L. and Dierks, T., 2007. The heparanome—the enigma of encoding and decoding heparan sulfate sulfation. *Journal of biotechnology*, 129, 290-307.

Landemore, G., Stefani, P., Quillec, M., Lecoq-Guilbert, P., Billotte, C. and Izard, J., 1999. Uneven distribution and size of rabbit lens capsule proteoglycans. *The Histochemical Journal*, 31, 161-167.

Lang, R. A. (2004). Pathways regulating lens induction in the mouse. *International Journal of Developmental Biology*, 48, 783–791.

Laterra, J., Silbert, J. E., & Culp, L. A. (1983). Cell surface heparan sulfate mediates some adhesive responses to glycosaminoglycan-binding matrices, including fibronectin. *The Journal of cell biology*, 96(1), 112-123.

Lau, C.S.L., Yu, C.B.O., Wong, H.K., Fan, D.S.P., Mak, H.T., Wong, K.W., Lam, D.S.C., Pang, C.P., Choy, K.W., 2010. Allelic imbalance at 13q31 is associated with reduced GPC6 in Chinese with sporadic retinoblastoma. *Br J Ophthalmol* 94, 357–362.

Laurie, G. W., Bing, J. T., Kleinman, H. K., Hassell, J. R., Aumailley, M., Martin, G. R., & Feldmann, R. J. (1986). localisation of binding sites for laminin, heparan sulfate proteoglycan and fibronectin on basement membrane (type IV) collagen. *Journal of molecular biology*, 189(1), 205-216.

Le, A.C., Musil, L.S., 2001. A novel role for FGF and extracellular signal-regulated kinase in gap junction-mediated intercellular communication in the lens. *J Cell Biology*, 154, 197-216.

Leadbeater WE, Gonzalez A-M, Logaras N, Berry M, Turnbull JE, Logan A. Intracellular trafficking in neurones and glia of fibroblast growth factor-2, fibroblast growth factor receptor 1 and heparan sulphate proteoglycans in the injured adult rat cerebral cortex. *J Neurochem*. 2006;96(4):1189-1200. doi:10.1111/j.1471-4159.2005.03632.x

Leadbeater, W.E., Gonzalez, A.-M., Logaras, N., Berry, M., Turnbull, J.E., Logan, A., 2006. Intracellular trafficking in neurones and glia of fibroblast growth factor-2, fibroblast growth factor receptor 1 and heparan sulphate proteoglycans in the injured adult rat cerebral cortex. *J. Neurochem*. 96, 1189–1200.

Leask, A., & Abraham, D. J. (2004). TGF- β signalling and the fibrotic response. *The FASEB Journal*, 18(7), 816-827.

Li, H. et al., 2019. Lens differentiation is controlled by the balance between PDGF and FGF signalling. M. E. Bronner, ed. *PLoS biology*, 17(2), p.e3000133.

Li, J.-P., Gong, F., Hagner-McWhirter, A., Forsberg, E., Abrink, M., Kisilevsky, R., Zhang, X., Lindahl, U., 2003. Targeted disruption of a murine glucuronyl C5-epimerase gene results in heparan sulfate lacking L-iduronic acid and in neonatal lethality. *Journal of Biological Chemistry* 278, 28363–28366.

Li, J.P., Kusche-Gullberg, M., 2016. Heparan Sulfate: Biosynthesis, Structure, and Function. *Int Rev Cell Mol Biol* 325, 215–273.

Li, N. et al., 2019. A Frizzled-Like Cysteine-Rich Domain in Glypican-3 Mediates Wnt Binding and Regulates Hepatocellular Carcinoma Tumor Growth in Mice. *Hepatology (Baltimore, Md.)*, 70(4), pp.1231–1245.

Li, X., Wang, F., Ren, M., Du, M., & Zhou, J. (2019). The effects of c-Src kinase on EMT signalling pathway in human lens epithelial cells associated with lens diseases. *BMC ophthalmology*, 19, 1-10.

Liang, Y., Häring, M., Roughley, P. J., Margolis, R. K., & Margolis, R. U. (1997). Glypican and biglycan in the nuclei of neurons and glioma cells: Presence of functional nuclear localisation

signals and dynamic changes in glypican during the cell cycle. *Journal of Cell Biology*, 139, 851–864.

Lin X. Functions of heparan sulfate proteoglycans in cell signalling during development. *Development*. 2004;131(24):6009-6021. doi:10.1242/dev.01522.

Lin, X., Buff, E.M., Perrimon, N., Michelson, A.M., 1999. Heparan sulfate proteoglycans are essential for FGF receptor signalling during *Drosophila* embryonic development. *Development* 126, 3715–3723.

Liu, C-Y., Kao, W.W-Y., 2015. Corneal Epithelial Wound Healing, *Progress in Molecular Biology and Translational Science* 134, 61–71.

Liu, I. H., Zhang, C., Min, J. K., & Cole, G. J. (2008). Retina development in zebrafish requires the heparan sulfate proteoglycan agrin. *Developmental Neurobiology*, 68, 877–898.

Liu, I.H., Zhang, C., Min, J.K., Cole, G.J., 2008. Retina development in zebrafish requires the heparan sulfate proteoglycan agrin. *Dev Neurobiol* 68, 877–898.

Liu, Y.C., Wierbowski, B.M., Salic, A., 2022. Hedgehog pathway modulation by glypican 3-conjugated heparan sulfate. *J Cell Sci* 135.

Liu, Z. Z., Taiyab, A., & West-Mays, J. A. (2021). MMP9 differentially regulates proteins involved in actin polymerization and cell migration during TGF- β -induced EMT in the lens. *International Journal of Molecular Sciences*, 22(21), 11988.

Lo WK, Biswas SK, Brako L, Shiels A, Gu S, Jiang JX. Aquaporin-0 targets interlocking domains to control the integrity and transparency of the eye lens. *Invest Ophthalmol Vis Sci*. 2014;55(3):1202-1212. doi:10.1167/iovs.13-13379.

Lo, W.K. et al., 2014. Aquaporin-0 targets interlocking domains to control the integrity and transparency of the eye lens. *Investigative Ophthalmology and Visual Science*, 55(3), pp.1202–1212.

Lord, M. S., Tang, F., Rnjak-Kovacina, J., Smith, J. G., Melrose, J., & Whitelock, J. M. (2018). The multifaceted roles of perlecan in fibrosis. *Matrix Biology*, 68, 150-166.

Lovicu FJ, McAvoy JW. Structural analysis of lens epithelial explants induced to differentiate into fibres by fibroblast growth factor (FGF). *Experimental Eye Research*. 1989;49(3):479-494. doi:10.1016/0014-4835(89)90056-0.

Lovicu, F. J., & McAvoy, J. W. (2001). FGF-induced lens cell proliferation and differentiation is dependent on MAPK (ERK1/2) signalling.

Lovicu, F. J., & McAvoy, J. W. (2005). Growth factor regulation of lens development. *Developmental biology*, 280(1), 1-14.

Lovicu, F. J., Ang, S., Chorazyczewska, M., & McAvoy, J. W. (2004). Deregulation of lens epithelial cell proliferation and differentiation during the development of TGF- β -induced anterior subcapsular cataract. *Dev Neurosci*, 26, 446–455.

Lovicu, F. J., McAvoy, J. W., & De Iongh, R. U. (2011). Understanding the role of growth factors in embryonic development: insights from the lens. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 366(1568), 1204-1218.

Lovicu, F. J., McAvoy, J. W., & Tsonis, P. A. (2008). Epithelial explants and their application to study developmental processes in the lens. *Animal Models in Eye Research*, 10, 134-147.

Lovicu, F.J. & McAvoy, J.W., 2001a. FGF-induced lens cell proliferation and differentiation is dependent on MAPK (ERK1/2) signalling. *Development*, 128(24), pp.5075–5084.

Lovicu, F.J. et al., 2014. Lens epithelial cell proliferation. In *Lens Epithelium and Posterior Capsular Opacification*. Tokyo: Springer Japan, pp. 59–80.

Lovicu, F.J., McAvoy, J.W., 1993. localisation of acidic fibroblast growth factor, basic fibroblast growth factor, and heparan sulphate proteoglycan in rat lens: Implications for lens polarity and growth patterns. *Investigative Ophthalmology & Visual Science* 34, 3355–3365.

Lugemwa, F. N., & Esko, J. D. (1991). Estradiol beta-D-xyloside, an efficient primer for heparan sulfate biosynthesis. *Journal of Biological Chemistry*, 266(11), 6674-6677.

Lunde, I. G., Herum, K. M., Carlson, C. C., & Christensen, G. (2016). Syndecans in heart fibrosis. *Cell and tissue research*, 365, 539-552.

Lyon, M., Rushton, G. and Gallagher, J.T., 1997. The interaction of the transforming growth factor- β s with heparin/heparan sulfate is isoform-specific. *J Biol Chem*, 272, 18000-18006.

Lyu, J., Joo, C.K., 2004. Wnt signalling enhances FGF2-triggered lens fibre cell differentiation. *Development* 131, 1813–1824.

Ma, B., Jing, R., Liu, J., Qi, T. and Pei, C., 2019. Gremlin is a potential target for posterior capsular opacification. *Cell Cycle*, 18, 1714-1726.

Ma, C., Moses, K., 1995. Wingless and patched are negative regulators of the morphogenetic furrow and can affect tissue polarity in the developing *Drosophila* compound eye. *Development* 121, 2279–2289.

Maccarana, M., Sakura, Y., Tawada, A., Yoshida, K., Lindahl, U., 1996. Domain structure of heparan sulfates from bovine organs. *Journal of Biological Chemistry* 271, 17804–17810.

Maday, S. et al., 2008. A PDZ-binding motif controls basolateral targeting of syndecan-1 along the biosynthetic pathway in polarized epithelial cells. *Traffic (Copenhagen, Denmark)*, 9(11), pp.1915–1924.

Maddala, R. et al., 2003. Growth factor induced activation of Rho and Rac GTPases and actin cytoskeletal reorganisation in human lens epithelial cells. *Molecular Vision*, 9(46), pp.329–336.

Maeda, N., Ishii, M., Nishimura, K., Kamimura, K., 2011. Functions of chondroitin sulfate and heparan sulfate in the developing brain. *Neurochemical Research* 36, 1228–1240.

Maher, P.A., 1996. Nuclear Translocation of fibroblast growth factor (FGF) receptors in response to FGF-2. *The Journal of Cell Biology* 134, 529–536.

Majima, K., 1995. Human lens epithelial cells proliferate in response to exogenous EGF and have EGF and EGF receptor. *Ophthalmic Res.* 27, 356–365.

Mamuya, F. A., Wang, Y., Roop, V. H., Scheiblin, D. A., Zajac, J. C., & Duncan, M. K. (2014). The roles of α V integrins in lens EMT and posterior capsular opacification. *Journal of cellular and molecular medicine*, 18(4), 656-670.

Mann, S., Kröger, S., 1996. Agrin is synthesized by retinal cells and colocalises with gephyrin. *Molecular and Cellular Neuroscience*. 8, 1–13.

Marneros, A. G., Keene, D. R., Hansen, U., Fukai, N., Moulton, K., Goletz, P. L., Moiseyev, G., Pawlyk, B. S., Halfter, W., Dong, S., Shibata, M., Li, T., Crouch, R. K., Bruckner, P., & Olsen, B. R. (2004). Collagen XVIII/endostatin is essential for vision and retinal pigment epithelial function. *The EMBO Journal*, 23, 89–99.

Marneros, A.G., Olsen, B.R., 2003. Age-Dependent Iris Abnormalities in Collagen XVIII/Endostatin Deficient Mice with Similarities to Human Pigment Dispersion Syndrome. *Investigative Ophthalmology & Visual Science* 44, 2367–2372.

Martín, C., Alonso Revuelta, M. I., Lamus Molina, J. F., Moro Balbás, J. A., Mano Bonín, A. D. L., Fernández Gómez, J. M. F., & Gato Casado, Á. L. (2017). Lens capsule HSPG-Perlecan regulates lens fibre differentiation during chick embryo development. *Open Journal of Veterinary Medicine*, 7(2), 9-22.

Matsuo I, Kimura-Yoshida C. Extracellular distribution of diffusible growth factors controlled by heparan sulfate proteoglycans during mammalian embryogenesis. *Philosophical Transactions: Biological Sciences*. 2014;369(1657):1-9. doi:10.2307/24501891.

Matsuo, I., Kimura-Yoshida, C., 2014. Extracellular distribution of diffusible growth factors controlled by heparan sulfate proteoglycans during mammalian embryogenesis. *Philosophical Transactions: Biological Sciences* 369, 1–9.

McAvoy JW. Binding of FGF-1 and FGF-2 to heparan sulphate proteoglycans of the mammalian lens capsule. *Growth Factors*. 1997;14(1):1-13. doi:10.3109/08977199709021506.

McAvoy, J. W., & Chamberlain, C. G. (1989). Fibroblast growth factor (FGF) induces different responses in lens epithelial cells depending on its concentration. *Development*, 107(2), 221-228.

McAvoy, J. W., Chamberlain, C. G., De Iongh, R. U., Richardson, N. A., & Lovicu, F. J. (1991). The role of fibroblast growth factor in eye lens development. *Annals of the New York Academy of Sciences*, 638, 256-274.

McAvoy, J. W., Chamberlain, C. G., de Longh, R. U., Hales, A. M., & Lovicu, F. J. (1999). Lens development. *Eye (Lond)*, 13, 425–437.

Mertens, G. et al., 1996. Heparan sulfate expression in polarized epithelial cells: the apical sorting of glypican (GPI-anchored proteoglycan) is inversely related to its heparan sulfate content. *The Journal of Cell Biology*, 132(3), pp.487–497.

Midorikawa, Y., Ishikawa, S., Iwanari, H., Imamura, T., Sakamoto, H., Miyazono, K., ... & Aburatani, H. (2003). Glypican-3, overexpressed in hepatocellular carcinoma, modulates FGF2 and BMP-7 signalling. *International journal of cancer*, 103(4), 455-465.

Midwood, K. S., Valenick, L. V., Hsia, H. C., & Schwarzbauer, J. E. (2004). Coregulation of fibronectin signalling and matrix contraction by tenascin-C and syndecan-4. *Molecular biology of the cell*, 15(12), 5670-5677.

Mii, Y., & Takada, S. (2020). Heparan Sulfate Proteoglycan Clustering in Wnt signalling and Dispersal. *Front. Cell Dev. Biol.*, 8, 631.

Miyake, M. et al., 2014. Clinical implications in the shift of syndecan-1 expression from the cell membrane to the cytoplasm in bladder cancer. *BMC Cancer*, 14(1), pp.885–7.

Mochizuki, T. & Masai, I., 2014. The lens equator: A platform for molecular machinery that regulates the switch from cell proliferation to differentiation in the vertebrate lens. *Development Growth & Differentiation*, 56(5), pp.387–401.

Mochizuki, T., Suzuki, S., Masai, I., 2014. Spatial pattern of cell geometry and cell-division orientation in zebrafish lens epithelium. *Biol Open* 3, 982–994.

Mohan, P. S., & Spiro, R. G. (1991). Characterization of heparan sulfate proteoglycan from calf lens capsule and proteoglycans synthesized by cultured lens epithelial cells. Comparison with other basement membrane proteoglycans. *Journal of Biological Chemistry*, 266, 8567–8575.

Moraescu, D., West-Mays, J. A., & Sheardown, H. D. (2010). Effect of delivery of MMP inhibitors from PDMS as a model IOL material on PCO markers. *Biomaterials*, 31(8), 2399-2407.

Morgan, M.R., Humphries, M.J., Bass, M.D., 2007. Synergistic control of cell adhesion by integrins and syndecans. *Nat Rev Mol Cell Biol* 8, 957–969.

Mu, H., Ohta, K., Kuriyama, S., Shimada, N., Tanihara, H., Yasuda, K., Tanaka, H., 2003. Equarin, a novel soluble molecule expressed with polarity at chick embryonic lens equator, is involved in eye formation. *Mech. Dev.* 120, 143–155.

Mundlos, S., Mortier, G., Bahlo, M., Allen, N. J., Lockhart, P. J. (2019). Pathogenic Variants in GPC4 Cause Keipert Syndrome. *The American Journal of Human Genetics*, 104, 914–924.

Muragaki, Y., Timmons, S., Griffith, C.M., Oh, S.P., Fadel, B., Quertermous, T., Olsen, B.R., 1995. Mouse Col18a1 is expressed in a tissue-specific manner as three alternative variants and is localised in basement membrane zones. *PNAS* 92, 8763–8767.

Murugan, S., & Cheng, C. (2022). Roles of eph-ephrin signalling in the eye lens cataractogenesis, biomechanics, and homeostasis. *Frontiers in Cell and Developmental Biology*, 10, 852236.

Muñoz, R., Moreno, M., Oliva, C., Orbenes, C., Larraín, J., 2006. Syndecan-4 regulates non-canonical Wnt signalling and is essential for convergent and extension movements in *Xenopus* embryos. *Nat Cell Biol* 8, 492–500.

Nagarajan, A., Malvi, P., Wajapeyee, N., 2018. Heparan sulfate and Heparan Sulfate Proteoglycans in cancer initiation and progression. *Front. Endocrinol.* 9, 646.

Nakato, H., Futch, T.A., Selleck, S.B., 1995. The division abnormally delayed (dally) gene: a putative integral membrane proteoglycan required for cell division patterning during postembryonic development of the nervous system in *Drosophila*. *Development* 121, 3687–3702.

Nathu, Z., Dwivedi, D. J., Reddan, J. R., Sheardown, H., Margetts, P. J., & West-Mays, J. A. (2009). Temporal changes in MMP mRNA expression in the lens epithelium during anterior subcapsular cataract formation. *Experimental eye research*, 88(2), 323-330.

Neill, T., Schaefer, L., Iozzo, R.V., 2015. Decoding the Matrix: Instructive Roles of Proteoglycan Receptors. *Biochemistry* 54, 4583–4598.

Neumayer, T., Hirnschall, N., Georgopoulos, M. and Findl, O., 2017. Natural course of posterior subcapsular cataract over a short time period. *Current Eye Research* 42, 1604-1607.

Oh, E., 2004. Syndecans-2 and -4; close cousins, but not identical twins. *Molecules and Cells* 17, 181–187.

Oh, E.S., Woods, A. & Couchman, J.R., 1997. Syndecan-4 proteoglycan regulates the distribution and activity of protein kinase C. *The Journal of biological chemistry*, 272(13), pp.8133–8136.

Ohlmann, A.V., Ohlmann, A., Welge-Lüssen, U., May, C.A., 2012. localisation of Collagen XVIII and Endostatin in the Human Eye. *Current Eye Research* 30, 27–34.

Okamoto, K., Honda, K., Doi, K., Ishizu, T., Katagiri, D., Wada, T., Tomita, K., Ohtake, T., Kaneko, T., Kobayashi, S., Nangaku, M., Tokunaga, K., Noiri, E., 2015. Glypican-5 Increases Susceptibility to Nephrotic Damage in Diabetic Kidney. *The American Journal of Pathology*. 185, 1889–1898.

Olivares, G.H., Carrasco, H., Aroca, F., Carvallo, L., Segovia, F., Larraín, J., 2009. Syndecan-1 regulates BMP signalling and dorso-ventral patterning of the ectoderm during early *Xenopus* development. *Developmental Biology* 329, 338–349.

Olivero, D.K. & Furcht, L.T., 1993. Type IV collagen, laminin, and fibronectin promote the adhesion and migration of rabbit lens epithelial cells in vitro. *Investigative Ophthalmology and Visual Science*, 34(10), pp.2825–2834.

Ong, M. D., Payne, D. M., & Garner, M. H. (2003). Differential protein expression in lens epithelial whole-mounts and lens epithelial cell cultures. *Experimental eye research*, 77(1), 35-49.

Onodera, S., 1991. Presence of the Basement Membrane Component-Heparan Sulfate Proteoglycan-in Bovine Lens Capsules. *Chemical and Pharmaceutical Bulletin*, 39, 1059-1061.

Ornitz, D. M. (2000). FGFs, heparan sulfate and FGFRs: Complex interactions essential for development. *BioEssays*, 22, 108–112.

Palma, V., Carrasco, H., Reinchisi, G., Olivares, G., Faunes, F., Larraín, J., 2011. Shh activity and localisation is regulated by perlecan. *Biological Research* 44, 63–67.

Pan, J., & Ho, M. (2021). Role of glypican-1 in regulating multiple cellular signalling pathways. *American Journal of Physiology-Cell Physiology*, 321, C846–C858.

Pan, Y., Carbe, C., Powers, A., Zhang, E.E., Esko, J.D., Grobe, K., Feng, G.-S., Zhang, X., 2008. Bud specific N-sulfation of heparan sulfate regulates Shp2-dependent FGF signalling during lacrimal gland induction. *Development* 135, 301–310.

Pan, Y., Woodbury, A., Esko, J.D., Grobe, K., Zhang, X., 2006. Heparan sulfate biosynthetic gene *Ndst1* is required for FGF signalling in early lens development. *Development* 133, 4933–4944.

Park, P. W., Reizes, O., & Bernfield, M. (2000). Cell surface heparan sulfate proteoglycans: selective regulators of ligand-receptor encounters. *Journal of Biological Chemistry*, 275(39), 29923-29926.

Park, P.J., Shukla, D., 2013. Role of heparan sulfate in ocular diseases. *Experimental Eye Research* 110, 1–9.

Park, P.W., Reizes, O., Bernfield, M., 2000. Cell surface heparan sulfate proteoglycans: selective regulators of ligand-receptor encounters. *Journal of Biological Chemistry* 275, 29923–29926.

Parmigiani, C. and McAvoy, J., 1984. Localisation of laminin and fibronectin during rat lens morphogenesis. *Differentiation*, 28, 53-61.

Parmigiani, C.M. & McAvoy, J.W., 2009. The roles of laminin and fibronectin in the development of the lens capsule. *Current Eye Research*, 10(6), pp.501–511.

Parmigiani, C.M. and McAvoy, J.W., 1991. The roles of laminin and fibronectin in the development of the lens capsule. *Current eye research*, 10, 501-511.

Partovian, C., Ju, R., Zhuang, Z.W., Martin, K.A., Simons, M., 2008. Syndecan-4 regulates subcellular localisation of mTOR Complex2 and Akt activation in a PKC alpha-dependent manner in endothelial cells. *Mol. Cell* 32, 140–149.

Passos-Bueno, M.R., Suzuki, O.T., Armelin-Correa, L.M., Sertié, A.L., Errera, F.I.V., Bagatini, K., Kok, F., Leite, K.R.M., 2006. Mutations in collagen 18A1 and their relevance to the human phenotype. *An Acad Bras Cienc* 78, 123–131.

Pataki, C., 2015. Wnt signalling Cascades and the Roles of Syndecan Proteoglycans. *Journal of Histochemistry & Cytochemistry* 63, 465–480.

Patel, V.N., Likar, K.M., Zisman-Rozen, S., Cowherd, S.N., Lassiter, K.S., Sher, I., Yates, E.A., Turnbull, J.E., Ron, D., Hoffman, M.P., 2008. Specific heparan sulfate structures modulate FGF10-mediated submandibular gland epithelial morphogenesis and differentiation. *Journal of Biological Chemistry* 283, 9308–9317.

Pathania, M., Wang, Y., Simirskii, V. N., & Duncan, M. K. (2016). $\beta 1$ -integrin controls cell fate specification in early lens development. *Differentiation*, 92, 133–147.

Pathania, M.; Wang, Y.; Simir V. N.; Duncan, M. K. $\beta 1$ -integrin controls cell fate specification in early lens development. *Differentiation* 2016, 92, 133–147.

Peek, R., McAvoy, J.W., Lubsen, N.H., Schoenmakers, J.G.G., 1992. Rise and fall of crystallin gene messenger levels during fibroblast growth factor induced terminal differentiation of lens cells. *Developmental Biology* 152, 152–160.

Pegge, J., Tatsinkam, A. J., Rider, C. C., & Bell, E. (2020). Heparan sulfate proteoglycans regulate BMP signalling during neural crest induction. *Dev. Biol*, 460, 108–114.

Persson, A., Ellervik, U., & Mani, K. (2018). Fine-tuning the structure of glycosaminoglycans in living cells using xylosides. *Glycobiology*, 28(7), 499-511.

Pesudovs, K., Lansingh, V. C., Kempen, J. H., Steinmetz, J. D., Briant, P. S., Varma, R., ... & Bourne, R. R. (2021). Cataract-related blindness and vision impairment in 2020 and trends over time in relation to VISION 2020: the Right to Sight: an analysis for the Global Burden of Disease Study. *Investigative Ophthalmology & Visual Science*, 62(8), 3523-3523.

Peterson, J.A., Sheibani, N., David, G., Garcia-Pardo, A., Peters, D.M., 2005. Heparin II domain of fibronectin uses $\alpha 4 \beta 1$ integrin to control focal adhesion and stress fibre formation, independent of syndecan-4. *Journal of Biological Chemistry* 280, 6915–6922.

Pillai-Kastoori, L., Wen, W., Wilson, S. G., Strachan, E., Lo-Castro, A., Fichera, M., Musumeci, S. A., Lehmann, O. J., & Morris, A. C. (2014). Sox11 Is Required to Maintain Proper Levels of Hedgehog signalling during Vertebrate Ocular Morphogenesis. *PLoS Genetics*, 104491.

Polisetti, N., Zenkel, M., Menzel-Severing, J., Kruse, F.E., Schlötzer-Schrehardt, U., 2016. Cell Adhesion Molecules and Stem Cell-Niche-Interactions in the Limbal Stem Cell Niche. *Stem Cells* 34, 203–219.

Poulain, F.E., Yost, H.J., 2015. Heparan sulfate proteoglycans: a sugar code for vertebrate development? *Development* 142, 3456–3467.

Pratt, T., Conway, C.D., Tian, N.M.-L., Price, D.J., Mason, J.O., 2006. Heparan sulphation patterns generated by specific heparan sulfotransferase enzymes direct distinct aspects of retinal axon guidance at the optic chiasm. *J. Neurosci.* 26, 6911–6923.

Puri, S., Coulson-Thomas, Y.M., Gesteira, T.F., Coulson-Thomas, V.J., 2020. Distribution and Function of Glycosaminoglycans and Proteoglycans in the Development, Homeostasis and Pathology of the Ocular Surface. *Front. Cell Developmental Biology* 8, 731.

Qiao, D., Yang, X., Meyer, K., Friedl, A., 2008. Glypican-1 regulates anaphase promoting complex/cyclosome substrates and cell cycle progression in endothelial cells. *Molecular Biology of the Cell* 19, 2789–2801.

Qin, Y., Zhu, Y., Luo, F., Chen, C., Chen, X., Wu, M., 2017. Killing two birds with one stone: dual blockade of integrin and FGF signalling through targeting syndecan-4 in postoperative capsular opacification. *Cell Death Dis* 8, e2920.

Qu, X., Carbe, C., Tao, C., Powers, A., Lawrence, R., van Kuppevelt, T. H., Cardoso, W. V., Grobe, K., Esko, J. D., & Zhang, X. (2011). Lacrimal gland development and Fgf10-Fgfr2b signalling are controlled by 2-O- and 6-O-sulfated heparan sulfate. *Journal of Biological Chemistry*, 286, 14435–14444.

Qu, X., Pan, Y., Carbe, C., Powers, A., Grobe, K., Zhang, X., 2012.

Glycosaminoglycan-dependent restriction of FGF diffusion is necessary for lacrimal gland development. *Development* 139, 2730–2739.

Qu, X.; Carbe, C.; Tao, C.; Powers, A.; Lawrence, R.; van Kuppevelt, T. H.; Cardoso, W. V.; Grobe, K.; Esko, J. D.; Zhang, X. Lacrimal gland development and Fgf10-Fgfr2b signalling are controlled by 2-O- and 6-O-sulfated heparan sulfate. *Journal of Biological Chemistry* 2011, 286, 14435–14444.

Quarto, N.; Amalric, F. Heparan sulfate proteoglycans as transducers of FGF-2 signalling. *Journal of Cell Science* 1994, 107 (Pt 11), 3201–3212.

Rapraeger, A. C. (2000). Syndecan-Regulated Receptor signalling. *The Journal of Cell Biology*, 149, 995–997.

Rapraeger, A., Jalkanen, M., Bernfield, M., 1986. Cell surface proteoglycan associates with the cytoskeleton at the basolateral cell surface of mouse mammary epithelial cells. *The Journal of Cell Biology* 103, 2683–2696.

Rapraeger, A.C., 2001. Molecular interactions of syndecans during development. *Seminars in Cell & Developmental Biology* 12, 107–116.

Raulinajtys-Grzybek, M., Grabska-Liberek, I., Opala, A., Słomka, M., & Chrobot, M. (2020). Budget impact analysis of lens material on the posterior capsule opacification (PCO) as a complication after the cataract surgery. *Cost Effectiveness and Resource Allocation*, 18, 1-12.

Rehn, M., Pihlajaniemi, T., 1995. Identification of three N-terminal ends of type XVIII collagen chains and tissue-specific differences in the expression of the corresponding transcripts. The longest form contains a novel motif homologous to rat and *Drosophila* frizzled proteins. *Journal of Biological Chemistry* 270, 4705–4711.

Reneker, L.W., Overbeek, P.A., 1996. Lens-Specific Expression of PDGF-A Alters Lens Growth and Development. *Developmental Biology* 180, 554–565.

Richardson, N.A., McAvoy, J.W. & Chamberlain, C.G., 1992. Age of rats affects response of lens epithelial explants to fibroblast growth factor. *Experimental Eye Research*, 55(5), pp.649–656.

Richardson, T. P., Trinkaus-Randall, V., & Nugent, M. A. (2001). Regulation of heparan sulfate proteoglycan nuclear localisation by fibronectin. *Journal of cell science*, 114(9), 1613-1623.

Rider, C.C. and Mulloy, B., 2017. Heparin, heparan sulphate and the TGF- β cytokine superfamily. *Molecules*, 22, 713.

Ringvall, M., Kjellén, L., 2010. Mice Deficient in Heparan Sulfate N-Deacetylase/N-Sulfotransferase 1. *Progress in Molecular Biology and Translational Science* 93, 35–58.

Robinson, M. L. (2006). An essential role for FGF receptor signalling in lens development. *Seminars in Cell & Developmental Biology*, 17, 726–740.

Roper, J. A., Williamson, R. C., & Bass, M. D. (2012). Syndecan and integrin interactomes: large complexes in small spaces. *Current Opinion in Structural Biology*, 22, 583–590.

Rossi M. Heparan sulfate chains of perlecan are indispensable in the lens capsule but not in the kidney. *The EMBO Journal*. 2003;22(2):236-245. doi:10.1093/emboj/cdg019.

Rossi, M. (2003). Heparan sulfate chains of perlecan are indispensable in the lens capsule but not in the kidney. *The EMBO Journal*, 22, 236–245.

Ruiz, X.D., Mlakar, L.R., Yamaguchi, Y., Su, Y., Larregina, A.T., Pilewski, J.M., Feghali-Bostwick, C.A., 2012. Syndecan-2 Is a Novel Target of Insulin-Like Growth Factor Binding Protein-3 and Is Over-Expressed in Fibrosis. *PLoS One* 7, e43049.

Rychkova, N., Stahl, S., Gaetzner, S., Felbor, U., 2005. Non-heparan sulfate-binding interactions of endostatin/collagen XVIII in murine development. *Developmental Dynamics* 232, 399–407.

Rønning, S.B. et al., 2015. Syndecan-4 regulates muscle differentiation and is internalized from the plasma membrane during myogenesis A. Asakura, ed. *PLoS ONE*, 10(6), p.e0129288.

Saarela, J., Rehn, M., Oikarinen, A., Autio-Harmainen, H., Pihlajaniemi, T., 1998. The short and long forms of type XVIII collagen show clear tissue specificities in their expression and location in basement membrane zones in humans. *Am. J. Pathol.* 153, 611–626.

Safaiyan, F., Kolset, S.O., Prydz, K., Gottfridsson, E., Lindahl, U., Salmivirta, M., 1999. Selective effects of sodium chlorate treatment on the sulfation of heparan sulfate. *Journal of Biological Chemistry* 274, 36267–36273.

Saint-Geniez, M., Kurihara, T., D'Amore, P.A., 2009. Role of cell and matrix-bound VEGF isoforms in lens development. *Investigative Ophthalmology & Visual Science* 50, 311–321.

Sakane, H., Yamamoto, H., Matsumoto, S., Sato, A. and Kikuchi, A., 2012. localisation of glypican-4 in different membrane microdomains is involved in the regulation of Wnt signalling. *Journal of cell science*, 125, 449-460.

Salmerón-Sánchez, M., & Dalby, M. J. (2016). Synergistic growth factor microenvironments. *Chemical Communications*, 52(91), 13327-13336.

Saoncella, S., Echtermeyer, F., Denhez, F., Nowlen, J.K., Mosher, D.F., Robinson, S.D., Hynes, R.O. and Goetinck, P.F., 1999. Syndecan-4 signals cooperatively with integrins in a Rhoddependent manner in the assembly of focal adhesions and actin stress fibres. *Proceedings of the National Academy of Sciences*, 96, 2805-2810.

Saravanan, C., Cao, Z., Head, S.R., Panjwani, N., 2019. Analysis of differential expression of glycosyltransferases in healing corneas by glycogene microarrays. *Glycobiology* 29, 188–189.

Sarrazin, S., Lamanna, W. C., & Esko, J. D. (2011). Heparan sulfate proteoglycans. *Cold Spring Harbour Perspective Biology*, 3, 1–33.

Schaefer, L., 2014. Proteoglycans, key regulators of cell-matrix dynamics. *Matrix biology : journal of the International Society for Matrix Biology*, 35, pp.1–2.

Schrage, Y.M., Hameetman, L., Szuhai, K., Cleton-Jansen, A.-M., Taminiau, A.H.M., Hogendoorn, P.C.W., Bovée, J.V.M.G., 2009. Aberrant heparan sulfate proteoglycan localisation, despite normal exostosin, in central chondrosarcoma. *Am. J. Pathol.* 174, 979–988.

Schröder, E., Gebel, L., Ereemeev, A.A., Morgner, J., Grum, D., Knauer, S.K., Bayer, P., Mueller, J.W., 2012. Human PAPS Synthase Isoforms Are Dynamically Regulated Enzymes with Access to Nucleus and Cytoplasm. *PLoS One* 7, e29559.

Schuksz, M., Fuster, M. M., Brown, J. R., Crawford, B. E., Ditto, D. P., Lawrence, R., & Esko, J. D. (2008). Surfen, a small molecule antagonist of heparan sulfate. *Proceedings of the National Academy of Sciences*, 105(35), 13075-13080.

Schulz, M.W. et al., 1993. Acidic and basic FGF in ocular media and lens: implications for lens polarity and growth patterns. *Development*, 118(1), pp.117–126.

Schulz, M.W., Chamberlain C.G., McAvoy, J.W., 1997. Binding of FGF-1 and FGF-2 to heparan sulphate proteoglycans of the mammalian lens capsule. *Growth Factors* 14, 1–13.

Seidel, C., Børset, M., Hjertner, Ø., Cao, D., Abildgaard, N., Hjorth-Hansen, H., Sanderson, R.D., Waage, A., Sundan, A., 2000. High levels of soluble syndecan-1 in myeloma-derived bone marrow: modulation of hepatocyte growth factor activity. *Blood* 96, 3139–3146.

Seppinen, L.; Pihlajaniemi, T. The multiple functions of collagen XVIII in development and disease. *Matrix Biology* 2011, 30, 83–92.64.

Shi, W., Kaneiwa, T., Cydzik, M., Gariepy, J., Filmus, J., 2020. Glypican-6 stimulates intestinal elongation by simultaneously regulating Hedgehog and non-canonical Wnt signalling. *Matrix Biology* 88, 19–32.

Shi, Y., De Maria, A., Lubura, S., Šikić, H., Bassnett, S., 2014. The penny pusher: A cellular model of lens growth. *Investigative Ophthalmology & Visual Science* 56, 799–809.

Shiau, C.E., Hu, N., Bronner-Fraser, M., 2010. Altering Glypican-1 levels modulates canonical Wnt signalling during trigeminal placode development. *Developmental Biology* 348, 107–118.

Shigemitsu, T., Majima, Y., & Shimizu, Y. (1998). Immunohistochemical studies on factors involved in after cataract. *Nippon Ganka Gakkai Zasshi*, 102(8), 531-539.

Shihan, M. H., Kanwar, M., Wang, Y., Jackson, E. E., Faranda, A. P., & Duncan, M. K. (2020). Fibronectin has multifunctional roles in posterior capsular opacification (PCO). *Matrix Biology*, 90, 79-108.

Shimokawa, K., Kimura-Yoshida, C., Nagai, N., Mukai, K., Matsubara, K., Watanabe, H., Matsuda, Y., Mochida, K., Matsuo, I., 2011. Cell Surface Heparan Sulfate Chains Regulate Local Reception of FGF signalling in the Mouse Embryo. *Dev. Cell* 21, 257–272.

Shirai K, Kitano-Izutani A, Miyamoto T, Tanaka S Saika S. 2014. Wound healing and epithelial-mesenchymal transition in the lens epithelium: roles of growth factors and extracellular matrix Lens epithelium and posterior capsular opacification. New York: Springer, Inc, p 159–174.

Shirai K, Saika S, Tanaka T, Okada Y, Flanders KC, Ooshima A, Ohnishi Y. 2006. A new model of anterior subcapsular cataract: involvement of TGF- β /SMAD signalling. *Mol Vis* 12:681–691.

Shirai, K., Tanaka, S. I., Lovicu, F. J., & Saika, S. (2018). The murine lens: A model to investigate in vivo epithelial–mesenchymal transition. *Developmental Dynamics*, 247(3), 340-345.

Shirke S, Faber SC, Hallem E, et al. Misexpression of IGF-I in the mouse lens expands the transitional zone and perturbs lens polarization. *Mech Dev*. 2001;101(1-2):167-174.
doi:10.1016/S0925-4773(00)00584-0.

Shu, D. Y., Wojciechowski, M. C., & Lovicu, F. J. (2017). Bone morphogenetic protein-7 suppresses TGF- β 2-induced epithelial-mesenchymal transition in the lens: implications for cataract prevention. *Investigative Ophthalmology & Visual Science*, 58(2), 781-796.

Shu, D.Y., Lovicu, F.J., 2017. Myofibroblast transdifferentiation: The dark force in ocular wound healing and fibrosis. *Progress in Retinal and Eye Research* 60, 44–65.

Shworak, N.W., 2011. Modulation of Growth Factor signalling by Heparan Sulfate Proteoglycans. In Modern Concepts in Angiogenesis. PUBLISHED BY IMPERIAL COLLEGE PRESS AND DISTRIBUTED BY WORLD SCIENTIFIC PUBLISHING CO., pp. 119–146.

Simirskii, V.N., Duncan, M.K., Paltsev, M.A., Suchkov, S.V., 2013. β 1 integrin as the integrating component in cell-cell cooperation for maintenance of lens transparency. Doklady Biochemistry and Biophysics 453, 297–299.

Simirskii, V.N., Wang, Y. and Duncan, M.K., 2007. Conditional deletion of β 1-integrin from the developing lens leads to loss of the lens epithelial phenotype. Developmental biology, 306, 658-668.

Smetsers, T. F. C. M., van de Westerlo, E. M. A., Dam, Ten, G. B., Clarijs, R., Versteeg, E. M. M., van Geloof, W. L., Veerkamp, J. H., van Muijen, G. N. P., & van Kuppevelt, T. H. (2003). localisation and characterization of melanoma-associated glycosaminoglycans: differential expression of chondroitin and heparan sulfate epitopes in melanoma. Cancer Research, 63, 2965–2970.

Smith, S.M.-L. et al., 2007. Heparan and chondroitin sulfate on growth plate perlecan mediate binding and delivery of FGF-2 to FGF receptors. Matrix biology : journal of the International Society for Matrix Biology, 26(3), pp.175–184

Smits, N.C., Kobayashi, T., Srivastava, P.K., Skopelja, S., Ivy, J.A., Elwood, D.J., Stan, R.V., Tsongalis, G.J., Sellke, F.W., Gross, P.L. and Cole, M.D., 2017. HS3ST1 genotype regulates antithrombin's inflammomodulatory tone and associates with atherosclerosis. Matrix Biol, 63, 69-90.

Song, H.H., Shi, W., Xiang, Y.-Y., Filmus, J., 2005. The loss of glypican-3 induces alterations in Wnt signalling. *Journal of Biological Chemistry* 280, 2116–2125.

Song, X., Sato, Y., Felemban, A., Ito, A., Hossain, M., Ochiai, H., Yamamoto, T., Sekiguchi, K., Tanaka, H., Ohta, K., 2012. Equarin is involved as an FGF signalling modulator in chick lens differentiation. *Developmental Biology* 368, 109–117.

Song, X., Sato, Y., Sekiguchi, K., Tanaka, H., Ohta, K., 2013. Equarin is involved in cell adhesion by means of heparan sulfate proteoglycan during lens development. *Developmental Dynamics* 242, 23–29.

Soulintzi, N., & Zagris, N. (2007). Spatial and temporal expression of perlecan in the early chick embryo. *Cells Tissues Organs*, 186, 243–256.

Souza, A. T. P., Lopes, H. B., Oliveira, F. S., Weffort, D., Freitas, G. P., Adolpho, L. F., Fernandes, R. R., Rosa, A. L., & Beloti, M. M. (2021). The extracellular matrix protein Agrin is expressed by osteoblasts and contributes to their differentiation. *Cell Tissue Research*, 386, 335–347.

Stepp, M.A., Daley, W.P., Bernstein, A.M., Pal-Ghosh, S., Tadvalkar, G., Shashurin, A., Palsen, S., Jurjus, R.A., Larsen, M., 2010. Syndecan-1 regulates cell migration and fibronectin fibril assembly. *Experimental Cell Research* 316, 2322–2339.

Stepp, M.A., Gibson, H.E., Gala, P.H., Iglesia, D.D.S., Pajooohesh-Ganji, A., Pal-Ghosh, S., Brown, M., Aquino, C., Schwartz, A.M., Goldberger, O., Hinkes, M.T., Bernfield, M., 2002. Defects in keratinocyte activation during wound healing in the syndecan-1-deficient mouse. *J. Cell. Sci.* 115, 4517–4531.

Stewart, M. D., & Sanderson, R. D. (2014). Heparan sulfate in the nucleus and its control of cellular functions. *Matrix Biology*, 35, 56–59.

Stigliano, I. et al., 2009. Glypican-3 regulates migration, adhesion and actin cytoskeleton organisation in mammary tumor cells through Wnt signalling modulation. *Breast Cancer Research and Treatment*, 114(2), pp.251–262.

Strate, I., Tessadori, F., & Bakkers, J. (2015). Glypican4nt and Bmp signalling. *Development*, 142, 1767–1776.

Stum, M., Davoine, C.S., Vicart, S., Guillot-Noël, L., Topaloglu, H., Carod-Artal, F.J., Kayserili, H., Hentati, F., Merlini, L., Urtizberea, J.A. and Hammouda, E.H., 2006. Spectrum of HSPG2 (Perlecan) mutations in patients with Schwartz-Jampel syndrome. *Human mutation*, 27, 1082-1091.

Stump, R. J., Ang, S., Chen, Y., von Bahr, T., Lovicu, F. J., Pinson, K., ... & McAvoy, J. W. (2003). A role for Wnt/ β -catenin signalling in lens epithelial differentiation. *Developmental biology*, 259(1), 48-61.

Sugiyama, Y.; Lovicu, F. J.; McAvoy, J. W. Planar cell polarity in the mammalian eye lens. *Organogenesis* 2011, 7, 191–201.

Sulochana, K. N., Ramakrishnan, S., Vasanthi, S. B., Madhavan, H. N., Arunagiri, K., & Punitham, R. (1997). First report of congenital or infantile cataract in deranged proteoglycan metabolism with released xylose. *British journal of ophthalmology*, 81(4), 319-323.

Sun, C., Liu, M., Sun, P., Yang, M., Yates, E.A., Guo, Z., Fernig, D.G., 2019. Sulfated polysaccharides interact with fibroblast growth factors and protect from denaturation. *FEBS Open Bio* 9, 1477–1487.

Szatmári, T. et al., 2017. Molecular targets and signalling pathways regulated by nuclear translocation of syndecan-1. *BMC cell biology*, 18(1), pp.34–20.

Taiyab, A., & West-Mays, J. (2022). Lens Fibrosis: Understanding the Dynamics of Cell Adhesion signalling in Lens Epithelial-Mesenchymal Transition. *Frontiers in Cell and Developmental Biology*, 10, 886053.

Taketo, M., Schroeder, A. C., Mobraaten, L. E., Gunning, K. B., Hanten, G., Fox, R. R., ... & Hansen, C. T. (1991). FVB/N: an inbred mouse strain preferable for transgenic analyses. *Proceedings of the National Academy of Sciences*, 88(6), 2065-2069.

Tao, C., Zhang, X., 2016. Retinal Proteoglycans Act as Cellular Receptors for Basement Membrane Assembly to Control Astrocyte Migration and Angiogenesis. *Cell Reports* 17, 1832–1844.

Tholozan, F.M., Gribbon, C., Li, Z., Goldberg, M.W., Prescott, A.R., McKie, N. and Quinlan, R.A., 2007. FGF-2 release from the lens capsule by MMP-2 maintains lens epithelial cell viability. *Molecular Biology of the Cell*, 18, 4222-4231.

Toler, T. L., Sutton, V. R., Agolini, E., Rinelli, M., Capolino, R., Martinelli, D., Zampino, G., Dumić, M., Reardon, W., Shaw-Smith, C., Leventer, R. J., Delatycki, M. B., Kleefstra, T.,

Tian, F., 2014. Rapamycin-induced apoptosis in HGF-stimulated lens epithelial cells by AKT/mTOR, ERK and JAK2/STAT3 pathways. *Int J Mol Sci* 15, 13833–13848.

Tillo, M., Charoy, C., Schwarz, Q., Maden, C.H., Davidson, K., Fantin, A., Ruhrberg, C., 2016. 2-and 6-O-sulfated proteoglycans have distinct and complementary roles in cranial axon guidance and motor neuron migration. *Development* 143, 1907–1913.

Tsen, G., Halfter, W., Kroger, S., Cole, G.J., 1995. Agrin is a heparan sulfate proteoglycan. *Journal of Biological Chemistry* 270, 3392–3399.

Tsonis, P. A.; Vergara, M. N.; Spence, J. R.; Madhavan, M.; Kramer, E. L.; Call, M. K.; Santiago, W. G.; Vallance, J. E.; Robbins, D. J.; Del Rio-Tsonis, K. A novel role of the hedgehog pathway in lens regeneration. *Developmental Biology*. 2004, 267, 450–461.

Tsoyi, K., Chu, S. G., Patino-Jaramillo, N. G., Wilder, J., Villalba, J., Doyle-Eisele, M., McDonald, J., Liu, X., El-Chemaly, S., Perrella, M. A., & Rosas, I. O. (2018). Syndecan-2 attenuates radiation-induced pulmonary fibrosis and inhibits fibroblast activation by regulating

PI3K/AKT/ROCK pathway via CD148. *American Journal of Physiology-Cell Physiology*, 58, 208–215.

Tumova, S., Woods, A. & Couchman, J.R., 2000. Heparan sulfate chains from glypican and syndecans bind the Hep II domain of fibronectin similarly despite minor structural differences. *Journal of Biological Chemistry*, 275(13), pp.9410–9417.

Udomsinprasert, W., Angkathunyakul, N., Klaikeaw, N., Vejchapipat, P., Poovorawan, Y., & Honsawek, S. (2020). Hepatic glypican-3 and alpha-smooth muscle actin overexpressions reflect severity of liver fibrosis and predict outcome after successful portoenterostomy in biliary atresia. *Surgery*, 167(3), 560-568.

Uechi, G. et al., 2014. Proteomic View of Basement Membranes from Human Retinal Blood Vessels, Inner Limiting Membranes, and Lens Capsules. *Journal of proteome research*, 13(8), pp.3693–3705.

Ursell, P. G., Dhariwal, M., Majirska, K., Ender, F., Kalson-Ray, S., Venerus, A., ... & Bouchet, C. (2018). Three-year incidence of Nd: YAG capsulotomy and posterior capsule opacification and its relationship to monofocal acrylic IOL biomaterial: a UK Real World Evidence study. *Eye*, 32(10), 1579-1589.

Utani, A., 2003. The Laminin $\alpha 3$ LG4 Domain Promotes Cell Adhesion through Syndecans. *Connective Tissue*, 35(4), pp.187–192.

Vallet, S. D., Clerc, O., & Ricard-Blum, S. (2020). Glycosaminoglycan-Protein Interactions: The First Draft of the Glycosaminoglycan Interactome. *Journal of Histochemistry and Cytochemistry*, 21, 22155420946403.

Vallet, S.D., Clerc, O., Ricard-Blum, S., 2021. Glycosaminoglycan-Protein Interactions: The First Draft of the Glycosaminoglycan Interactome. *J. Histochem. Cytochem.* 69, 93-104.

Van Den Born J, Salmivirta K, Henttinen T, et al. Novel heparan sulfate structures revealed by monoclonal antibodies. *J Biol Chem.* 2005;280(21):20516-20523. doi:10.1074/jbc.M502065200.

Van Den Born, J. et al., 2005. Novel heparan sulfate structures revealed by monoclonal antibodies. *The Journal of biological chemistry*, 280(21), pp.20516–20523.

VanSlyke, J.K., Boswell, B.A., Musil, L.S., 2018. Fibronectin regulates growth factor signalling and cell differentiation in primary lens cells. *J Cell Science*, 131, 22.

Waghmare, I., Wang, X., Page-McCaw, A., 2020. Dally-like protein sequesters multiple Wnt ligands in the *Drosophila* gerarium. *Developmental Biology* 464, 88–102.

Walker, J. & Menko, A.S., 2009. Integrins in lens development and disease. *Experimental Eye Research*, 88(2), pp.216–225.

Walker, J. L., Wolff, I. M., Zhang, L., & Menko, A. S. (2007). Activation of SRC kinases signals induction of posterior capsule opacification. *Investigative ophthalmology & visual science*, 48(5), 2214-2223.

Wang, Q. et al., 2009. MAPK/ERK1/2 and PI3-kinase signalling pathways are required for vitreous-induced lens fibre cell differentiation. *Experimental Eye Research*, 88(2), pp.293–306.

Wang, Q., McAvoy, J. W., & Lovicu, F. J. (2010). Growth factor signalling in vitreous humour–induced lens fibre differentiation. *Investigative ophthalmology & visual science*, 51(7), 3599-3610.

Wang, Q., Stump, R., McAvoy, J.W., Lovicu, F.J., 2009. MAPK/ERK1/2 and PI3-kinase signalling pathways are required for vitreous-induced lens fibre cell differentiation. *Experimental Eye Research* 88, 293–306.

Wang, S., Qiu, M., Xia, W., Xu, Y., Mao, Q., Wang, J., Dong, G., Xu, L., Yang, X., Yin, R., 2016. Glypican-5 suppresses Epithelial-Mesenchymal Transition of the lung adenocarcinoma by competitively binding to Wnt3a. *Oncotarget* 7, 79736–79746.

Wawersik, S., Purcell, P., Rauchman, M., Dudley, A.T., Robertson, E.J., Maas, R., 1999. BMP7 acts in murine lens placode development. *Developmental Biology* 207, 176–188.

Weber, G.F., Menko, A.S., 2006. Actin filament organisation regulates the induction of lens cell differentiation and survival. *Developmental Biology* 295, 714–729.

Webster Jr, E.H., Searls, R.L., Hilfer, S.R. and Zwaan, J., 1987. Accumulation and distribution of sulfated materials in the maturing mouse lens capsule. *The Anatomical Record*, 218, 329-337.

Wederell, E. D., & De Iongh, R. U. (2006). Extracellular matrix and integrin signalling in lens development and cataract. *Seminars in Cell & Developmental Biology*, 17, 759–776.

Wen, C., Wang, C., Hu, C., Qi, T., Jing, R., Wang, Y., ... & Pei, C. (2022). REPS2 downregulation facilitates FGF-induced adhesion and migration in human lens epithelial cells through FAK/Cdc42 signalling and contributes to posterior capsule opacification. *Cellular Signalling*, 97, 110378.

West-Mays, J. A., Pino, G., & Lovicu, F. J. (2010). Development and use of the lens epithelial explant system to study lens differentiation and cataractogenesis. *Progress in retinal and eye research*, 29(2), 135-143.

Whitelock, J. M., Melrose, J., & Iozzo, R. V. (2008). Diverse cell signalling events modulated by perlecan. *Biochemistry*, 47(43), 11174-11183.

Wilcox-Adelman, S.A., Denhez, F. and Goetinck, P.F., 2002. Syndecan-4 modulates focal adhesion kinase phosphorylation. *Journal of Biological Chemistry*, 277, 32970-32977.

Wiley, L. A., Shui, Y. B., & Beebe, D. C. (2010). Visualizing lens epithelial cell proliferation in whole lenses. *Molecular vision*, 16, 1253.

Williams, E.H., Pappano, W.N., Saunders, A.M., Kim, M.-S., Leahy, D.J., Beachy, P.A., 2010. Dally-like core protein and its mammalian homologues mediate stimulatory and inhibitory effects on Hedgehog signal response. *Proc. Natl. Acad. Sci. U.S.A.* 107, 5869–5874.

Wilson, S. E. (2022). Defective perlecan-associated basement membrane regeneration and altered modulation of transforming growth factor beta in corneal fibrosis. *Cellular and Molecular Life Sciences*, 79(3), 144.

Winkler, J., Wirbelauer, C., Frank, V. and Laqua, H., 2001. Quantitative distribution of glycosaminoglycans in young and senile (cataractous) anterior lens capsules. *Experimental Eye Research*, 72, 311-318.

Winzen, U., Cole, G. J., & Halfter, W. (2003). Agrin is a chimeric proteoglycan with the attachment sites for heparan sulfate/chondroitin sulfate located in two multiple serine-glycine clusters. *Journal of Biological Chemistry*, 278, 30106–30114.

Wishart, T. F. L., & Lovicu, F. J. (2021). An atlas of heparan sulfate proteoglycans in the postnatal rat lens. *Investigative Ophthalmology. Vis. Sci*, 62, 5–5.

Wishart, T. F. L., & Lovicu, F. J. (2023). Heparan sulfate proteoglycans (HSPGs) of the ocular lens. *Progress in Retinal and Eye Research*, 93, 101118.

Wishart, T. F., & Lovicu, F. J. (2023). Spatiotemporal Localisation of Heparan Sulphate Proteoglycans throughout Mouse Lens Morphogenesis. *Cells*, 12(10), 1364.

Wistow, G., Bernstein, S.L., Wyatt, M.K., Behal, A., Touchman, J.W., Bouffard, G., Smith, D., Peterson, K., 2002. Expressed sequence tag analysis of adult human lens for the NEIBank

Project: over 2000 non-redundant transcripts, novel genes and splice variants. *Molecular Vision* 8, 171–184.

Won, J.-H., Tsogtbaatar, O., Tsogtbartarr, O., Son, W., Singh, A., Choi, K.-W., Cho, K.-O., 2015. Cell type-specific responses to wingless, hedgehog and decapentaplegic are essential for patterning early eye-antenna disc in *Drosophila*. *PLoS One* 10, e0121999.

Wong, K. O.; Wong, K. P. Direct measurement and regulation of 3“-phosphoadenosine 5-”phosphosulfate (PAPS) generation in vitro. *Biochemical Pharmacology* 1996, 52, 1187–1194.

Wormstone IM, Wang L, Liu CSC. Posterior capsule opacification. *Exp Eye Res.* 2009, 88: 257–69.

Wormstone, I. M., & Eldred, J. A. (2016). Experimental models for posterior capsule opacification research. *Experimental eye research*, 142, 2-12.

Wormstone, I. M., & Wride, M. A. (2011). The ocular lens: a classic model for development, physiology and disease. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 366(1568), 1190-1192.

Wormstone, I.M., 2002. Posterior capsule opacification: a cell biological perspective. *Experimental Eye Research* 74, 337–347.

Wride, M. A. (2011). Lens fibre cell differentiation and organelle loss: many paths lead to clarity. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 366(1568), 1219-1233.

Wu, W. et al., 2014. A gradient of matrix-bound FGF-2 and perlecan is available to lens epithelial cells. *Experimental Eye Research*, 120, pp.10–14.

Xian, X., 2010. Syndecans as receptors and organizers of the extracellular matrix. *Cell Tissue Res.* 339, 31–46.

Yan, D., Lin, X., 2009. Shaping Morphogen Gradients by Proteoglycans. *Cold Spring Harb Perspect Biol* 1, a002493–a002493.

Yang, N. (2016). Syndecan-1-induced ECM fibre alignment requires integrin $\alpha\text{v}\beta 3$ and syndecan-1 ectodomain and heparan sulfate chains. *PLOS ONE*, 11, e0150132.

Ylikärppä, R., Eklund, L., Sormunen, R., Kontiola, A.I., Utriainen, A., Määttä, M., Fukai, N., Olsen, B.R., Pihlajaniemi, T., 2003. Lack of type XVIII collagen results in anterior ocular defects. *The FASEB Journal* 17, 2257–2259.

Yoneda, A., Couchman, J.R., 2003. Regulation of cytoskeletal organisation by syndecan transmembrane proteoglycans. *Matrix Biology* 22, 25–33.

Young, R.W., Ocumpaugh, D.E., 1966. Autoradiographic studies on the growth and development of the lens capsule in the rat. *Investigative Ophthalmology* 5, 583–589.

Yu, P., Pearson, C.S., Geller, H.M., 2018. Flexible Roles for Proteoglycan Sulfation and Receptor signalling. *Trends in Neurosciences* 41, 47–61.

Zelenka, P. S. (2004). Regulation of cell adhesion and migration in lens development. *International Journal of Developmental Biology*, 48, 857–865.

Zelenka, P. S., & Arpitha, P. (2008). Coordinating cell proliferation and migration in the lens and cornea. In *Seminars in cell & developmental biology* (Vol. 19, No. 2, pp. 113-124). Academic Press.

Zhang Y, Li J, Partovian C, Sellke FW, Simons M. Syndecan-4 modulates basic fibroblast growth factor 2 signalling in vivo. *American Journal of Physiology - Heart and Circulatory Physiology*. 2003;284(6 53-6):H2078-H2082.

Zhang, X., Wang, F., Sheng, J., 2016b. “Coding” and ‘Decoding’: hypothesis for the regulatory mechanism involved in heparan sulfate biosynthesis. *Carbohydr. Res.* 428, 1–7.

Zhang, Y. et al., 2003. Syndecan-4 modulates basic fibroblast growth factor 2 signalling in vivo. *American Journal of Physiology - Heart and Circulatory Physiology*, 284(6 53-6), pp.H2078–H2082.

Zhang, Y., Fan, J., Ho, J.W.K., Hu, T., Kneeland, S.C., Fan, X., Xi, Q., Sellarole, M.A., de Vries, W.N., Lu, W., Lachke, S.A., Lang, R.A., John, S.W.M., Maas, R.L., 2016a. Crim1 regulates integrin signalling in murine lens development. *Development* 143, 356–366.

Zhang, Y., Ryan, D.S., Bower, K.S., Ilan, N., Vlodavsky, I. and Laurie, G.W., 2010. Focus on molecules: heparanase. *Experimental Eye Research*, 91, 476-477.

Zhang, Y., You, J., Ren, W., Lin, X., 2013. *Drosophila* glypicans Dally and Dally-like are essential regulators for JAK/STAT signalling and Unpaired distribution in eye development. *Developmental Biology* 375, 23–32.

Zhao G, Bailey CG, Feng Y, Rasko J, Lovicu FJ. Negative regulation of lens fibre cell differentiation by RTK antagonists Spry and Spred. *Experimental Eye Research*. 2018;170:148-159. doi:10.1016/j.exer.2018.02.025.

Zhao Y, Zheng D, Cvekl A. A comprehensive spatial-temporal transcriptomic analysis of differentiating nascent mouse lens epithelial and fibre cells. *Experimental Eye Research*. 2018;175:56-72. doi:10.1016/j.exer.2018.06.004.

Zhao, G. et al., 2018. Negative regulation of lens fibre cell differentiation by RTK antagonists Spry and Spred. *Experimental Eye Research*, 170, pp.148–159.

Zhao, H. et al., 2008. Fibroblast growth factor receptor signalling is essential for lens fibre cell differentiation. *Developmental Biology*, 318(2), pp.276–288.

Zhao, Y., Zheng, D. & Cvekl, A., 2018. A comprehensive spatial-temporal transcriptomic analysis of differentiating nascent mouse lens epithelial and fibre cells. *Experimental Eye Research*, 175, pp.56–72.

Zhou, F., 2018. Glypican-3: A promising biomarker for hepatocellular carcinoma diagnosis and treatment. *Medicinal Research Reviews* 38, 741–767.

Zhou, M., Li, X.M. and Lavker, R.M., 2006. Transcriptional profiling of enriched populations of stem cells versus transient amplifying cells: a comparison of limbal and corneal epithelial basal cells. *Journal of Biological Chemistry*, 281, 19600-19609.

Zhou, Y. & Shiels, A., 2018. EphA2 and Efna5 participate in lens cell pattern-formation. *Differentiation*, 102, pp.1–9.

Zhou, Z., Wang, J., Cao, R., Morita, H., Soininen, R., Chan, K.M., Liu, B., Cao, Y., Tryggvason, K., 2004. Impaired angiogenesis, delayed wound healing and retarded tumor growth in perlecan heparan sulfate-deficient mice. *Cancer Res* 64, 4699–4702.

Zhu Z, Friess H, Kleeff J, et al. Glypican-3 expression is markedly decreased in human gastric cancer but not in esophageal cancer. *Am J Surg*. 2002;184(1):78-83.
doi:10.1016/s0002-9610(02)00884-x.

Zhu, Z. et al., 2002. Glypican-3 expression is markedly decreased in human gastric cancer but not in esophageal cancer. *American journal of surgery*, 184(1), pp.78–83.

Zoeller, J.J., Whitelock, J.M., Iozzo, R.V., 2009. Perlecan regulates developmental angiogenesis by modulating the VEGF-VEGFR2 axis. *Matrix Biol.* 28, 284–291.

Zong, F. et al., 2009. Syndecan-1 and FGF-2, but Not FGF Receptor-1, Share a Common Transport Route and Co-localise with Heparanase in the Nuclei of Mesenchymal Tumor Cells S. Wölfl, ed. *PLoS ONE*, 4(10), p.e7346.

APPENDIX 1

The following manuscripts represent work which was a major contribution to this thesis. The original manuscripts are attached below as they were published.

- **TFL Wishart** and FJ Lovicu (2023). Spatiotemporal Localisation of Heparan Sulphate Proteoglycans throughout Mouse Lens Morphogenesis. *Cells* (DOI: 10.3390/cells12101364).
- **TFL Wishart** and Frank J. Lovicu (2021). An Atlas of Heparan Sulfate Proteoglycans in the Postnatal Rat Lens. *Investigative Ophthalmology & Visual Science*, (DOI: 10.1167/iovs.62.14.5)
- **TFL Wishart** and FJ Lovicu (2022). Heparan sulfate proteoglycans (HSPGs) of the ocular lens. *Progress in Retinal and Eye Research* (DOI: 10.1016/j.preteyeres.2022.101118).

Article

Spatiotemporal Localisation of Heparan Sulphate Proteoglycans throughout Mouse Lens Morphogenesis

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Abstract: Heparan sulphate proteoglycans (HSPGs) consist of a core protein decorated with sulphated HS-glycosaminoglycan (GAG) chains. These negatively charged HS-GAG chains rely on the activity of PAPSS synthesising enzymes for their sulfation, which allows them to bind to and regulate the activity of many positively charged HS-binding proteins. HSPGs are found on the surfaces of cells and in the pericellular matrix, where they interact with various components of the cell microenvironment, including growth factors. By binding to and regulating ocular morphogens and growth factors, HSPGs are positioned to orchestrate growth factor-mediated signalling events that are essential for lens epithelial cell proliferation, migration, and lens fibre differentiation. Previous studies have shown that HS sulfation is essential for lens development. Moreover, each of the full-time HSPGs, differentiated by thirteen different core proteins, are differentially localised in a cell-type specific manner with regional differences in the postnatal rat lens. Here, the same thirteen HSPG-associated GAGs and core proteins as well as PAPSS2, are shown to be differentially regulated throughout murine lens development in a spatiotemporal manner. These findings suggest that HS-GAG sulfation is essential for growth factor-induced cellular processes during embryogenesis, and the unique and divergent localisation of different lens HSPG core proteins indicates that different HSPGs likely play specialized roles during lens induction and morphogenesis.

Keywords: lens development; heparan sulphate proteoglycan (HSPG); PAPSS; syndecan; glypican



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1. Introduction

During early embryogenesis, the morphogenesis of the eye lens begins with the proximal interaction between the surface head ectodermal cells with the evaginating optic vesicle, resulting in a thickening of this apposing ectoderm to become the lens placode. The apical constriction of these cells leads to placode invagination to form the lens pit, which will separate from the neighbouring head ectoderm to form the hollow lens vesicle. The cells at the anterior aspect of the lens vesicle will differentiate to form the lens epithelium, while the posterior lens vesicle cells elongate to form the primary lens fibre cells. The elongation of these primary fibres that extend to reach the overlying epithelium rids the vesicle of its lumen to form the basic structure of the lens [1,2]. From this point onwards, the lens continues to grow, as proliferating epithelia at the lens equator exit the cell cycle and elongate and differentiate to form secondary fibre cells, displacing the older fibre cells towards the centre of the lens [1,2]. As fibre cells mature, there is autophagy of their nuclear material and organelles, and the formation of specialized membrane interdigitations, as they acquire their distinctive transparency, a characteristic that allows the lens to perform its primary function to transmit and focus light onto the retina [3].

Lens development and maintenance are governed by the interplay of a complex network of signalling molecules within the ocular microenvironment [4]. Extensive research conducted over several decades has led to the identification of these crucial molecules

and elucidation of their distinct and complementary functions in regulating specific morphogenic events and cellular behaviour [5,6]. Secreted primarily from the different ocular tissues, including the lens, retina and ciliary body, these diffusible growth factors, including members of the fibroblast growth factor (FGF) [7–9], Wnt [10–12], bone morphogenetic protein (BMP) [13,14] and Hedgehog (Hh) [15,16] families, that are found in the ocular milieu, influence lens cell behaviour (i.e., proliferation, migration, differentiation, and cell death) [4]. For the defined stages of lens morphogenesis, these factors need to be tightly controlled in a spatial and temporal manner to regulate the induction, and anteroposterior, left–right, and dorsoventral patterning of the lens [5,6]. While the various effects of growth factors on lens cell behaviour are well known, and often overlapping, the mechanisms governing the movement of these factors through the extracellular space, their precise arrival and signal transmission within target cells, remains unknown. Recent studies in mice and other animal models have highlighted the significance of heparan sulphate proteoglycans (HSPGs) localised to the cell membrane and extracellular matrix (ECM) of tissues, in facilitating the signalling and distribution of growth factors, respectively [17].

HSPGs are composed of a specific core protein structure, covalently linked to a small number of heparan sulphate (HS) glycosaminoglycan (GAG) chains. Of the thirteen full-time HSPGs, they are divided into three major families depending on this core protein structure: the transmembrane family (i.e., syndecans), glycerophosphatidylinositol (GPI)-anchored family (i.e., glypicans) and the secreted family (e.g., perlecan, agrin, and collagen XVIII) [17,18]. The syndecans and glypicans are generally localised to the cell surface, whereas perlecan, agrin, and collagen XVIII are more commonly found in the ECM and basement membranes [17].

In the synthesis of HS-GAG chains, HS sulfotransferases facilitate the transfer of sulphate ions (SO_4^-) from the sulphate-donor molecule, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), to HS-GAGs. PAPS is a crucial and rate-limiting sulphate donor molecule that regulates all post-translational sulfation reactions in the golgi apparatus and is produced by cellular PAPS synthetases (PAPSS1/2) [19,20]. The negatively charged sulphate groups of the HS-GAG chains interact with the positively charged lysine/arginine-rich regions of many proteins [17], allowing HSPGs to interact with and modulate the activity of various growth factors including FGFs [21,22], BMPs [23,24], Wnts [25,26], Hh [27,28], and ECM molecules, such as integrins [29,30], laminin [31], and fibronectin [32].

The downstream effects of HSPG–protein interactions include the activation of high-affinity receptor signalling complexes, and the mediation of intracellular signalling cascades leading to the activation of key effector genes and changes in the cell behaviour and phenotype [17,33]. The pleiotropic modulation of growth factor receptor signalling by HSPGs (altering ligand density and activity) can locally stabilise mitogen and morphogen gradients at the tissue level [33]. These gradients are critical for organogenesis and for the maintenance of normal tissue homeostasis [34]. As the activity of HSPGs primarily depends on its fine structure (i.e., sulphation) and location (i.e., the cell surface or ECM) of their HS-GAG glycosaminoglycan chains, the specific activity of HSPGs in a given cell-type or tissue, at a given time can be determined by its HSPG profile: the localisation of HSPG core proteins and its complement of HS-GAG sulfation enzymes [17,33]. Previously, we have shown all HSPG core proteins are present in the postnatal rat lens, localised to its epithelia, fibres and basement membrane, the lens capsule, in unique and distinct expression patterns that align with key functional regions [35]. The distinct spatial and temporal expression of HSPGs implies that differentiating lens cells may acquire a unique set of HSPGs, leading to specific patterns of HS–protein interactions and function during lens morphogenesis. Here, we report for the first time the comprehensive spatial and temporal localisation of all HSPGs in the developing murine lens. Taken together with the current literature, we highlight putative roles for specific HSPGs during key stages of lens development, as well as provide support for the requirement of HS sulphation in growth factor-induced lens cellular processes.

2. Materials and Methods

2.1. Animals

Animal experimentation protocols were authorized by The University of Sydney Animal Ethics Committee and were conducted in accordance with the guidelines established by the National Health and Medical Research Council (Australia) as well as the Association for Research in Vision and Ophthalmology's declaration for the Use of Animals in Ophthalmic and Vision Research. Mouse conception was recorded as midnight on the day of mating and embryos were routinely collected post conception at embryonic (E) day 9.5 (E9.5), E10.5, E11.5, E14.5, and E16.5, representing key stages of lens development (see Figure 1). Pregnant FvB/N mice were sacrificed by asphyxiation with carbon dioxide, and embryos were extracted and sacrificed by decapitation. Embryonic heads were collected for histological processing. Ten-day-old (P10) albino Wistar rats (*Rattus norvegicus*) were sacrificed by asphyxiation with carbon dioxide followed by cervical dislocation.

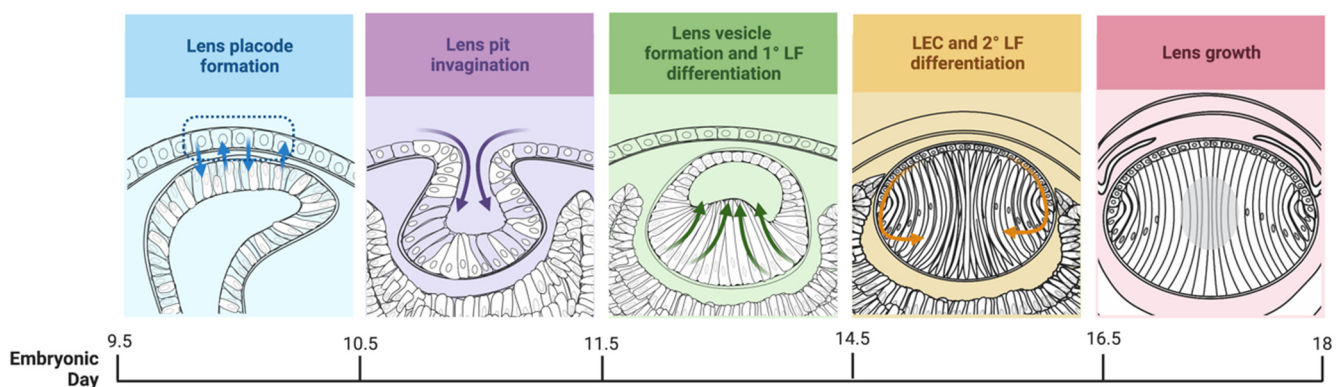


Figure 1. Representative stages of murine lens development. Arrows in lens placode formation indicate inductive interactions. Other arrows indicate cell movements/growth.

2.2. Immunolabelling of Heparan Sulphate Proteoglycans

Intact embryonic heads were collected and fixed for 24 h in 10% neutral buffered formalin (NBF; Sigma, Castle Hill, NSW, Australia) before processing for routine paraffin embedding. Paraffin-embedded tissues were serially sectioned at 5–6 μm . Mid-sagittal sections of embryonic heads from at least three independent animals were used for immunolabelling and for periodic acid–Schiff's (PAS) staining to highlight lens morphology. Monoclonal antibodies were used to detect all highly sulphated HSPGs as previously described [35]. Embryonic head sections were first labelled for highly sulphated forms of HS-GAG (HS-GAG F58-10E4, 370255; Amsbio LLC, Abingdon, UK). We also labelled for sulphated chondroitin sulphate (CS)-GAGs (CS-56, C8035; Sigma), as some HSPGs (syndecans -1/-3, agrin and collagen XVIII) carry CS-GAG chains alongside their predominant HS-GAG chains. To characterize the spatial localisation of the different HSPGs in the developing murine lens, we immunolabelled mid-sagittal sections using commercially available antibodies (see Supplementary Table S1). For all immunofluorescent labelling studies, cell nuclei were counter-labelled with bisbenzimidazole (Hoechst 33258; Sigma). Images in figures are oriented with the ventral side positioned to the right and the dorsal side to the left. Controls were included to account for any effects of autofluorescence (unlabelled tissue controls), secondary antibodies (chain-specific isotype and secondary antibody alone controls), co-labelling effects (single label controls), and nuclear staining (no Hoechst controls). Tissue samples from at least three individual animals (up to 6 eyes) at different stages of development were used for each label/antibody investigated.

A light microscope equipped with epifluorescence (Leica DMLB 100S, DFC-450C camera and LAS software v4.8.0, Leica Microsystems, Wetzlar, Germany) was used for PAS-staining (brightfield). For immunolabelling (immunofluorescence) studies, images were captured using a ZEISS LSM-800 microscope with an AxioCam 506 camera and ZEN

Blue Edition v2.3 software (Carl Zeiss, Jena, Germany), or a ZEISS AxioScan.Z1 microscope with a Hamamatsu ORCA-flash 4.0 v2 camera and ZEN SlideScan 2012 software (Carl Zeiss). All images were saved as uncompressed tagged information field format (TIFF) files at 16-bit depth. Image processing was performed using Photoshop v21.1.1 (Adobe, Inc., San Jose, CA, USA) and ImageJ (FIJI) v2.0 (Wayne Rasband, NIH, Bethesda, MD, USA) on the Mac OS X 10.15.6 (Apple Inc., Cupertino, CA, USA) and Windows 10 (Microsoft Inc., Seattle, WA, USA) operating systems.

3. Results

A summary of the labelling for HS-GAG, CS-GAG, PAPSS2, and all thirteen HSPG core proteins during murine lens development is outlined in Table 1.

3.1. PAPSS2 Is Localised to the Developing Mouse Lens

We first immunolabelled the PAPSS2 (HSPG sulfation) in the developing mouse lens (Figure 2). PAPSS2 reactivity was absent from all early eye structures, including cells comprising the lens placode (E9.5, Figure 2(A1)), and lens pit (E10.5, Figure 2(A2)). It was not until E11.5, in the lens vesicle that PAPSS2 immunolabelling appeared, strongly localised to the elongating primary lens fibre cells (Figure 2(A3)). This fibre-specific label persisted and was also observed in the early secondary lens fibre cells at E14.5 (Figure 2(A4–A7)) and E16.5 (Figure 2(A8–A10)). At E16.5, PAPSS2 reactivity was also evident in the anterior lens epithelium but was not present in the lens capsule (Figure 2(A9)).

3.2. Heparan Sulphate Is the Predominant Sulphated Glycosaminoglycan Expressed throughout Murine Lens Development

HSPG core proteins can carry HS-GAG GAG chains or a combination of both HS-GAG and CS-GAG chains. To determine which of these two GAG species represents the major “active” sulphated fraction of HSPGs in the developing lens, the distribution of HS-GAG and CS-GAG was compared during key stages of lens development (Figure 3(A1–A8,B1–B8)) using antibodies specific for the sulphated domains on HS-GAG (HS-10E4, Figure 3(C1–C8)) or CS-GAG (CS-56, Figure 3(D1–D8)) chains. HS-GAG chain labelling was first observed at very low levels in the cells of the lens placode (E9.5, Figure 3(C1)) with stronger labelling in the lens pit (E10.5, Figure 3(C2)). At E11.5 (Figure 3(C3)), HS-GAG labelling was prominent in the developing lens capsule, anterior lens epithelium and ubiquitously in elongating primary lens fibre cells. HS-GAG reactivity was maintained in the lens capsule and cytoplasm of anterior epithelial cells at E14.5 (Figure 3(C4)) and E16.5 (Figure 3(C5–C8)). The HS-GAG chain labelling was the strongest throughout the cytoplasm of secondary lens fibre cells (Figure 3(C4)), with decreased reactivity at the germinative and transitional zones (Figure 3(C4,C8)). The concentrated immunolabelling of HS-GAG was also observed at the posterior tips (E14.5, Figure 3(C4)) and in the nuclei (Figure 3(C4,C7)) of elongating secondary fibre cells. Although absent from the extracellular matrix of the early developing eye (E9.5 and E10.5; Figure 3(B1,B2)), HS-GAG was strongly associated with the matrix connecting the posterior lens vesicle and early retina (Figure 3(C3)), and the developing vitreous humour from E14.5 (Figure 3(C4)). In contrast to the localisation of active HS-GAG chains in early lens development, CS-GAG chain labelling was primarily observed in the pericellular matrix (Figure 3(D1–D3)). CS-GAG immunolabelling was completely absent from the lens placode (Figure 3(D1)), lens pit (Figure 3(D2)), and lens vesicle (E11.5, Figure 3(D3)). Similarly, CS-GAG reactivity was first observed at low levels in the E14.5 lens capsule, anterior lens epithelium, and anterior pole of the secondary lens fibre cells (Figure 3(D4)). CS-GAG immunostaining was strongly correlated with the extracellular matrix at all early stages of eye development, particularly in the preplacode matrix (E9.5; Figure 3(D1)), and the interstitial matrix connecting the invaginating lens pit and optic cup (E10.5; Figure 3(D2)) and surrounding the lens vesicle (E11.5; Figure 3(D3)). CS-GAG was weakly evident in the ocular humours in the later stages of eye development (E14.5 and E16.5; Figure 3(D4–D6)). CS-GAG immunolabelling was absent from

the lens capsule at E16.5 (Figure 3(D5,D6,D8)), and decreased in the fibre cells relative to E14.5 (Figure 3(D7,D8)). The CS-GAG labelling remained in the anterior lens epithelium (Figure 3(D5)) and decreased in the germinative and transitional zones (Figure 3(D8)).

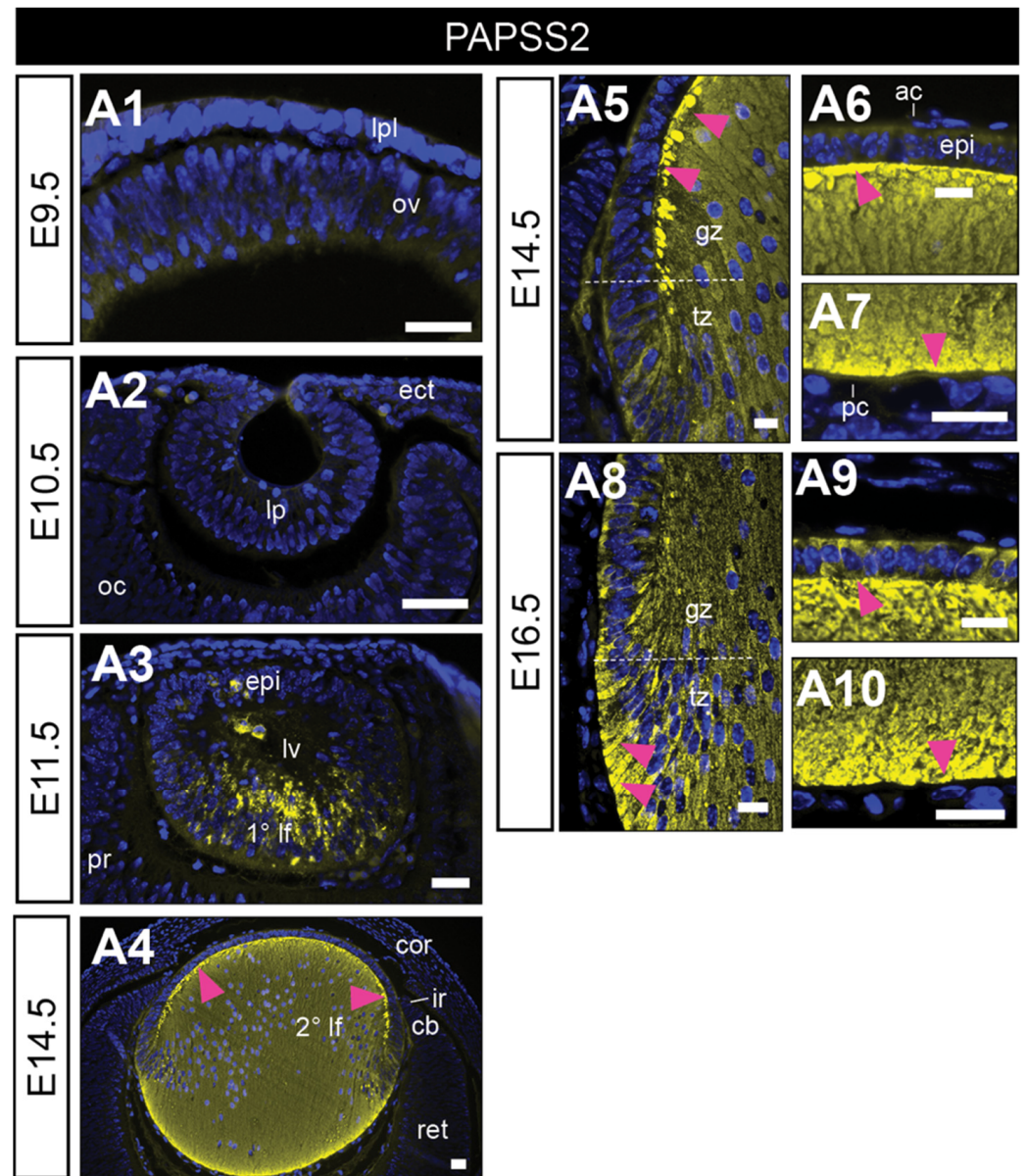


Figure 2. Immunolocalisation of PAPS synthetase-2 in the developing murine eye. Mid-sagittal mouse (FvB/N) eye sections from embryonic day-9.5 (E9.5; (A1), E10.5 (A2), E11.5 (A3), E14.5 (A4–A7) and E16.5 (A8–A10)) labelled for 3'-Phosphoadenosine 5'-Phosphosulphate Synthase 2 (PAPSS2; yellow). Nuclei counterstained with Hoechst (blue). Dotted white lines indicate lens equator (A5,A8). Magenta arrowheads indicate localisation at lens fibre tips (A5–A10). Lens placode (lpl), optic vesicle (ov), head ectoderm (ect), lens pit (lp), optic cup (oc), lens vesicle (lv), anterior lens epithelium (epi), primary lens fibre cells (1° lf), secondary lens fibre cells (2° lf), cornea (cor), retina (ret), ciliary body (cb), iris (ir), germinative zone (gz), transitional zone (tz), anterior lens capsule (ac), and posterior lens capsule (pc). Scale bars = 25 µm. Images in figures are oriented with ventral side positioned to the right and the dorsal side to the left.

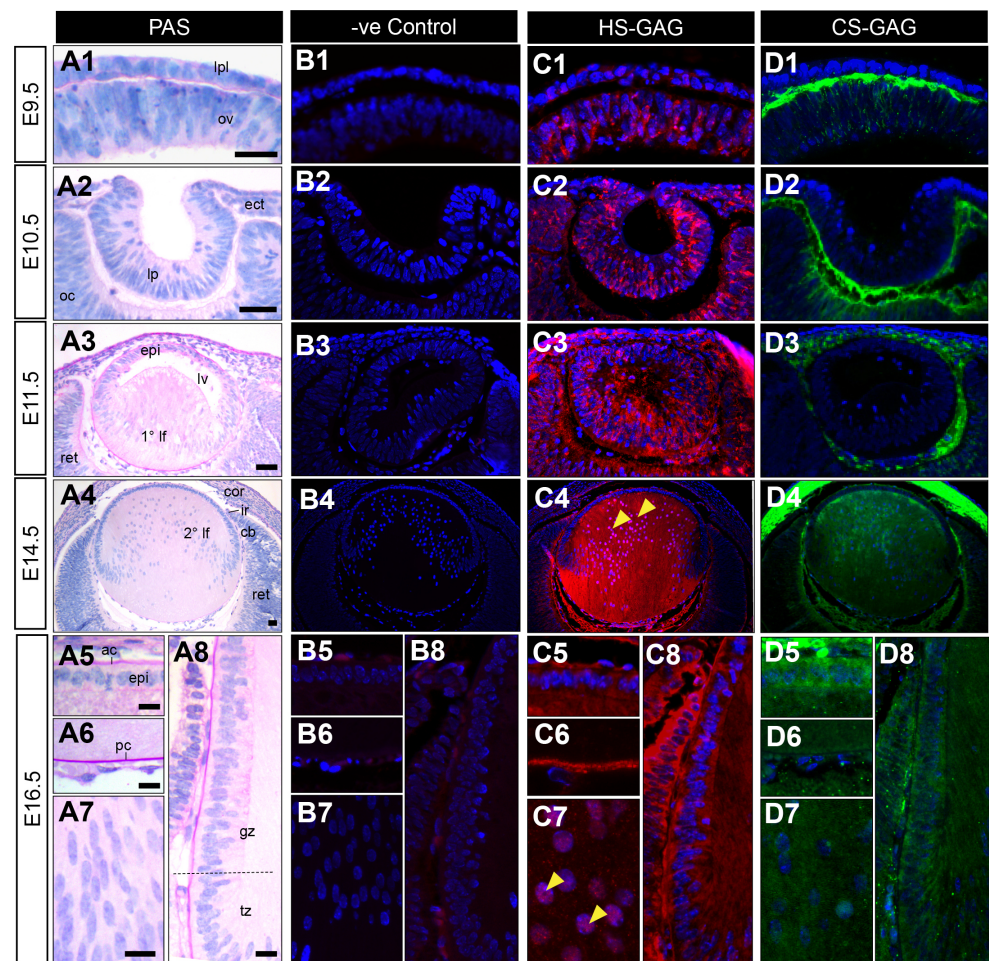


Figure 3. Distribution of sulphated GAGs in mouse eye development. Eye sections from embryonic day 9.5 (E9.5; (A1,B1,C1,D1), E10.5 (A2,B2,C2,D2), E11.5 (A3,B3,C3,D3), E14.5 (A4,B4,C4,D4), and E16.5 (A5–A8,B5–B8,C5–C8,D5–D8)) FvB/N mice. Mid-sagittal eye sections stained with periodic acid–Schiff’s (PAS, (A1–A8)) show cell morphology. Sections stained with a secondary antibody only show representative negative controls (negative control; (B1–B8)). Sections labelled the highly sulphated “active” forms of heparan sulphate (HS-GAG, red, (C1–C8)) or chondroitin sulphate (CS-GAG, green, (D1–D8)) glycosaminoglycan chains. Nuclei counterstained with Hoechst (blue). Yellow arrowheads indicate nuclear localisation (C4,C7). Dotted lines indicate the lens equator (A8). The lens placode (lpl), head ectoderm (ect), preplacodal matrix (ppm); optic vesicle (ov), lens pit (lp), optic cup (oc), lens vesicle (lv), anterior lens epithelium (epi), primary lens fibre cells (1° lf), secondary lens fibre cells (2° lf), cornea (cor), retina (ret), ciliary body (cb), iris (ir), germinative zone (gz), transitional zone (tz), anterior lens capsule (ac), posterior lens capsule (pc). Scale bars = 25 μ m. Images in the figures are oriented with a ventral side positioned to the right and the dorsal side to the left.

3.3. Syndecan HSPGs Are Differentially Spatially Localised in the Lens throughout Development

The different syndecan HSPGs differ in their expression, both temporally and spatially, at all major stages of eye development. Syndecan-1 remains absent from the developing lens until E16.5, where it is strongly localised to the lens capsule and posterior vessels of the tunica vasculosa lentis (tv1). Weak syndecan-1 staining is also observed in a subset of anterior lens epithelial cells and anterior secondary fibre cell tips (Figure 4(A5)). Syndecan-2 (Figure 4(B1)), -3 (Figure 4(C1)) and -4 (Figure 4(D1)) are found at E9.5 in both the optic vesicle and the lens placode. The expressions of these syndecans persist at embryonic day 10.5, with strong labelling in the lens pit and optic cup (Figure 4(B2,C2,D2)). Syndecan-2 strongly stained throughout the invaginating lens pit, while syndecan-3 and -4 labelled particularly strong in the posterior cells of the lens pit (Figure 4(C2,D2)). At E11.5, the prospective

lens epithelial cells and elongating primary lens fibre cells showed strong labelling of syndecan-2 (Figure 4(B3)) and -3 (Figure 4(C3)). For syndecan-2, this label was restricted to the primary lens fibres and absent from the anterior aspect of the lens (Figure 4(D3)). Syndecan-1 was absent from the lens up to E11.5, where it first appears to localise to the inner aspect of the retina (Figure 4(A3)). At E14.5, the localisation of each of the syndecans was distinct from each other. Syndecan-1 was primarily localised to the differentiating retinal ganglion cells, and the lens capsule and blood vessels (Figure 4(A4)). Syndecan-2 was strongly localised to the lens capsule, tvl, lens epithelial cells, and nuclei of fibre cells. It also showed strong localisation in the cornea and developing iris (Figure 4(B4)). Syndecan-3 (Figure 4(D3)) and -4 (Figure 4(D4)) were both present in the lens epithelium and fibres in different patterns. Syndecan-3 staining was primarily observed in the secondary lens fibres, concentrated on the anterior fibre tips. It was absent from newer secondary lens fibre cells (Figure 4(C4)). On the other hand, syndecan-4 staining was primarily observed in early secondary fibre cells, with distinct staining at the anterior and posterior fibre cell tips (Figure 4(D4)). These expression patterns were maintained at E16.5 for syndecan-2 (Figure 4(B5–B8)), -3 (Figure 4(C5–C8)), and -4 (Figure 4(D5–D8)). At E16.5, syndecan-1 was still absent from the lens cells, but strongly localised to the lens capsule (Figure 4(A5–A8)).

3.4. Glypican HSPGs Differ in Temporal Expression at Key Stages of Lens/Eye Development

At E9.5, glypican-4 is exclusively localised to the pre-placodal matrix (Figure 5(D1)), while glypican-2, -5, and -6 are present in both the lens placode cells and optic vesicle (Figure 5(B1,C1,E1,F1)). Glypican-1 was absent at this early stage (Figure 5(A1)). By E10.5, all glypicans were present in the lens pit and optic cup, apart from glypican-4, that was localised to the extracellular matrix between the lens pit and optic cup (Figure 5(D2)). The label for glypican-3 and -5 was concentrated upon the basal aspect of the lens pit in the presumptive primary lens fibre cells (Figure 5(C2,E2)). Glypican-1, -2, -5, and -6 were also present in the surface head ectoderm (Figure 5(A2,B2,E2,F2)). All glypicans were present in the lens vesicle (Figure 5(A2,B2,C2,E2,F2)), except for glypican-4, that was primarily localised to the lens capsule, differentiating retinal ganglion cells, and posterior lens blood vessels (Figure 5(D3)). At E11.5, glypican-3 stained all lens vesicle cells diffusely, with more intense staining in the presumptive lens epithelial cells (Figure 5(C3)). Glypican-1, -2, -5, and -6 were predominantly localised to the posterior elongating primary lens fibre cells (Figure 5(A3,B3,E3,F3)). These glypicans were mostly localised in a concentrated region at the posterior tips of the primary lens fibres, apart from glypican-6, that strongly labelled all fibre cells (Figure 5(F3)). From E14.5, the expression of glypicans was generally consistent. Glypican-1 (Figure 5(A4–A8)), -2 (Figure 5(B4–B8)), -3 (Figure 5(C4–C8)), and -5 (Figure 5(E4–E8)) were predominantly localised to the lens epithelium and newly differentiating secondary lens fibre cells, where perinuclear staining was observed for all four glypicans (Figure 5(A7,B7,C7,E7)). Glypican-4 was absent from lens cells, with some punctate staining in the anterior lens epithelium, and strong localisation to the lens capsule (Figure 5(D4–D8)). Glypican-6 was absent from the epithelium and transitional zone, but strongly labelled the cytoplasm of the fibre cells (Figure 5(F4–F8)).

Postnatally, in the 10-day-old rat lens, labelling for glypican-5 was localised to both epithelial and fibre cells, with stronger labelling at the germinative and transitional zones (Supplementary Figure S1(B1–B4)). Glypican-5 was also found in the corneal epithelium and endothelium (Supplementary Figure S1(B5,B7)), ciliary non-pigmented epithelium (Supplementary Figure S1(B8)), and in remnant blood vessels of the tunica vasculosa lentis (Supplementary Figure S1(B4)). Glypican-5 was absent from the lens capsule (Supplementary Figure S1(B1,B2,B4)).

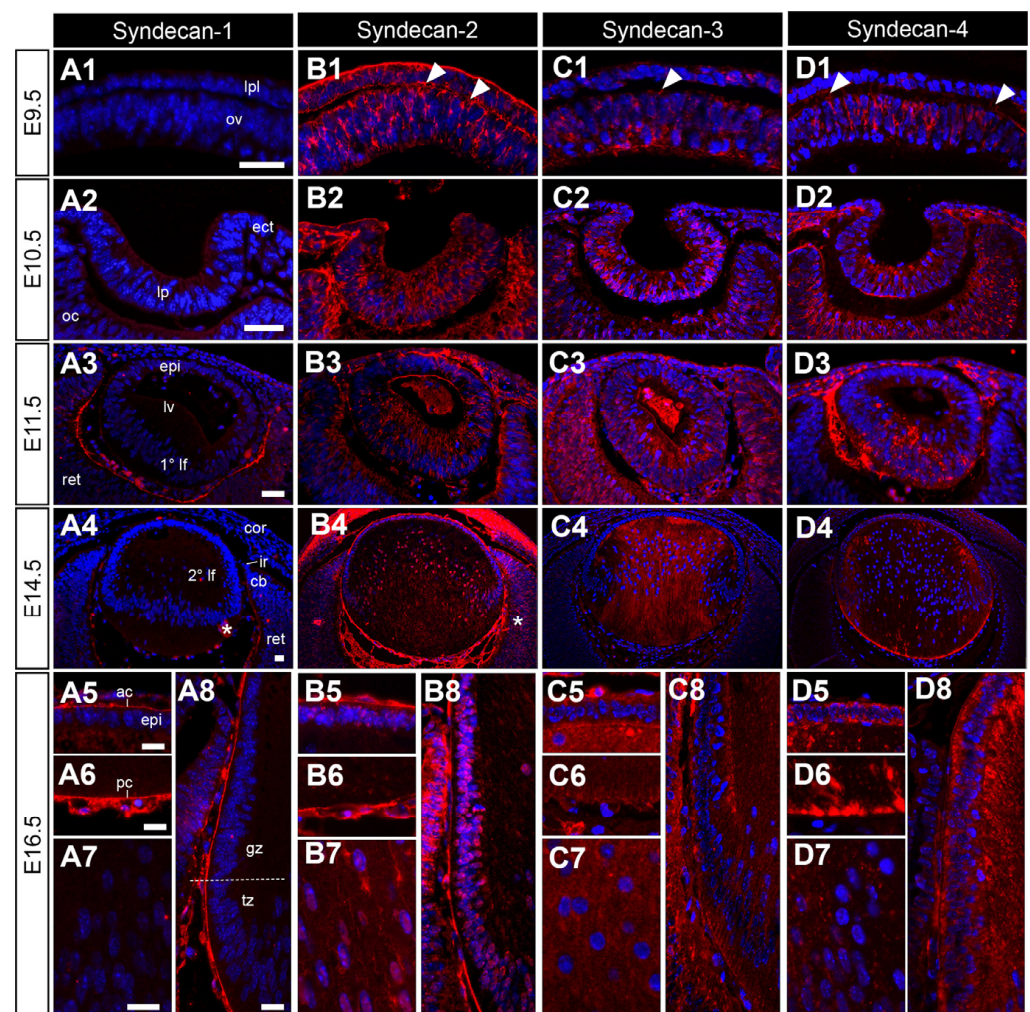


Figure 4. Distribution of syndecan core proteins in the developing mouse eye. Mid-sagittal sections from embryonic day-9.5 (E9.5; (A1,B1,C1,D1)), E10.5 (A2,B2,C2,D2), E11.5 (A3,B3,C3,D3), E14.5 (A4,B4,C4,D4) and E16.5 (A5–A8,B5–B8,C5–C8,D5–D8) FvB/N mice. Sections labelled for syndecan-1 (A1–A8), syndecan-2 (B1–B8), syndecan-3 (C1–C8), or syndecan-4 (D1–D8) HSPG core proteins (red). Nuclei counterstained with Hoechst (blue). Labelling of cell nuclei indicated in (B7,B8). White arrowheads indicate the staining of lens fibre cell tips (C4–C6,C8,D3, D4,D6–D8). The dotted line indicates the lens equator (A8). Lens placode (lpl), head ectoderm (ect), preplacodal matrix (ppm), optic vesicle (ov), lens pit (lp), optic cup (oc), lens vesicle (lv), anterior lens epithelium (epi), primary lens fibre cells (1° lf), secondary lens fibre cells (2° lf), cornea (cor), retina (ret), ciliary body (cb), iris (ir), germinative zone (gz), transitional zone (tz), anterior lens capsule (ac), and posterior lens capsule (pc). Scale bars = 25 µm. * denotes artefact. Images in the figures are oriented with the ventral side positioned to the right and the dorsal side to the left.

3.5. Localisation of High-Molecular-Weight HSPGs Extends beyond the Lens Capsule

Immunoreactivity for perlecan is weak in early lens structures (Figure 6(A1,A2)) but labelling appears to be stronger in the matrix and basement membranes of the E11.5 eye (Figure 6(A3)). At this stage, perlecan is observed in the anterior cells of the lens vesicle (Figure 6(A3)). This pattern of staining is maintained in the fully formed lens at E14.5 and E16.5, with perlecan mostly restricted to the lens capsule and anterior lens epithelium (Figure 6(A4–A8)). Diffuse perlecan staining was observed in the nuclei of some differentiating lens fibre cells (Figure 6(A7)). Both collagen XVIII/endostatin (Figure 6(B1,B2)) and agrin (Figure 6(C1,C2)) were observed in the cells of the lens placode and lens pit. Collagen XVIII/endostatin reactivity was restricted to a punctate label in the primary lens fibres

of the lens vesicle (Figure 6(B3)). This label persisted in the E14.5 lens (Figure 6(B4)). At E16.5, collagen XVIII/endostatin labelled in condensed patches throughout the cytoplasm of both lens epithelial and fibre cells (Figure 6(B5–B8)). On the other hand, agrin stained strongly throughout the cells of the lens vesicle, concentrated apically in the presumptive anterior lens epithelial cells, and basally in the new elongating primary lens fibre cells (Figure 6(C3)). From E14.5, agrin immunoreactivity was strongly visible throughout the cytoplasm of the central lens fibre cells (Figure 6(C4)), and this label persisted in the E16.5 lens (Figure 6(C5–C8)). Agrin was clearly associated with a subset of fibre cell nuclei (Figure 6(C4,C7)). Agrin staining was almost completely absent from the anterior lens epithelium and transitional zone (Figure 6(C4,C5,C8) and Table 1).

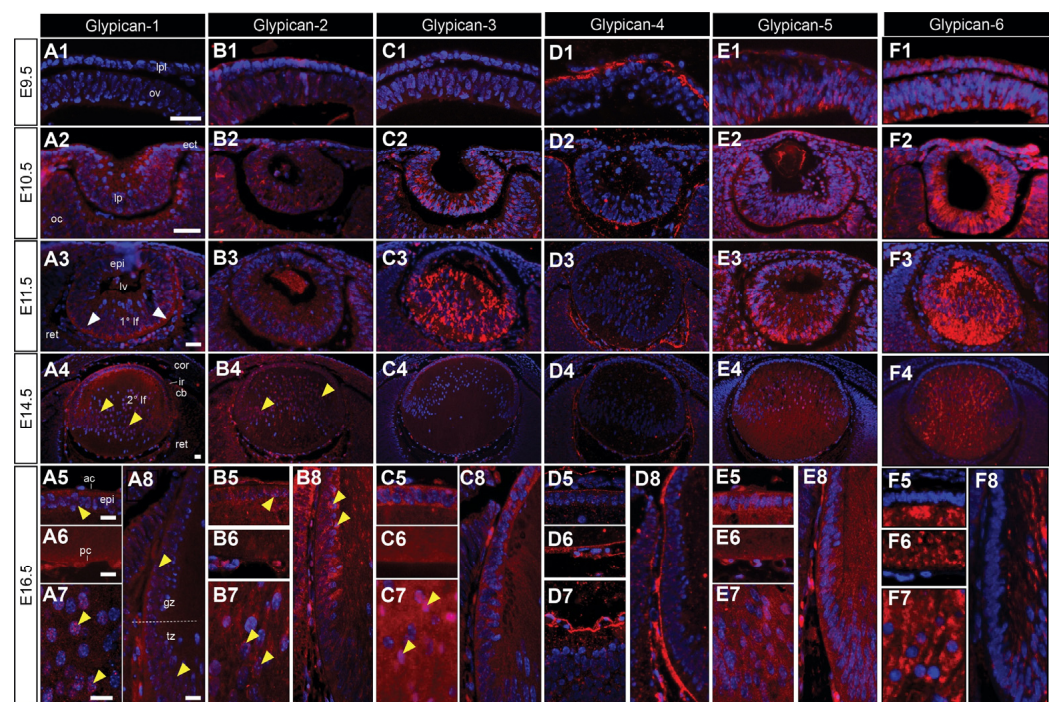


Figure 5. Distribution of glypican core proteins in the developing mouse eye. Mid-sagittal sections from embryonic day-9.5 (E9.5; (A1–F1)), E10.5 (A2–F2), E11.5 (A3–F3), E14.5 (A4–F4), and E16.5 (A5–A8, B5–B8, C5–C8, D5–D8, E5–E8, F5–F8) FvB/N mice. Sections labelled for glypican-1 (A1–A8), glypican-2 (B1–B8); glypican-3 (C1–C8), glypican-4 (D1–D8), glypican-5 (E1–E8), or glypican-6 (F1–F8) HSPG core proteins (red). Nuclei counterstained with Hoechst dye (blue). Yellow arrowheads indicate nuclear localisation (A4, A5, A7, A8, B4, B5, B7, B8, C7). White arrowheads indicate the staining of lens fibre cell tips (A3). Dotted lines indicate lens equator (A8). Lens placode (lpl), head ectoderm (ect), preplacodal matrix (ppm), optic vesicle (ov), lens pit (lp), optic cup (oc), lens vesicle (lv), anterior lens epithelium (epi), primary lens fibre cells (1° lf), secondary lens fibre cells (2° lf), cornea (cor), retina (ret), ciliary body (cb), iris (ir), germinative zone (gz), transitional zone (tz), anterior lens capsule (ac), and posterior lens capsule (pc). Scale bars = 25 μ m. Images in figures are oriented with ventral side positioned to the right and the dorsal side to the left.

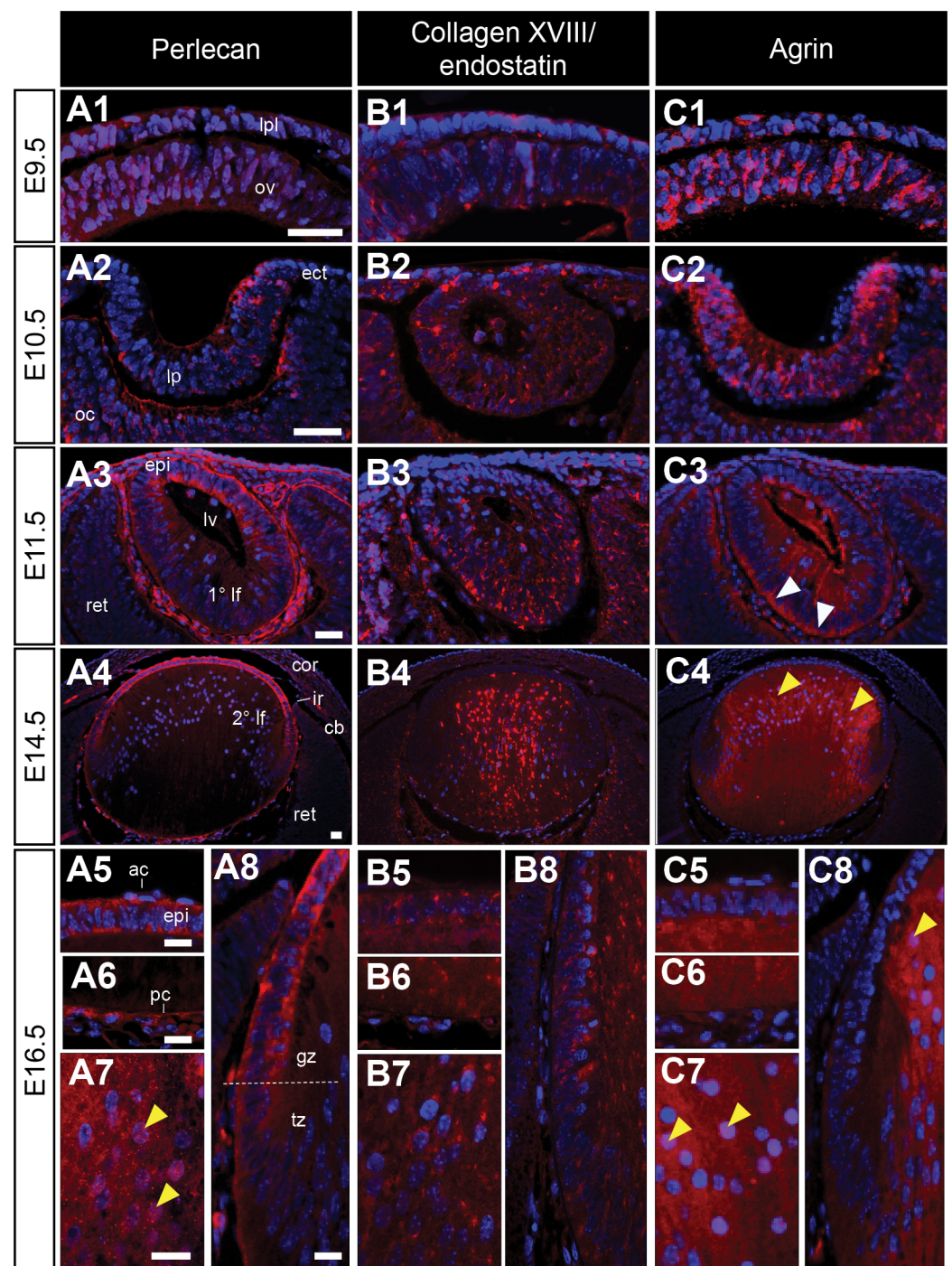


Figure 6. Distribution of high-molecular-weight HSPG core proteins in the developing mouse eye. Mid-sagittal sections from embryonic day-9.5 (E9.5; (A1–C1)), E10.5 (A2–C2), E11.5 (A3–C3), E14.5 (A4–C4), and E16.5 (A5–A8, B5–B8, C5–C8) FvB/N mice. Sections labelled for perlecan (A1–A8), collagen-XVIII/endostatin (B1–B8), or agrin (C1–C8) HSPG core proteins (red). Nuclei counterstained with Hoechst dye (blue). Yellow arrowheads indicate nuclear localisation (A7, C4, C7, C8). White arrowheads indicate the staining of the lens fibre cell tips (C3). Dotted lines indicate the lens equator (A8). The lens placode (lpl), head ectoderm (ect), optic vesicle (ov), lens pit (lp), optic cup (oc), lens vesicle (lv), anterior lens epithelium (epi), primary lens fibre cells (1° lf), secondary lens fibre cells (2° lf), cornea (cor), retina (ret), ciliary body (cb), iris (ir), germinative zone (gz), transitional zone (tz), anterior lens capsule (ac), and posterior lens capsule (pc). Scale bars = 25 µm. Images in figures are oriented with ventral side positioned to the right and the dorsal side to the left.

Table 1. Summary of HSPG immunolocalisation in mouse eye lens development.

Embryonic Day		GAG	Other	Core Proteins
E9.5	Lens placode	HS		Sdc2, Sdc3, Sdc4, Gpc2, Gpc4, Gpc5, Gpc6, perlecan, collagen XVIII, agrin
	Optic vesicle	HS, CS		Sdc2, Sdc3, Sdc4, Gpc2, Gpc5, Gpc6, perlecan, collagen XVIII, agrin
	Preplacodal matrix	CS		Sdc2, Sdc4, Gpc4, Agrin
	Lens pit	HS		Sdc2, Sdc3, Sdc4, Gpc1, Gpc2, Gpc3, Gpc5, Gpc6, perlecan, collagen XVIII, agrin
E10.5	Head ectoderm	HS		Sdc2, Sdc3, Sdc4, Gpc1, Gpc2, Gpc3, Gpc4, Gpc5, Gpc6, perlecan, agrin
	Optic cup	HS, CS		Sdc2, Sdc3, Sdc4, Gpc1, Gpc2, Gpc3, Gpc4, Gpc5, Gpc6, perlecan, collagen XVIII, agrin
	Extracellular matrix (lens pit-optic cup)	CS		Sdc2, Gpc4, perlecan?
	Anterior lens epithelium	HS	PAPSS2	Sdc2, Sdc3, Sdc4, Gpc1, Gpc2, Gpc3, perlecan, collagen XVIII, agrin
E11.5	1° lens fibre cells	HS	PAPSS2	Sdc2, Sdc3, Sdc4, Gpc1, Gpc2, Gpc3, Gpc5, Gpc6, perlecan, collagen XVIII, agrin
	Lens capsule	HS		Sdc1, Sdc2, Gpc4, perlecan
	Anterior lens epithelium	HS *, CS		Sdc2 *, Sdc3, Sdc4, Gpc1, Gpc2, Gpc3, Gpc4, perlecan, collagen XVIII
	2° lens fibre cells	HS *, CS	PAPSS2	Sdc2 *, Sdc3, Sdc4, Gpc1, Gpc2, Gpc3, Gpc5, Gpc6, collagen XVIII, agrin
E14.5	Lens capsule	HS, CS		Sdc1, Sdc2, Gpc2, Gpc4, perlecan
	Tunica vasculosa lentis	HS, CS		Sdc1, Sdc2, Gpc1, Gpc2, Gpc4, Gpc5, perlecan
	Anterior lens epithelium	HS *, CS		Sdc2 *, Sdc3, Sdc4, Gpc1 *, Gpc2 *, Gpc3 *, Gpc5, perlecan, collagen XVIII, agrin
	2° lens fibre cells	HS *, CS	PAPSS2	Sdc2 *, Sdc3, Sdc4, Gpc1 *, Gpc2 *, Gpc3 *, Gpc5, Gpc6, perlecan *, collagen XVIII, agrin
E16.5	Lens capsule	HS		Sdc1, Sdc2, Gpc4, perlecan
	Tunica vasculosa lentis	HS, CS		Sdc1, Sdc2, Gpc1, Gpc2, Gpc4, Gpc5, perlecan

* Nuclear localisation in cells.

4. Discussion

While there are many reports to date studying vertebrate lens morphogenesis and the growth factors involved, less is understood about how these different growth factors are positioned to drive this development. Given the important role of HSPGs in regulating many growth factors, together with recent studies implicating HSPGs as essential for the induction and development of the eye, here we present the most comprehensive spatiotemporal labelling of all the different HSPG core proteins and their associated GAGs throughout murine lens development. This study complements and extends from our earlier report on the localisation of HSPGs in the postnatal rat lens [35], wherein we described how the defined patterns of all HSPGs associate with lens cellular activity. Of note is that our earlier study reported the absence of glypican 5 in the postnatal lens, of which we now have corrected and clearly show that it is indeed also present in the lens when using a more selective and effective commercial antibody. Given that all HSPGs are expressed in the lens, by linking the defined localisation of their respective HSPG core proteins, along with their reported mode of activity, it can provide valuable insights into their functional roles [36,37].

The activity of HSPGs is largely dependent on the distinct sulfotransferases of their HS chains, which source sulphate from PAPS produced by PAPSS1/2 synthetases [38,39]. Transcriptome data identify both *papss1* and *papss2* in the lens [40], with strong PAPSS2 protein labelling in the developing murine and postnatal rat eye, specifically in the lens [38]. Here, we found that PAPSS2 was absent in very early lens induction (i.e., lens placode, lens pit), with strong reactivity first appearing in the lens vesicle, distinctly labelling the elongating primary lens fibre cells. This fibre-specific label persisted and was later observed in secondary lens fibres at E14.5 and E16.5 when the differentiating lens epithelium also began to label strongly for PAPSS2. The weak to no immunolabelling for PAPSS2 in the lens placode and pit is of interest as we see the strong labelling of not only HS-GAG but many HSPGs that require PAPSS for their sulfation. The absence of PAPSS2 suggests that the activity of HSPGs in early lens induction may be largely mediated by HSPG core protein–protein interactions, and is independent of GAG–protein interactions, implying an expected low sulfation and binding potential of these HSPGs with ocular growth factors. An example of this is glypican-4, that only carries HS chains but does not overlap in expression with HS-GAG. Alternatively, it is also plausible that the other PAPSS isoform, PAPSS1 (encoded by the *papss1* gene), is the predominant sulphate donor at this early stage of lens development, consistent with transcript expression profiles from published embryonic mouse lens datasets [41–44].

The selectivity of interactions between HSPGs and proteins is not solely determined by the type of core protein (such as syndecan or glypican) or its location (for example, on the cell surface or in the extracellular matrix), but also by the precise structure (i.e., sulphation) of the HS-GAG chains. Although HSPGs are primarily composed of HS-GAGs, certain species such as syndecan-1/-3 [45,46], agrin [47], and collagen XVIII [48] may also carry CS-GAGs. Lens cells produce some proteoglycans carrying both HS-GAG and CS-GAG, although HS-GAG is the most common [35,49,50]. Consistent with this, here we report that HS-GAG, and not CS-GAG, is the predominant GAG associated with HSPGs throughout murine lens development. Unlike HS-GAG, CS-GAG was absent from the lens until E14.5, indicating that HS-GAGs are likely the major species regulating the early events of lens induction, invagination, and cell fate specification. The presence of CS-GAG in the pre-placodal and peri-lenticular matrix throughout development may suggest a role in cell–matrix interactions or morphogen gradient formation. In contrast, HS-GAG was absent from the ocular matrix throughout the early stages of lens development, instead appearing and localising predominantly to the presumptive and differentiating lens cells. These patterns were often associated with regions of lens cell activity (i.e., lens placode, posterior aspect of lens pit/vesicle and lens transitional zone). The expression patterns of HS-GAG and CS-GAG in the developing lens suggest that both GAG types may have overlapping and distinct roles in regulating functional cellular processes. For instance, fibre differentiation may be regulated by both GAG types, while morphogen gradient formation in the lens capsule may be predominantly regulated by HS-GAG. In other tissues, such as the developing brain, HS-GAG and CS-GAG have been shown to serve distinct functional roles, with CS-GAG stabilizing existing synaptic connections and HS-GAG stimulating the formation of new synapses [51]. These functional differences are likely due to the selective interaction of each GAG type with different proteins in a structure-dependent manner [52]. The reported differential localisation of HS-GAG and CS-GAG [51,53,54] is consistent with their distinct functional roles [55].

Most of the HSPG core proteins that we examined localised to the cells of the surface head ectoderm and optic vesicle; however, syndecan-1 and glypican-1 were both absent at this stage. Despite being a traditionally cell-associated HSPG, glypican-4 was absent from the head ectoderm and optic vesicle but was strongly localised to the pre-placodal matrix. Similarly, syndecan-2, which is traditionally a cell-associated HSPG, was also observed in the pre-placodal matrix, along with the surface head ectoderm and optic vesicle, consistent with previous reports of syndecan-2 being a matrix-associated HSPG in ocular tissues [35,56]. The pre-placodal matrix is thought to prevent this defined region

of proliferative head ectodermal cells from spreading, so that their continued proliferation and progressive cell crowding thickens this region of the ectoderm to form the lens placode [57]. The regulated signalling activities of several mitogens and morphogens, such as BMPs, Hh, FGFs, and Wnts, orchestrate the inductive events associated with the optic vesicle-presumptive lens interaction [58,59], and all these growth factors have been shown to be regulated by the HSPGs that we localise to the lens placode [60–64]. FGF, Hh, BMP, and Wnt-signalling are each essential regulators in eye development, with their on/off switching essential for different stages of lens development [6], and defects in their signalling activity shown to compromise lens development. While the sustained inductive signalling of BMP and FGF are essential for lens placode development and the early stages of lens specification [65], these early stages also require the inhibition of Hh- [16] and Wnt- [66] signalling, with heightened Hh-signalling activity shown to suppress lens formation in zebrafish [67]. Later in lens morphogenesis, Wnt-signalling is activated to promote epithelial cell adhesion, integrity, and polarity, as well as fibre differentiation [11,66,68,69]. Constitutively active Hh-signalling through its receptor, patched, promotes epithelial maintenance at the expense of fibre differentiation [70]. Thus, the inhibition of Hh-signalling, once again with fibre cell differentiation, is essential for normal lens development. BMP-signalling promotes apical constriction and pro-survival signals during lens placode invagination and drives proliferation of lens epithelial cells and cell cycle exit at the transitional zone, that is bolstered by FGF-signalling driving lens epithelial cell proliferation and fibre differentiation [13,14,71].

The spatiotemporal regulation of the ocular morphogens, together with other extracellular signalling proteins, forms the basis of lens patterning, polarity, growth, and cell fate specification [1,6,72], that are regulated by the many HSPGs we report to be expressed herein. Many of these same HSPGs regulate other matrix-related proteins, including β 1-integrins and fibronectin [73–75], that are also co-localised to the early lens placode cells, and play an essential role in this inductive process [76–79].

Glypicans are predominantly expressed during embryonic development, and increasing evidence suggests that they are major regulators of morphogenic signalling and gradient events in development, controlling left/right and dorsal/ventral patterning, as well as controlling tissue size and growth cues [80]. Genetic and biochemical studies have shown that glypicans can stimulate or inhibit the signalling pathways triggered by Wnts, Hh, BMPs, and FGFs, that have been mentioned to be responsible for patterning and tissue formation during embryogenesis, depending on the context of the tissue to drive growth patterning events [80]. Not surprisingly, mutations in genes encoding glypicans often result in developmental abnormalities or overgrowth [80]. For instance, Glypican-3 has been shown to inhibit hedgehog signalling during development by competitively binding to the ligand, preventing it from binding to patched, through a HS-GAG independent process [61]. Similarly, Glypicans-1, -3, and -4 negatively regulate BMP-signalling by competing with BMP receptors for ligand binding in a similar fashion [81,82]. On the other hand, glypicans seem to both positively and negatively regulate Wnt-signalling in different developmental systems. Glypican-4 promotes cardiac specification and differentiation by attenuating canonical Wnt- and BMP-signalling [81], while glypican-1 can act as a Wnt-coreceptor to activate Wnt-signalling [82]. For different morphogens, glypicans seem to mainly activate or enhance signalling. Glypicans can interact with FGFs and their receptors to stabilize their assembly and enhance downstream ERK1/2 or PI3K/Akt, as well as bind to TGF- β and its receptors to promote SMAD-signalling [82].

As the mouse lens placode and optic vesicle invaginate at E10.5, we find that most of the HSPG core proteins localise to the cells of the lens pit, with syndecan-1 and glypican-4 yet to appear. Glypican-4 was predominantly localised to the surrounding matrix together with syndecan-2 and perlecan. Here, we begin to observe subtle changes between some of the core proteins, such as syndecan-4, that appears to distinctly label the posterior tips of lens pit cells, with the majority of the other core proteins localising diffusely throughout all the lens pit cells. BMP-4/-7 activity [83] and the downstream activation of GTPases

(RhoA, Rac1, ROCK) drive changes in cytoskeletal architecture essential for cell elongation, apical constriction, and epithelial invagination during lens pit formation [84]. Glypican-3 is well established as a primary regulator of cellular responses of BMP-4/-7 in developing tissues [85], although emerging evidence suggests that other HSPGs, such as agrin [86] and syndecan-3 [87], may also play tissue-specific roles in BMP regulation. On the other hand, the syndecans are major regulators of RhoA/Rac1/ROCK GTPase-signalling and cytoskeletal modifications, through interactions with the intracellular domains of their core proteins [88–90].

As the mouse lens acquires more of an antero-posterior polarity with the formation of the lens vesicle at E11.5, we see that the acute change in posterior lens vesicle cell elongation to form the first primary fibres is associated with much HSPG activity. In the first instance, we not only have PAPSS2 appearing in these cells, but also co-localising with HS-GAG and the core proteins of most HSPGs, suggesting their putative roles in the differentiation, attachment, and elongation of primary lens fibre cells. Syndecan-1 continues to remain absent not only from lens cells but from all developing eye structures. Given that PAPSS2 appears distinctly at this developmental stage, overlapping with many HSPGs and HS-GAGs, this suggests a marked rise in HS sulfotransferase activity that may be essential to drive primary lens fibre differentiation. With the differentiation of the primary fibres, we also have the concomitant differentiation of the anterior lens vesicle cells to form the epithelium. Lens epithelial cell differentiation is known to be regulated by Wnt/beta-catenin signalling [68] which in turn is regulated by glypican-3 [91], that is perfectly positioned for this, given the strong reactivity we observe in the anterior lens cells.

While we show that glypicans appear to generally localise in similar patterns to syndecans, the glypicans display temporal differences in their onset. This may be indicative of different core proteins regulating different developmental/cell fate specification/patterning events. It may be the case that the variable spatiotemporal localisation of the different HSPGs, particularly glypicans, throughout lens development is representative at least in part by their differential regulation of the morphogenetic switches in signalling events [80]. Although no lens-specific phenotypes have been reported for animals deficient in glypicans, compensatory regulation between closely related HSPGs (i.e., glypicans-2/-4 and -3/-5) is likely. Our understanding of this family of HSPGs and how they function in different tissues is still very limited.

The larger secreted HSPGs, perlecan [35,92–94], collagen XVIII [35,92], and agrin [35,95] are recognized as the predominant HSPGs in the postnatal lens capsule; however, we have previously reported different patterns of other HSPG core proteins (syndecans-1/-2/-4 and glypicans-2/-4) in the postnatal rat lens capsule: syndecan-2 and perlecan are abundant throughout the lens capsule, while syndecan-4, glypican-2, glypican-4, collagen XVIII/endostatin, and agrin are restricted to the outer reticular lamina of the lens capsule [35]. In contrast, syndecan-1-labelling appeared to be uniquely limited to the basal lamina. In the developing mouse eye, we only observed a subset of these HSPGs in the lens capsule (syndecans-1/-2/-4, glypican-4 and perlecan), suggesting that some HSPGs (i.e., glypican-2, collagen XVIII, and agrin) are deposited in the capsule at later stages. Of the minor HSPG core proteins we observed in the developing lens, three are syndecans, that bind to several structural and matrix regulatory proteins found in the lens capsule (e.g., collagen IV, laminin, fibronectin and integrins), and have previously been implicated as key regulators of ECM assembly, cell–matrix interactions, and cell adhesion [38].

Perlecan exists in an antero-posterior gradient in the lens capsule, consistent with complementary antero-posterior gradients of FGF bioavailability and signalling activity [96,97]. Perlecan HSPGs are also required for the structural integrity of the lens capsule [36], as it is shown here to be progressively deposited to the capsule over the course of lens development, consistent with its label in the embryonic chick lens [98]. While the other large secreted high-molecular-weight HSPGs, collagen XVIII, and agrin are often reported to be associated with ECM and basement membranes, here we report that both these core proteins are present from the earliest stages of lens development, primarily within the cells

of the presumptive and developing lens, and are not observed in the lens capsule, as we previously reported in the postnatal rat lens [38]. Rather, they are primarily associated with the lens epithelium, and show particularly strong staining in lens fibre cells. Mice deficient for *col18a1*, encoding the collagen XVII core protein, have a widespread malformation of ocular tissues [99,100], with the overexpression of the endostatin domain of Collagen XVIII leading to cataract and structurally compromised basement membranes [101,102]. The overexpression of agrin also produces a dysgenic phenotype that is specific to the eye [95]. Similarly, the knock-down of agrin in zebrafish embryos results in a small eye and lens phenotype, leading to decreased HS-GAG staining in the lens, but not retina, suggesting that other HSPGs may be upregulated in response to this loss of agrin [103]. The knockdown of agrin in zebrafish also leads to selectively decreased FGF-signalling and the downstream activity of MAPK/ERK in the lens, providing strong evidence that agrin is likely regulating FGF-ERK signalling activity in the developing lens [103]. Interestingly, the strong pattern of HSPG-labelling in the posterior lens vesicle cells mirrors that of phospho-ERK1/2 staining [104]. Since FGF/ERK-signalling is required for lens fibre cell elongation [105], and agrin is strongly localised to both the rapidly elongating primary and secondary lens fibres, it may be playing an important role in FGF-induced lens fibre differentiation. Notably, agrin is absent from the transitional zone of the lens, where cells exit the cell cycle and begin to differentiate into fibres, instead of selectively staining the elongated/ing lens fibres.

Here, we report on the immunolabelling of HS-GAG and specific core proteins in the nuclei of lens epithelial cells (syndecan-2) and fibre cells (syndecan-2, glypican-1, glypican-5, perlecan, and agrin). Some of these HSPGs have previously been reported in the cell nuclei of other tissues, such as glypican-1 (neurons, glial cells); [106] and agrin (developing rat brain cells) [107], while other HSPGs (i.e., glypican-5), to the best of our knowledge, are reported herein for the first time in the nuclei of lens cells. HS-GAG and HSPG core proteins in select cell types have been proposed to play roles in the transport of cargo or growth factor/receptor complexes to the cell nucleus (e.g., FGF/FGFR) to regulate gene expression [35,108]. The presence or absence of HSPGs correlates with cellular function, phenotype, and changes in cell cycle phases, suggesting that their placement in the nucleus may control cellular function [35,108]. Here, we report that most HSPGs associated with lens cell nuclei are present in the transitional zone or in new fibre cells, where the lens epithelial cells exit the cell cycle and undergo major morphological changes to differentiate into fibre cells, suggesting that these specific HSPGs may play a regulatory role in these processes. Further investigations are necessary to elucidate the specific functional roles of HSPGs in lens cells. The current study is the first to comprehensively map all HSPG localisation in a developing tissue during organogenesis. The unique patterns of expression observed here for HSPG core proteins in the developing lens imply that, during differentiation, lens cells acquire a distinct set of HSPGs that may interact selectively with proteins, resulting in distinct patterns of HS-protein binding and signalling regulation. Moreover, our findings suggest that different HSPGs may play distinct and overlapping roles at different stages of lens induction and morphogenesis. In conjunction with earlier research, our findings also suggest that HS-GAG sulfation is necessary for growth factor-induced cellular processes in the lens, and that PAPSS2 plays a distinct temporal role as a sulphate donor. By further characterizing and specifically modulating the different sulphated HSPGs expressed in the lens, we hope to better understand how HSPGs regulate different growth factor-mediated signalling, and its respective lens cell behaviour. With this, the specific roles of HSPGs in modulating growth factor activity could facilitate the development of innovative approaches for manipulating cellular behaviour. Such strategies could have broader implications beyond the context of lens biology and diseases, extending to other growth factor-mediated pathologies.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells12101364/s1>, Figure S1: Distribution of glypican-5 core protein in the 10-day-old rat eye; Table S1: Primary antibodies used for GAG and core protein immunolocalisation.

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References

- McAvoy, J.W.; Chamberlain, C.G.; de Longh, R.U.; Hales, A.M.; Lovicu, F.J. Lens development. *Eye* **1999**, *13*, 425–437. [\[CrossRef\]](#)
- Lovicu, F.J.; Ang, S.; Chorazyczewska, M.; McAvoy, J.W. Deregulation of lens epithelial cell proliferation and differentiation during the development of TGF β -induced anterior subcapsular cataract. *Dev. Neurosci.* **2004**, *26*, 446–455. [\[CrossRef\]](#)
- Wormstone, M.I.; Wride, M.A. The ocular lens: A classic model for development, physiology and disease. *Philos. Trans. R. Soc. B Biol. Sci.* **2011**, *366*, 1190–1192. [\[CrossRef\]](#)
- Lovicu, F.J.; McAvoy, J.W. Growth factor regulation of lens development. *Dev. Biology* **2005**, *280*, 1–14. [\[CrossRef\]](#)
- Cvekl, A.; Ashery-Padan, R. The cellular and molecular mechanisms of vertebrate lens development. *Development* **2014**, *141*, 4432–4447. [\[CrossRef\]](#)
- Cvekl, A.; Zhang, X. Signaling and Gene Regulatory Networks in Mammalian Lens Development. *Trends Genet.* **2017**, *33*, 677–702. [\[CrossRef\]](#)
- McAvoy, J.W.; Chamberlain, C.G.; de Longh, R.U.; Richardson, N.A.; Lovicu, F.J. The role of fibroblast growth factor in eye lens development. *Ann. N.Y. Acad. Sci.* **1991**, *638*, 256–274. [\[CrossRef\]](#)
- Faber, S.C.; Dimanlig, P.; Makarenkova, H.P.; Shirke, S.; Ko, K.; Lang, R.A. Fgf receptor signaling plays a role in lens induction. *Development* **2001**, *128*, 4425–4438. [\[CrossRef\]](#)
- Robinson, M.L. An essential role for FGF receptor signaling in lens development. *Semin. Cell Dev. Biol.* **2006**, *17*, 726–740. [\[CrossRef\]](#)
- De Longh, R.U.; Abud, H.E.; Hime, G.R. WNT/Frizzled signaling in eye development and disease. *Front. Bioscience* **2006**, *11*, 2442–2464. [\[CrossRef\]](#)
- Chen, Y.; Stump, R.J.W.; Lovicu, F.J.; Shimono, A.; McAvoy, J.W. Wnt signaling is required for organization of the lens fiber cell cytoskeleton and development of lens three-dimensional architecture. *Dev. Biol.* **2008**, *324*, 161–176. [\[CrossRef\]](#)
- Sugiyama, Y.; Lovicu, F.J.; McAvoy, J.W. Planar cell polarity in the mammalian eye lens. *Organogenesis* **2011**, *7*, 191–201. [\[CrossRef\]](#) [\[PubMed\]](#)
- Faber, S.C.; Robinson, M.L.; Makarenkova, H.P.; Lang, R.A. Bmp signaling is required for development of primary lens fiber cells. *Development* **2002**, *129*, 3727–3737. [\[CrossRef\]](#)
- Shu, D.Y.; Lovicu, F.J. Insights into Bone Morphogenetic Protein—(BMP-) Signaling in Ocular Lens Biology and Pathology. *Cells* **2021**, *10*, 2604. [\[CrossRef\]](#)
- Tsonis, P.A.; Vergara, M.N.; Spence, J.R.; Madhavan, M.; Kramer, E.L.; Call, M.K.; Santiago, W.G.; Vallance, J.E.; Robbins, D.J.; Del Rio-Tsonis, K. A novel role of the hedgehog pathway in lens regeneration. *Dev. Biol.* **2004**, *267*, 450–461. [\[CrossRef\]](#) [\[PubMed\]](#)
- Pillai-Kastoori, L.; Wen, W.; Wilson, S.G.; Strachan, E.; Lo-Castro, A.; Fichera, M.; Musumeci, S.A.; Lehmann, O.J.; Morris, A.C. Sox11 Is Required to Maintain Proper Levels of Hedgehog Signaling during Vertebrate Ocular Morphogenesis. *PLoS Genet.* **2014**, *10*, e1004491. [\[CrossRef\]](#)
- Sarrazin, S.; Lamanna, W.C.; Esko, J.D. Heparan sulfate proteoglycans. *Cold Spring Harb. Perspect. Biol.* **2011**, *3*, a004952. [\[CrossRef\]](#) [\[PubMed\]](#)
- Dreyfuss, J.L.; Regatieri, C.V.; Jarrouge, T.R.; Cavalheiro, R.P.; Sampaio, L.O.; Nader, H.B. Heparan sulfate proteoglycans: Structure, protein interactions and cell signaling. *An. Acad. Bras. Cienc.* **2009**, *81*, 409–429. [\[CrossRef\]](#)
- Wong, K.O.; Wong, K.P. Direct measurement and regulation of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) generation in vitro. *Biochem. Pharmacol.* **1996**, *52*, 1187–1194. [\[CrossRef\]](#)
- Schröder, E.; Gebel, L.; Ereemeev, A.A.; Morgner, J.; Grum, D.; Knauer, S.K.; Bayer, P.; Mueller, J.W. Human PAPS Synthase Isoforms Are Dynamically Regulated Enzymes with Access to Nucleus and Cytoplasm. *PLoS ONE* **2012**, *7*, e29559. [\[CrossRef\]](#)
- Quarto, N.; Amalric, F. Heparan sulfate proteoglycans as transducers of FGF-2 signalling. *J. Cell Sci.* **1994**, *107 Pt 11*, 3201–3212. [\[CrossRef\]](#) [\[PubMed\]](#)

22. Ornitz, D.M. FGFs, heparan sulfate and FGFRs: Complex interactions essential for development. *BioEssays* **2000**, *22*, 108–112. [\[CrossRef\]](#)
23. Ashikari-Hada, S.; Habuchi, H.; Kariya, Y.; Itoh, N.; Reddi, A.H.; Kimata, K. Characterization of Growth Factor-binding Structures in Heparin/Heparan Sulfate Using an Octasaccharide Library. *J. Biol. Chem.* **2004**, *279*, 12346–12354. [\[CrossRef\]](#)
24. Pegge, J.; Tatsinkam, A.J.; Rider, C.C.; Bell, E. Heparan sulfate proteoglycans regulate BMP signalling during neural crest induction. *Dev. Biol.* **2020**, *460*, 108–114. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Fuerer, C.; Habib, S.J.; Nusse, R. A study on the interactions between heparan sulfate proteoglycans and Wnt proteins. *Dev. Dyn.* **2010**, *239*, 184–190. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Mii, Y.; Takada, S. Heparan Sulfate Proteoglycan Clustering in Wnt Signaling and Dispersal. *Front. Cell Dev. Biol.* **2020**, *8*, 631. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Carrasco, H.; Olivares, G.H.; Faunes, F.; Oliva, C.; Larraín, J. Heparan sulfate proteoglycans exert positive and negative effects in Shh activity. *J. Cell. Biochem.* **2005**, *96*, 831–838. [\[CrossRef\]](#)
28. Palma, V.; Carrasco, H.; Reinchisi, G.; Olivares, G.; Faunes, F.; Larraín, J. SHh activity and localization is regulated by perlecan. *Biol. Res.* **2011**, *44*, 63–67. [\[CrossRef\]](#)
29. Couchman, J.R.; Woods, A. Syndecan-4 and integrins: Combinatorial signaling in cell adhesion. *J. Cell Sci.* **1999**, *112*, 3415–3420. [\[CrossRef\]](#)
30. Yang, N. Syndecan-1-induced ECM fiber alignment requires integrin $\alpha v \beta 3$ and syndecan-1 ectodomain and heparan sulfate chains. *PLoS ONE* **2016**, *11*, e0150132. [\[CrossRef\]](#)
31. Carulli, S.; Beck, K.; Dayan, G.; Boulesteix, S.; Lortat-Jacob, H.; Rousselle, P. Cell surface proteoglycans syndecan-1 and -4 bind overlapping but distinct sites in laminin $\alpha 3$ LG45 protein domain. *J. Biol. Chem.* **2012**, *287*, 12204–12216. [\[CrossRef\]](#) [\[PubMed\]](#)
32. Tumova, S.; Woods, A.; Couchman, J.R. Heparan sulfate chains from glypican and syndecans bind the Hep II domain of fibronectin similarly despite minor structural differences. *J. Biol. Chem.* **2000**, *275*, 9410–9417. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Bishop, J.R.; Schuksz, M.; Esko, J.D. Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature* **2007**, *446*, 1030–1037. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Hufnagel, L.; Kreuger, J.; Cohen, S.M.; Shraiman, B.I. On the role of glypicans in the process of morphogen gradient formation. *Dev. Biol.* **2006**, *300*, 512–522. [\[CrossRef\]](#)
35. Wishart, T.F.L.; Lovicu, F.J. An atlas of heparan sulfate proteoglycans in the postnatal rat lens. *Investig. Ophthalmol. Vis. Sci.* **2021**, *62*, 5. [\[CrossRef\]](#)
36. Rossi, M. Heparan sulfate chains of perlecan are indispensable in the lens capsule but not in the kidney. *EMBO J.* **2003**, *22*, 236–245. [\[CrossRef\]](#)
37. Pan, Y.; Woodbury, A.; Esko, J.D.; Grobe, K.; Zhang, X. Heparan sulfate biosynthetic gene *Ndst1* is required for FGF signaling in early lens development. *Development* **2006**, *133*, 4933–4944. [\[CrossRef\]](#)
38. Wishart, T.F.L.; Lovicu, F.J. Heparan sulfate proteoglycans (HSPGs) of the ocular lens. *Prog. Retin. Eye Res.* **2023**, *93*, 101118. [\[CrossRef\]](#)
39. Qu, X.; Carbe, C.; Tao, C.; Powers, A.; Lawrence, R.; van Kuppevelt, T.H.; Cardoso, W.V.; Grobe, K.; Esko, J.D.; Zhang, X. Lacrimal gland development and *Fgf10-Fgfr2b* signaling are controlled by 2-O- and 6-O-sulfated heparan sulfate. *J. Biol. Chem.* **2011**, *286*, 14435–14444. [\[CrossRef\]](#)
40. Kakrana, A.; Yang, A.; Anand, D.; Djordjevic, D.; Ramachandruni, D.; Singh, A.; Huang, H.; Ho, J.W.K.; Lachke, S.A. ISyTE 2.0: A database for expression-based gene discovery in the eye. *Nucleic Acids Res.* **2018**, *46*, D875–D885. [\[CrossRef\]](#)
41. Khan, S.Y.; Hackett, S.F.; Lee, M.-C.W.; Pourmand, N.; Talbot, C.C.; Riazuddin, S.A. Transcriptome Profiling of Developing Murine Lens Through RNA Sequencing. *Investig. Ophthalmol. Vis. Sci.* **2015**, *56*, 4919–4926. [\[CrossRef\]](#) [\[PubMed\]](#)
42. Hoang, T.V.; Kumar, P.K.R.; Sutharzan, S.; Tsonis, P.A.; Liang, C.; Robinson, M.L. Comparative transcriptome analysis of epithelial and fiber cells in newborn mouse lenses with RNA sequencing. *Mol. Vis.* **2014**, *20*, 1491–1517. [\[PubMed\]](#)
43. Cavaleiro, G.R.; Matos-Rodrigues, G.E.; Zhao, Y.; Gomes, A.L.; Anand, D.; Predes, D.; de Lima, S.; Abreu, J.G.; Zheng, D.; Lachke, S.A.; et al. N-myc regulates growth and fiber cell differentiation in lens development. *Dev. Biol.* **2017**, *429*, 105–117. [\[CrossRef\]](#)
44. Audette, D.S.; Anand, D.; So, T.; Rubenstein, T.B.; Lachke, S.A.; Lovicu, F.J.; Duncan, M.K. Prox1 and fibroblast growth factor receptors form a novel regulatory loop controlling lens fiber differentiation and gene expression. *Development* **2016**, *143*, 318–328. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Kokenyesi, R.; Bernfield, M. Core protein structure and sequence determine the site and presence of heparan sulfate and chondroitin sulfate on syndecan-1. *J. Biol. Chem.* **1994**, *269*, 12304–12309. [\[CrossRef\]](#) [\[PubMed\]](#)
46. Gould, S.E.; Upholt, W.B.; Kosher, R.A. Characterization of Chicken Syndecan-3 as a Heparan Sulfate Proteoglycan and Its Expression during Embryogenesis. *Dev. Biol.* **1995**, *168*, 438–451. [\[CrossRef\]](#) [\[PubMed\]](#)
47. Winzen, U.; Cole, G.J.; Halfter, W. Agrin is a chimeric proteoglycan with the attachment sites for heparan sulfate/chondroitin sulfate located in two multiple serine-glycine clusters. *J. Biol. Chem.* **2003**, *278*, 30106–30114. [\[CrossRef\]](#)
48. Dong, S.; Cole, G.J.; Halfter, W. Expression of collagen XVIII and localization of its glycosaminoglycan attachment sites. *J. Biol. Chem.* **2003**, *278*, 1700–1707. [\[CrossRef\]](#)
49. Heathcote, J.G.; Orkin, R.W. Biosynthesis of Sulphated Macromolecules by Rabbit Lens Epithelium. I. Identification of the Major Macromolecules Synthesized by Lens Epithelial Cells in vitro. *J. Cell. Biol.* **1984**, *99*, 852–860. [\[CrossRef\]](#)

50. Mohan, P.S.; Spiro, R.G. Characterization of heparan sulfate proteoglycan from calf lens capsule and proteoglycans synthesized by cultured lens epithelial cells. Comparison with other basement membrane proteoglycans. *J. Biol. Chem.* **1991**, *266*, 8567–8575. [\[CrossRef\]](#)
51. Maeda, N.; Ishii, M.; Nishimura, K.; Kamimura, K. Functions of chondroitin sulfate and heparan sulfate in the developing brain. *Neurochem. Res.* **2011**, *36*, 1228–1240. [\[CrossRef\]](#) [\[PubMed\]](#)
52. Vallet, S.D.; Clerc, O.; Ricard-Blum, S. Glycosaminoglycan-Protein Interactions: The First Draft of the Glycosaminoglycan Interactome. *J. Histochem. Cytochem.* **2020**, *69*, 93–104. [\[CrossRef\]](#) [\[PubMed\]](#)
53. Clark, S.J.; Keenan, T.D.L.; Fielder, H.L.; Collinson, L.J.; Holley, R.J.; Merry, C.L.R.; van Kuppevelt, T.H.; Day, A.J.; Bishop, P.N. Mapping the differential distribution of glycosaminoglycans in the adult human retina, choroid, and sclera. *Investig. Ophthalmol. Vis. Sci.* **2011**, *52*, 6511–6521. [\[CrossRef\]](#) [\[PubMed\]](#)
54. Smetsers, T.F.C.M.; van de Westerloo, E.M.A.; ten Dam, G.B.; Clarijs, R.; Versteeg, E.M.M.; van Geloof, W.L.; Veerkamp, J.H.; van Muijen, G.N.P.; van Kuppevelt, T.H. Localization and characterization of melanoma-associated glycosaminoglycans: Differential expression of chondroitin and heparan sulfate epitopes in melanoma. *Cancer Res.* **2003**, *63*, 2965–2970. [\[PubMed\]](#)
55. Karamanos, N.K.; Piperigkou, Z.; Theocharis, A.D.; Watanabe, H.; Franchi, M.; Baud, S.; Brézillon, S.; Götte, M.; Passi, A.; Vigetti, D.; et al. Proteoglycan chemical diversity drives multifunctional cell regulation and therapeutics. *Chem. Rev.* **2018**, *118*, 9152–9232. [\[CrossRef\]](#) [\[PubMed\]](#)
56. Filla, M.S.; David, G.; Weinreb, R.N.; Kaufman, P.L.; Peters, D.M. Distribution of syndecans 1–4 within the anterior segment of the human eye: Expression of a variant syndecan-3 and matrix-associated syndecan-2. *Exp. Eye Res.* **2004**, *79*, 61–74. [\[CrossRef\]](#)
57. Huang, J.; Rajagopal, R.; Liu, Y.; Dattilo, L.K.; Shaham, O.; Ashery-Padan, R.; Beebe, D.C. The mechanism of lens placode formation: A case of matrix-mediated morphogenesis. *Dev. Biol.* **2011**, *355*, 32–42. [\[CrossRef\]](#)
58. Lang, R.A. Pathways regulating lens induction in the mouse. *Int. J. Dev. Biol.* **2004**, *48*, 783–791. [\[CrossRef\]](#)
59. Gunhaga, L. The lens: A classical model of embryonic induction providing new insights into cell determination in early development. *Philos. Trans. Biol. Sci.* **2011**, *366*, 1193–1203. [\[CrossRef\]](#)
60. Rapraeger, A.C. Syndecan-Regulated Receptor Signaling. *J. Cell. Biol.* **2000**, *149*, 995–997. [\[CrossRef\]](#)
61. Capurro, M.I.; Xu, P.; Shi, W.; Li, F.; Jia, A.; Filmus, J. Glypican-3 inhibits Hedgehog signaling during development by competing with patched for Hedgehog binding. *Dev. Cell* **2008**, *14*, 700–711. [\[CrossRef\]](#) [\[PubMed\]](#)
62. Bányai, L.; Sonderegger, P.; Patthy, L. Agrin binds BMP2, BMP4 and TGFβ1. *PLoS ONE* **2010**, *5*, e10758. [\[CrossRef\]](#)
63. Seppinen, L.; Pihlajaniemi, T. The multiple functions of collagen XVIII in development and disease. *Matrix Biol.* **2011**, *30*, 83–92. [\[CrossRef\]](#)
64. Kerever, A.; Mercier, F.; Nonaka, R.; de Vega, S.; Oda, Y.; Zalc, B.; Okada, Y.; Hattori, N.; Yamada, Y.; Arikawa Hirasawa, E. Perlecan is required for FGF-2 signaling in the neural stem cell niche. *Stem Cell Res.* **2014**, *12*, 492–505. [\[CrossRef\]](#)
65. Jin, H.; Fisher, M.; Grainger, R.M. Defining progressive stages in the commitment process leading to embryonic lens formation. *Genesis* **2012**, *50*, 728–740. [\[CrossRef\]](#)
66. Fujimura, N. WNT/β-Catenin Signaling in Vertebrate Eye Development. *Front. Cell. Dev. Biol.* **2016**, *4*, 138. [\[CrossRef\]](#)
67. Barth, K.A.; Wilson, S.W. Expression of zebrafish nk2. 2 is influenced by sonic hedgehog/vertebrate hedgehog-1 and demarcates a zone of neuronal differentiation in the embryonic forebrain. *Development* **1995**, *121*, 1755–1768. [\[CrossRef\]](#) [\[PubMed\]](#)
68. Stump, R.; Ang, S.; Chen, Y.J.; von Bahr, T.; Lovicu, F.J.; Pinson, K.; de Iongh, R.U.; Yamaguchi, T.P.; Sassoon, D.A.; McAvoy, J.W. A role for Wnt/beta-catenin signaling in lens epithelial differentiation. *Dev. Biol.* **2003**, *259*, 48–61. [\[CrossRef\]](#) [\[PubMed\]](#)
69. Dawes, L.J.; Sugiyama, Y.; Tanedo, A.S.; Lovicu, F.J.; McAvoy, J.W. Wnt-frizzled signaling is part of an FGF-induced cascade that promotes lens fiber differentiation. *Investig. Ophthalmol. Vis. Sci.* **2013**, *54*, 1582–1590. [\[CrossRef\]](#)
70. Kerr, C.L.; Huang, J.; Williams, T.; West-Mays, J.A. Activation of the hedgehog signaling pathway in the developing lens stimulates ectopic FoxE3 expression and disruption in fiber cell differentiation. *Investig. Ophthalmol. Vis. Sci.* **2012**, *53*, 3316–3330. [\[CrossRef\]](#)
71. Boswell, B.A.; Overbeek, P.A.; Musil, L.S. Essential role of BMPs in FGF-induced secondary lens fiber differentiation. *Dev. Biol.* **2008**, *324*, 202–212. [\[CrossRef\]](#) [\[PubMed\]](#)
72. Wederell, E.D.; De Iongh, R.U. Extracellular matrix and integrin signaling in lens development and cataract. *Semin. Cell. Dev. Biol.* **2006**, *17*, 759–776. [\[CrossRef\]](#) [\[PubMed\]](#)
73. Morgan, M.R.; Humphries, M.J.; Bass, M.D. Synergistic control of cell adhesion by integrins and syndecans. *Nat. Rev. Mol. Cell. Biol.* **2007**, *8*, 957–969. [\[CrossRef\]](#)
74. Kim, M.-S.; Saunders, A.M.; Hamaoka, B.Y.; Beachy, P.A.; Leahy, D.J. Structure of the protein core of the glypican Dally-like and localization of a region important for hedgehog signaling. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 13112–13117. [\[CrossRef\]](#) [\[PubMed\]](#)
75. Roper, J.A.; Williamson, R.C.; Bass, M.D. Syndecan and integrin interactomes: Large complexes in small spaces. *Curr. Opin. Struct. Biol.* **2012**, *22*, 583–590. [\[CrossRef\]](#) [\[PubMed\]](#)
76. Parmigiani, C.; McAvoy, J. Localisation of laminin and fibronectin during rat lens morphogenesis. *Differentiation* **1984**, *28*, 53–61. [\[CrossRef\]](#) [\[PubMed\]](#)
77. Zelenka, P.S. Regulation of cell adhesion and migration in lens development. *Int. J. Dev. Biol.* **2004**, *48*, 857–865. [\[CrossRef\]](#)
78. Simirskii, V.N.; Wang, Y.; Duncan, M.K. Conditional deletion of β1-integrin from the developing lens leads to loss of the lens epithelial phenotype. *Dev. Biol.* **2007**, *306*, 658–668. [\[CrossRef\]](#) [\[PubMed\]](#)
79. Pathania, M.; Wang, Y.; Simirskii, V.N.; Duncan, M.K. β1-integrin controls cell fate specification in early lens development. *Differentiation* **2016**, *92*, 133–147. [\[CrossRef\]](#)

80. Filmus, J. The function of Glypicans in the mammalian embryo. *Am. J. Physiol. Cell. Physiol.* **2022**, *322*, C694–C698. [\[CrossRef\]](#)
81. Strate, I.; Tessadori, F.; Bakkers, J. Glypican4 promotes cardiac specification and differentiation by attenuating canonical Wnt and Bmp signaling. *Development* **2015**, *142*, 1767–1776. [\[CrossRef\]](#) [\[PubMed\]](#)
82. Pan, J.; Ho, M. Role of glypican-1 in regulating multiple cellular signaling pathways. *Am. J. Physiol. Cell. Physiol.* **2021**, *321*, C846–C858. [\[CrossRef\]](#) [\[PubMed\]](#)
83. Jidigam, V.K.; Srinivasan, R.C.; Patthey, C.; Gunhaga, L. Apical constriction and epithelial invagination are regulated by BMP activity. *Biol. Open* **2015**, *4*, 1782–1791. [\[CrossRef\]](#)
84. Chauhan, B.K.; Lou, M.; Zheng, Y.; Lang, R.A. Balanced Rac1 and RhoA activities regulate cell shape and drive invagination morphogenesis in epithelia. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 18289–18294. [\[CrossRef\]](#)
85. Grisaru, S. Glypican-3 modulates BMP- and FGF-mediated effects during renal branching morphogenesis. *Dev. Biol.* **2001**, *231*, 31–46. [\[CrossRef\]](#) [\[PubMed\]](#)
86. Souza, A.T.P.; Lopes, H.B.; Oliveira, F.S.; Weffort, D.; Freitas, G.P.; Adolpho, L.F.; Fernandes, R.R.; Rosa, A.L.; Beloti, M.M. The extracellular matrix protein Agrin is expressed by osteoblasts and contributes to their differentiation. *Cell Tissue Res.* **2021**, *386*, 335–347. [\[CrossRef\]](#)
87. Fisher, M.C.; Li, Y.; Seghatoleslami, M.R.; Dealy, C.N.; Kosher, R.A. Heparan sulfate proteoglycans including syndecan-3 modulate BMP activity during limb cartilage differentiation. *Matrix Biol.* **2006**, *25*, 27–39. [\[CrossRef\]](#)
88. Bass, M.D.; Morgan, M.R.; Humphries, M.J. Integrins and syndecan-4 make distinct, but critical, contributions to adhesion contact formation. *Soft Matter* **2007**, *3*, 372–376. [\[CrossRef\]](#)
89. Okina, E.; Manon-Jensen, T.; Whiteford, J.R.; Couchman, J.R. Syndecan proteoglycan contributions to cytoskeletal organization and contractility. *Scand. J. Med. Sci. Sports* **2009**, *19*, 479–489. [\[CrossRef\]](#)
90. Tsoyi, K.; Chu, S.G.; Patino-Jaramillo, N.G.; Wilder, J.; Villalba, J.; Doyle-Eisele, M.; McDonald, J.; Liu, X.; El-Chemaly, S.; Perrella, M.A.; et al. Syndecan-2 attenuates radiation-induced pulmonary fibrosis and inhibits fibroblast activation by regulating PI3K/Akt/ROCK pathway via CD148. *Am. J. Cell. Mol. Biol.* **2018**, *58*, 208–215. [\[CrossRef\]](#)
91. Kolluri, A.; Ho, M. The Role of Glypican-3 in Regulating Wnt, YAP, and Hedgehog in Liver Cancer. *Front. Oncol.* **2019**, *9*, 708. [\[CrossRef\]](#)
92. Uechi, G.; Sun, Z.; Schreiber, E.M.; Halfter, W.; Balasubramani, M. Proteomic View of Basement Membranes from Human Retinal Blood Vessels, Inner Limiting Membranes, and Lens Capsules. *J. Proteome Res.* **2014**, *13*, 3693–3705. [\[CrossRef\]](#) [\[PubMed\]](#)
93. Wu, W.; Tholozan, F.M.; Goldberg, M.W.; Bowen, L.; Wu, J.; Quinlan, R.A. A gradient of matrix-bound FGF-2 and perlecan is available to lens epithelial cells. *Exp. Eye Res.* **2014**, *120*, 10–14. [\[CrossRef\]](#) [\[PubMed\]](#)
94. DeDreu, J.; Walker, J.L.; Menko, A.S. Dynamics of the lens basement membrane capsule and its interaction with connective tissue-like extracapsular matrix proteins. *Matrix Biol.* **2021**, *96*, 18–46. [\[CrossRef\]](#)
95. Fuerst, P.G.; Rauch, S.M.; Burgess, R.W. Defects in eye development in transgenic mice overexpressing the heparan sulfate proteoglycan agrin. *Dev. Biol.* **2007**, *303*, 165–180. [\[CrossRef\]](#) [\[PubMed\]](#)
96. Chamberlain, C.G.; McAvoy, J.W. Fibre differentiation and polarity in the mammalian lens: A key role for FGF. *Prog. Retin. Eye Res.* **1997**, *16*, 443–478. [\[CrossRef\]](#)
97. Hubka, K.M.; Carson, D.D.; Harrington, D.A.; Farach-Carson, M.C. Perlecan domain I gradients establish stable biomimetic heparin binding growth factor gradients for cell migration in hydrogels. *Acta Biomater.* **2019**, *97*, 385–398. [\[CrossRef\]](#)
98. Soulintzi, N.; Zagris, N. Spatial and temporal expression of perlecan in the early chick embryo. *Cells Tissues Organs* **2007**, *186*, 243–256. [\[CrossRef\]](#)
99. Fukai, N.; Eklund, L.; Marneros, A.G.; Oh, S.P.; Keene, D.R.; Tamarkin, L.; Niemelä, M.; Ilves, M.; Li, E.; Pihlajaniemi, T.; et al. Lack of collagen XVIII/endostatin results in eye abnormalities. *EMBO J.* **2002**, *21*, 1535–1544. [\[CrossRef\]](#)
100. Marneros, A.G.; Keene, D.R.; Hansen, U.; Fukai, N.; Moulton, K.; Goletz, P.L.; Moiseyev, G.; Pawlyk, B.S.; Halfter, W.; Dong, S.; et al. Collagen XVIII/endostatin is essential for vision and retinal pigment epithelial function. *EMBO J.* **2004**, *23*, 89–99. [\[CrossRef\]](#)
101. Elamaa, H.; Sormunen, R.; Rehn, M.; Soininen, R.; Pihlajaniemi, T. Endostatin Overexpression Specifically in the Lens and Skin Leads to Cataract and Ultrastructural Alterations in Basement Membranes. *Am. J. Pathol.* **2005**, *166*, 221–229. [\[CrossRef\]](#)
102. Aikio, M.; Hurskainen, M.; Brideau, G.; Hägg, P.; Sormunen, R.; Heljasvaara, R.; Gould, D.B.; Pihlajaniemi, T. Collagen XVIII short isoform is critical for retinal vascularization, and overexpression of the Tsp-1 domain affects eye growth and cataract formation. *Investig. Ophthalmol. Vis. Sci.* **2013**, *54*, 7450–7462. [\[CrossRef\]](#)
103. Liu, I.H.; Zhang, C.; Min, J.K.; Cole, G.J. Retina development in zebrafish requires the heparan sulfate proteoglycan agrin. *Dev. Neurobiol.* **2008**, *68*, 877–898. [\[CrossRef\]](#) [\[PubMed\]](#)
104. Zhao, H.; Yang, T.; Madakashira, B.P.; Thiels, C.A.; Bechtel, C.A.; Garcia, C.M.; Zhang, H.; Yu, K.; Ornitz, D.M.; Beebe, D.C.; et al. Fibroblast growth factor receptor signaling is essential for lens fiber cell differentiation. *Dev. Biol.* **2008**, *318*, 276–288. [\[CrossRef\]](#) [\[PubMed\]](#)
105. Lovicu, F.J.; McAvoy, J.W. FGF-induced lens cell proliferation and differentiation is dependent on MAPK (ERK1/2) signalling. *Development* **2001**, *128*, 5075–5084. [\[CrossRef\]](#)
106. Liang, Y.; Häring, M.; Roughley, P.J.; Margolis, R.K.; Margolis, R.U. Glypican and biglycan in the nuclei of neurons and glioma cells: Presence of functional nuclear localization signals and dynamic changes in glypican during the cell cycle. *J. Cell. Biol.* **1997**, *139*, 851–864. [\[CrossRef\]](#) [\[PubMed\]](#)

107. Expression of agrin in the developing and adult rat brain. *Neuroscience* **1997**, *76*, 581–596. [[CrossRef](#)]
108. Stewart, M.D.; Sanderson, R.D. Heparan sulfate in the nucleus and its control of cellular functions. *Matrix Biol.* **2014**, *35*, 56–59. [[CrossRef](#)]

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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 response²¹ 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

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 $\log(\Delta Ct)$ relative to *Gapdh* $\pm$ 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 μ m. 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 –1⁴⁷ and –3,⁴⁸ agrin,⁴⁹ and collagen XVIII/endostatin⁵⁰) 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 bisbenzimidazole (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 μ g/mL L-glutamine, 50 IU/mL penicillin, 50 μ g/mL streptomycin, 2.5 μ g/mL Amphostat B, and 0.1% bovine serum albumin (BSA; all from Sigma, Castle Hill, New South Wales, Australia) at 37°C in 5% CO₂. 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 (NaClO₃, in M199; Sigma)^{16,56} or with up to 10 μ M 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 NaClO₃)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 μ M) for 24 hours to assess cell viability. Explants treated with Surfen (5 μ M) 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 μ g/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 described¹⁷ 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, Wetzlar, 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 μ M) 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 differentiation⁵⁸), with or without Surfen (5 μ M), 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 μ M), were fixed (10% NBF for 5 minutes) 5 days after treatment and immunolabeled for markers of fiber differentiation: rabbit anti- β -crystallin²³ and mouse

anti-Aquaporin-0/MIP⁵⁹ (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 $\pm$ 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-Šidák 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 *Sdc2*, *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 –1⁴⁷ and –3,⁴⁸ agrin,⁴⁹ and collagen XVIII/endostatin⁵⁰) 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 CS⁵³ (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

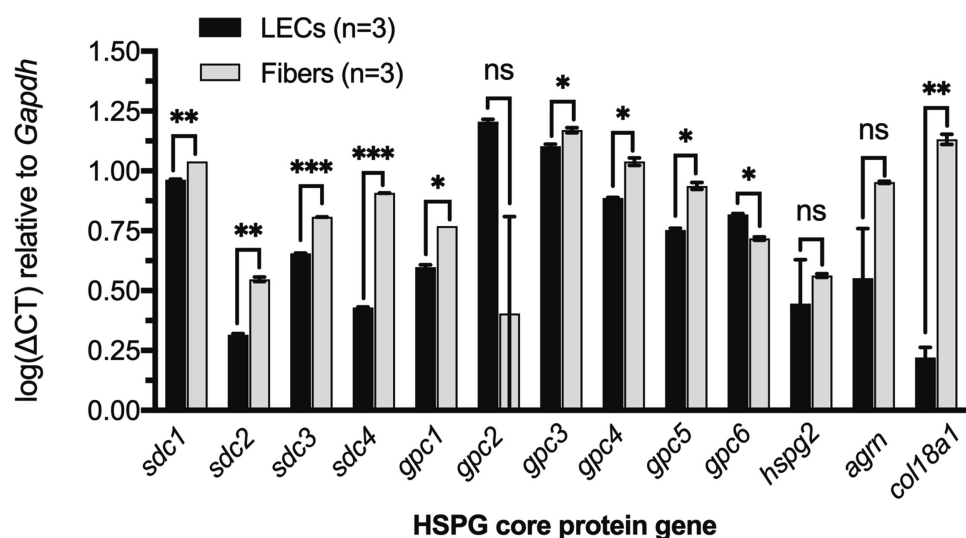


FIGURE 1. Relative expression of HSPG core protein transcripts in 10-day-old rat lens epithelial and fiber cells. Data are represented as $\log(\Delta Ct)$ of HSPG core protein transcript expression in lens epithelial cells (LECs; dark bar) or Fibers (grey bar), relative to *Gapdh* $\pm$ standard error 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-Šidák method. Significance threshold set at $P > 0.05$ ($P > 0.05 = ns$, not significant; $P < 0.05 = *$; $P < 0.01 = **$; $P < 0.001 = ***$).

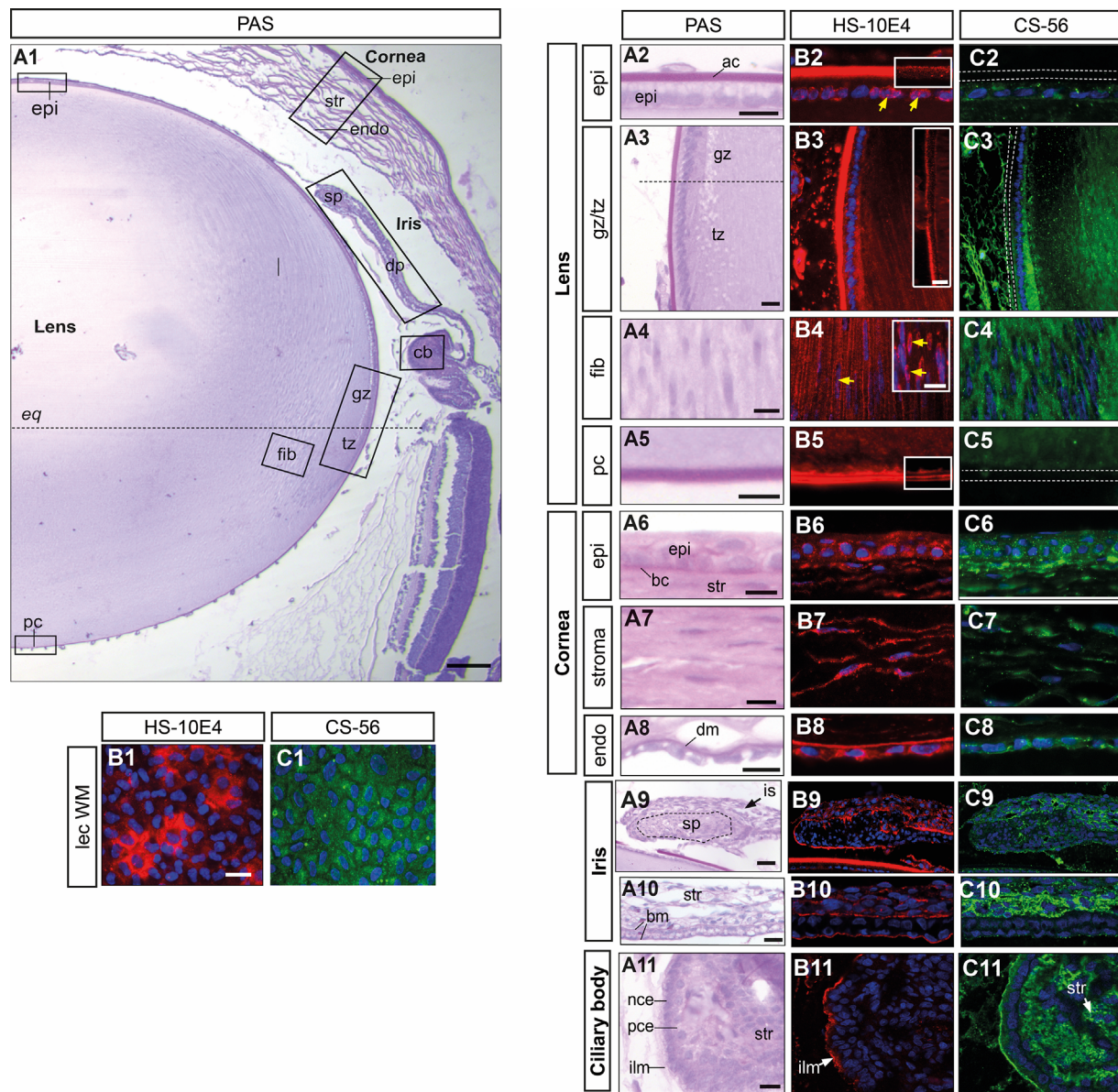


FIGURE 2. Distribution of sulfated glycosaminoglycans in the 10-day-old rat eye. Mid-sagittal eye sections stained with Periodic acid-Schiffs (PAS; A1–11) show morphology of regions of interest. Corresponding sections (B2–B11, C2–C11) and 10-day-old rat lens epithelial whole-mounts (lec WM; B1, C1) immunolabel the highly sulfated domains on heparan sulfate (HS; B1–11) or chondroitin sulfate (CS; C1–11) glycosaminoglycan chains. Yellow arrows (B2, B4) indicate nuclear localization. Dotted white lines demarcate outline of the lens capsule. Insets in B2, B3, and B5 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 μ m (A1), 25 μ m (A2–11, B1–11, C1–11).

cells (see Figs. 2B1, 2B2, 2B3) and on the cell membranes of newly elongating lens fiber cells (see Figs. 2B3, 2B4). 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, 2B4 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

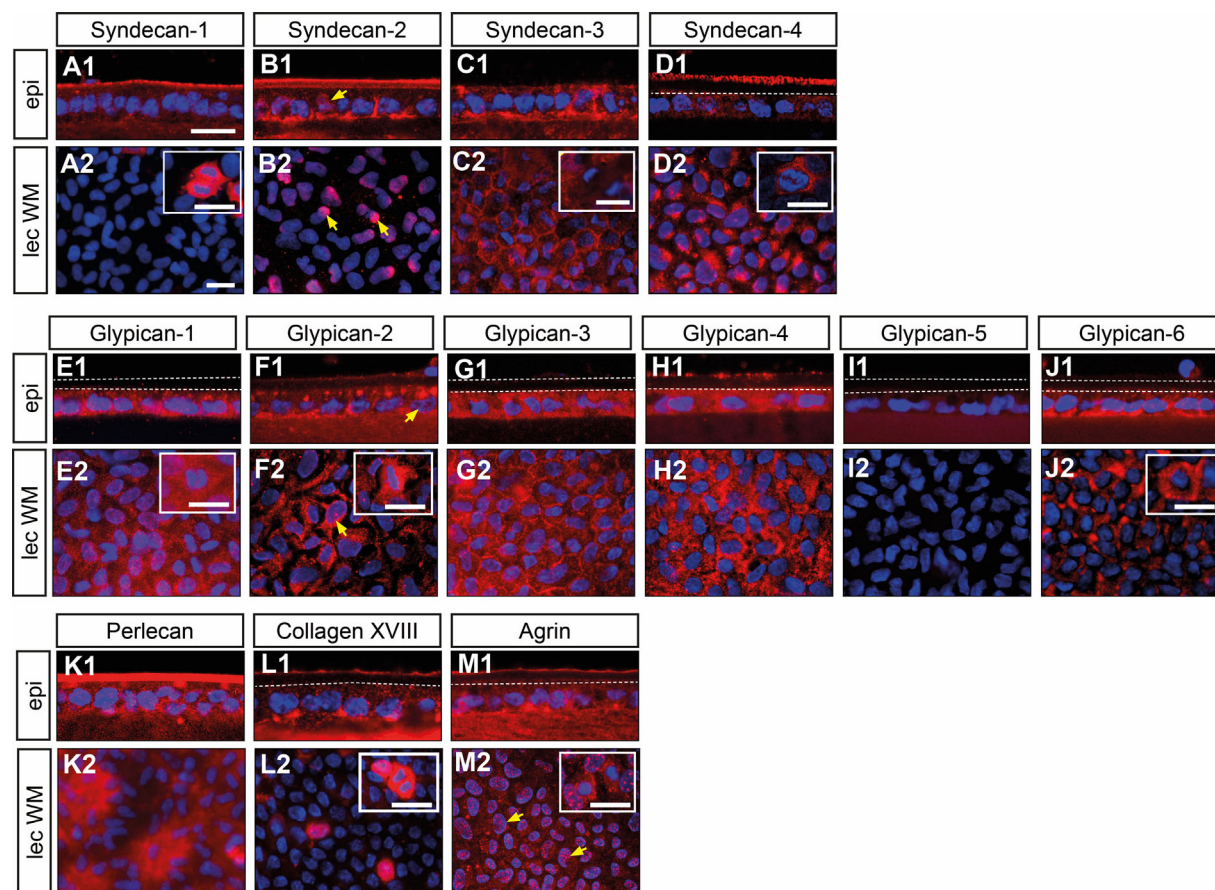


FIGURE 3. Anterior lens capsule and epithelia. Representative immunofluorescence (red) of HSPG core proteins for Syndecan-1 to -4 (A–D), Glypican-1 to -6 (E–J), Perlecan (K), Collagen XVIII/endostatin (L), and Agrin (M) in sections (1-series) or epithelial wholemounts (lec WM, 2-series) of 10-day-old 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 μ m.

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

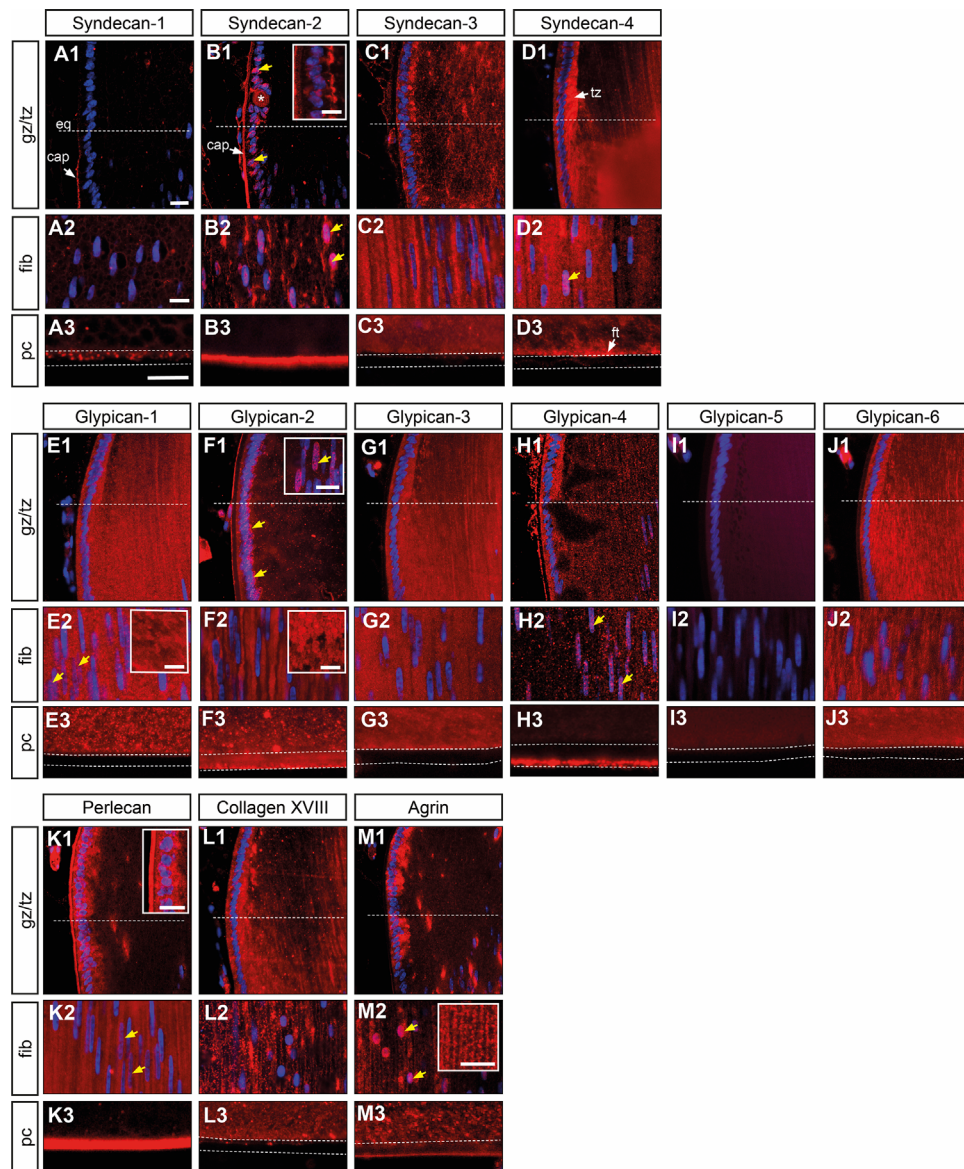


FIGURE 4. Equatorial lens, cortex and posterior capsule. Representative immunofluorescence (red) of HSPG core proteins for Syndecan-1 to -4 (A–D), Glypican-1 to -6 (E–J), Perlecan (K), Collagen XVIII (L), and Agrin (M) in sections of the equatorial germinative/transitional zone (gz/tz; 1-series), outer 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 μ m.

(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, telophase), 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 μ M 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 μ M 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 μ M ($P = 0.007$; 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 μ M 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 μ M ($P = 0.02$; see Fig. 5D), and completely with 5 μ M ($P < 0.001$; 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

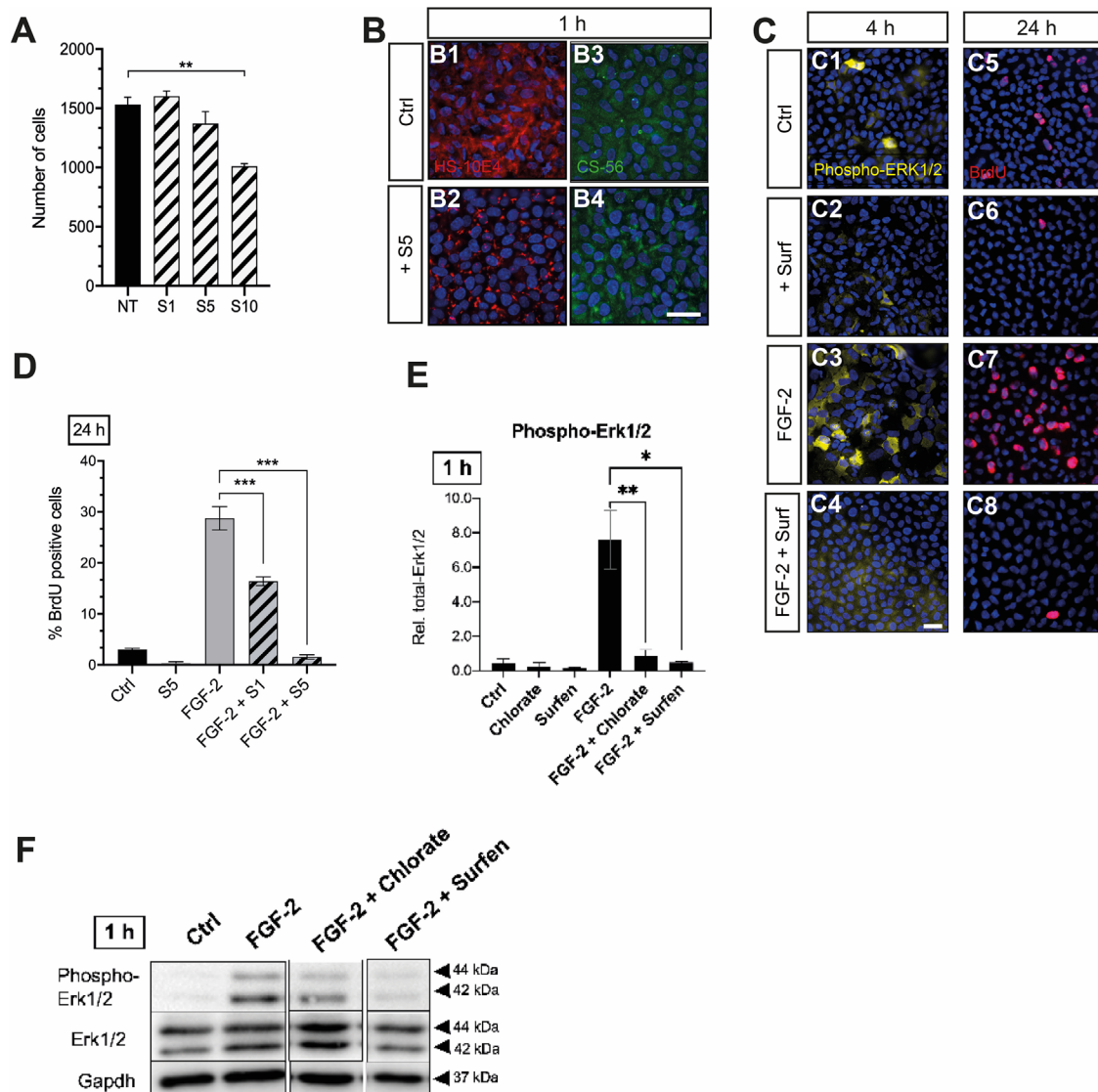


FIGURE 5. Surfen blocks FGF-2-induced lens epithelial cell proliferation and pERK1/2. The 10-day-old rat lens epithelial explants treated for 24 hours with increasing doses of Surfen (1 μ M, S1; 5 μ M, S5; or 10 μ M, S10) had decreased cell viability (**A**; number of cells). (**B**) Treatment with 5 μ M Surfen alone (**B2**, **B4**) for 1 hour decreased immunolabeling of heparan sulfate (HS-10E4; **B2**) relative to control explants (**B1**), but not chondroitin sulfate (CS-56; **B3**, **B4**). Surfen (up to 5 μ M) 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, C1–4). (**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 μ M) at 1 hour. Data are the mean $\pm$ SEM from at least three independent experiments, applying unpaired two-tailed Student's *t*-test analysis (* P = 0.014; ** P = 0.006). Scale bar = 25 μ m.

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

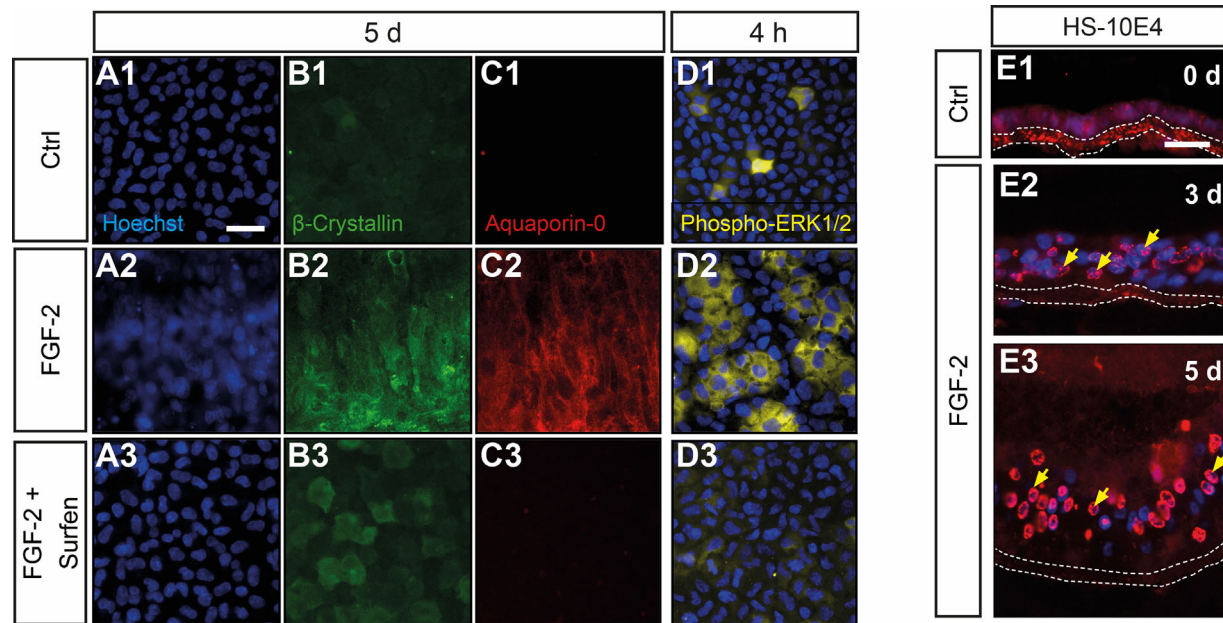


FIGURE 6. Sulfation of heparan sulfate is required for FGF-2-induced lens fiber differentiation. The 10-day-old rat lens epithelial explants were treated up to 5 days with a differentiating dose of FGF-2 (150 ng/mL), with or without Surfen (5 μ M). Relative to controls (A1–D1), explants treated with 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 μ m.

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 Assay⁶⁵ 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-4⁸⁵ 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 Pataki³⁹ 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 endometrium¹⁰² 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 integrins¹⁰⁵ 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-actin¹¹¹ and β 1 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 (PKC α) 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, β 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 β 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 func-

tions, such as proliferation and differentiation (reviewed in Stewart and Sanderson¹²⁴). 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 C α -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 β 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 RMRKK 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/MIP¹⁴¹ 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 signal-

ing 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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References

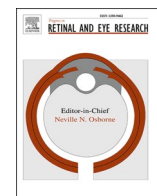
1. Wormstone IM, Wride MA. Introduction: The ocular lens: a classic model for development, physiology and disease. *Philosophic Trans Biologic Sci.* 2011;366(1568):1190–1192.
2. Mochizuki T, Masai I. The lens equator: A platform for molecular machinery that regulates the switch from cell proliferation to differentiation in the vertebrate lens. *Dev Growth Differ.* 2014;56(5):387–401.
3. Lovicu FJ, Iyengar L, Dawes LJ, McAvoy JW. Lens epithelial cell proliferation. In: *Lens Epithelium and Posterior Capsular Opacification*. Tokyo: Springer Japan; 2014:59–80.
4. Wride MA. Lens fibre cell differentiation and organelle loss: many paths lead to clarity. *Philosophic Trans Biologic Sci.* 2011;366(1568):1219–1233.
5. Iyengar L, Patkunanathan B, McAvoy JW, Lovicu FJ. Growth factors involved in aqueous humour-induced lens cell proliferation. *Growth Factors.* 2009;27(1):50–62.
6. Wang Q, McAvoy JW, Lovicu FJ. Growth Factor Signaling in Vitreous Humor-Induced Lens Fiber Differentiation. *Invest Ophthalmol Vis Sci.* 2010;51(7):3599–3610.
7. Shirke S, Faber SC, Hallem E, et al. Misexpression of IGF-I in the mouse lens expands the transitional zone and perturbs lens polarization. *Mech Dev.* 2001;101(1-2):167–174.
8. Majima K. Human lens epithelial cells proliferate in response to exogenous EGF and have EGF and EGF receptor. *Ophthalmic Res.* 1995;27(6):356–365.
9. Kok A, Lovicu FJ, Chamberlain CG, McAvoy JW. Influence of platelet-derived growth factor on lens epithelial cell proliferation and differentiation. *Growth Factors.* 2002;20(1):27–34.
10. Li H, Mao Y, Bouaziz M, et al. Lens differentiation is controlled by the balance between PDGF and FGF signaling. Bronner ME, ed. *PLoS Biol.* 2019;17(2):e3000133.
11. Choi J, Park SY, Joo CK. Hepatocyte growth factor induces proliferation of lens epithelial cells through activation of ERK1/2 and JNK/SAPK. *Invest Ophthalmol Vis Sci.* 2004;45(8):2696–2704.
12. Cheng C, Gong X. Diverse roles of Eph/ephrin signaling in the mouse lens. Buday L, ed. *PLoS One.* 2011;6(11):e28147.
13. Zhou Y, Shiels A. EphA2 and Efna5 participate in lens cell pattern-formation. *Differentiation.* 2018;102:1–9.
14. Belecky-Adams TL, Adler R, Beebe DC. Bone morphogenetic protein signaling and the initiation of lens fiber cell differentiation. *Development.* 2002;129(16):3795–3802.
15. Zhao H, Yang T, Madakashira BP, et al. Fibroblast growth factor receptor signaling is essential for lens fiber cell differentiation. *Dev Biol.* 2008;318(2):276–288.
16. Iyengar L, Lovicu FJ. Aqueous humour-induced lens epithelial cell proliferation requires FGF-signalling. *Growth Factors.* 2017;35(4-5):131–143.
17. Iyengar L, Patkunanathan B, Lynch OT, McAvoy JW, Rasko JEJ, Lovicu FJ. Aqueous humour- and growth factor-induced lens cell proliferation is dependent on MAPK/ERK1/2 and Akt/PI3-K signalling. *Exp Eye Res.* 2006;83(3):667–678.

18. Wang Q, Stump R, McAvoy JW, Lovicu FJ. MAPK/ERK1/2 and PI3-kinase signalling pathways are required for vitreous-induced lens fibre cell differentiation. *Exp Eye Res.* 2009;88(2):293–306.
19. Robinson ML. An essential role for FGF receptor signaling in lens development. *Seminars in Cell & Developmental Biology.* 2006;17(6):726–740.
20. Chamberlain CG, McAvoy JW. Fibre differentiation and polarity in the mammalian lens: A key role for FGF. *Progress in Retinal and Eye Research.* 1997;16(3):443–478.
21. Chamberlain CG, McAvoy JW. Induction of Lens fibre differentiation by acidic and basic fibroblast growth factor (FGF). *Growth Factors.* 1989;1(2):125–134.
22. Lovicu FJ, McAvoy JW. FGF-induced lens cell proliferation and differentiation is dependent on MAPK (ERK1/2) signalling. *Development.* 2001;128(24):5075–5084.
23. Lovicu FJ, McAvoy JW. FGF-induced lens cell proliferation and differentiation is dependent on MAPK (ERK1/2) signalling. *Development.* 2001;128(24):5075–5084.
24. de Iongh RU, Lovicu FJ, Hanneken A, Baird A, McAvoy JW. FGF receptor-1 (flg) expression is correlated with fibre differentiation during rat lens morphogenesis and growth. *Developmental Dynamics.* 1996;206(4):412–426.
25. Bishop JR, Schuksz M, Esko JD. Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature.* 2007;446(7139):1030–1037.
26. Esko JD, Prestegard JH, Linhardt RJ. Proteins That Bind Sulfated Glycosaminoglycans. In: Varki A, Cummings RD, Esko JD, et al., eds. *Essentials of Glycobiology*. 3rd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 2017:493–503.
27. Park PW, Reizes O, Bernfield M. Cell surface heparan sulfate proteoglycans: selective regulators of ligand-receptor encounters. *J Biol Chem.* 2000;275(39):29923–29926.
28. Forsten-Williams K, Chu CL, Fannon M, Buczek-Thomas JA, Nugent MA. Control of growth factor networks by heparan sulfate proteoglycans. *Annals of Biomedical Engineering.* 2008;36(12):2134–2148.
29. Matsuo I, Kimura-Yoshida C. Extracellular distribution of diffusible growth factors controlled by heparan sulfate proteoglycans during mammalian embryogenesis. *Philosophical Transactions: Biological Sciences.* 2014;369(1657):1–9.
30. Shworak NW. Modulation of Growth Factor Signaling by Heparan Sulfate Proteoglycans. In: Simons M, Rubanyi GM, eds. *Modern Concepts in Angiogenesis*. London, England, UK: Published by Imperial College Press and Distributed by World Scientific Publishing Co.; 2011:119–146.
31. Sun C, Liu M, Sun P, et al. Sulfated polysaccharides interact with fibroblast growth factors and protect from denaturation. *FEBS Open Bio.* 2019;9(8):1477–1487.
32. Lin X. Functions of heparan sulfate proteoglycans in cell signaling during development. *Development.* 2004;131(24):6009–6021.
33. Afratis NA, Nikitovic D, Multhaupt HAB, Theocharis AD, Couchman JR, Karamanos NK. Syndecans – key regulators of cell signaling and biological functions. *The FEBS Journal.* 2017;284(1):27–41.
34. Fico A, Maina F, Dono R. Fine-tuning of cell signaling by glypicans. *Cell Mol Life Sci.* 2011;68(6):923–929.
35. Schaefer L. Proteoglycans, key regulators of cell-matrix dynamics. *Matrix Biol.* 2014;35:1–2.
36. Poulain FE, Yost HJ. Heparan sulfate proteoglycans: a sugar code for vertebrate development? *Development.* 2015;142(20):3456–3467.
37. Rossi M. Heparan sulfate chains of perlecan are indispensable in the lens capsule but not in the kidney. *The EMBO Journal.* 2003;22(2):236–245.
38. Fuerst PG, Rauch SM, Burgess RW. Defects in eye development in transgenic mice overexpressing the heparan sulfate proteoglycan agrin. *Dev Biol.* 2007;303(1):165–180.
39. Couchman JR, Pataki CA. An Introduction to Proteoglycans and Their Localization. *Journal of Histochemistry & Cytochemistry.* 2012;60(12):885–897.
40. Ford-Perriss M, Turner K, Guimond S, et al. Localisation of specific heparan sulfate proteoglycans during the proliferative phase of brain development. *Developmental Dynamics.* 2003;227(2):170–184.
41. Hubka KM, Carson DD, Harrington DA, MC Farach-Carson. Perlecan domain I gradients establish stable biomimetic heparin binding growth factor gradients for cell migration in hydrogels. *Acta Biomaterialia.* 2019;97:385–398.
42. Wu W, Tholozan FM, Goldberg MW, Bowen L, Wu J, Quinlan RA. A gradient of matrix-bound FGF-2 and perlecan is available to lens epithelial cells. *Exp Eye Res.* 2014;120:10–14.
43. Zhang Y, Li J, Partovian C, Sellke FW, Simons M. Syndecan-4 modulates basic fibroblast growth factor 2 signaling in vivo. *American Journal of Physiology - Heart and Circulatory Physiology.* 2003;284(6 53-6):H2078–H2082.
44. Dealy CN, Seghatolleslami MR, Ferrari D, Kosher RA. FGF-Stimulated Outgrowth and Proliferation of Limb Mesoderm Is Dependent on Syndecan-3. *Dev Biol.* 1997;184(2):343–350.
45. Galli A, Roure A, Zeller R, Dono R. Glypican 4 modulates FGF signalling and regulates dorsoventral forebrain patterning in Xenopus embryos. *Development.* 2003;130(20):4919–4929.
46. Shimokawa K, Kimura-Yoshida C, Nagai N, et al. Cell Surface Heparan Sulfate Chains Regulate Local Reception of FGF Signaling in the Mouse Embryo. *Dev Cell.* 2011;21(2):257–272.
47. Kokenyesi R, Bernfield M. Core protein structure and sequence determine the site and presence of heparan sulfate and chondroitin sulfate on syndecan-1. *J Biol Chem.* 1994;269(16):12304–12309.
48. Gould SE, Upholt WB, Kosher RA. Characterization of Chicken Syndecan-3 as a Heparan Sulfate Proteoglycan and Its Expression during Embryogenesis. *Dev Biol.* 1995;168(2):438–451.
49. Winzen U, Cole GJ, Halfter W. Agrin is a chimeric proteoglycan with the attachment sites for heparan sulfate/chondroitin sulfate located in two multiple serine-glycine clusters. *Journal of Biological Chemistry.* 2003;278(32):30106–30114.
50. Dong S, Cole GJ, Halfter W. Expression of collagen XVIII and localization of its glycosaminoglycan attachment sites. *Journal of Biological Chemistry.* 2003;278(3):1700–1707.
51. Van Den Born J, Salmivirta K, Henttinen T, et al. Novel heparan sulfate structures revealed by monoclonal antibodies. *J Biol Chem.* 2005;280(21):20516–20523.
52. David G, Bai XM, Van der Schueren B, Cassiman JJ, Van den Berghe H. Developmental changes in heparan sulfate expression: in situ detection with mAbs. *The Journal of Cell Biology.* 1992;119(4):961–975.
53. Avnur Z, Geiger B. Immunocytochemical localization of native chondroitin-sulfate in tissues and cultured cells using specific monoclonal antibody. *Cell.* 1984;38(3):811–822.
54. West-Mays JA, Pino G, Lovicu FJ. Development and use of the lens epithelial explant system to study lens differentiation and cataractogenesis. *Progress in Retinal and Eye Research.* 2010;29(2):135–143.

55. McAvoy JW, Chamberlain CG. Fibroblast Growth-Factor (Fgf) Induces Different Responses in Lens Epithelial-Cells Depending on Its Concentration. *Development*. 1989;107(2):221–228.
56. Safaiyan F, Kolset SO, Prydz K, Gottfridsson E, Lindahl U, Salmivirta M. Selective effects of sodium chlorate treatment on the sulfation of heparan sulfate. *J Biol Chem*. 1999;274(51):36267–36273.
57. Schuksz M, Fuster MM, Brown JR, et al. Surfen, a small molecule antagonist of heparan sulfate. *Proc Natl Acad Sci USA*. 2008;105(35):13075–13080.
58. Iyengar L, Wang Q, Rasko JEJ, McAvoy JW, Lovicu FJ. Duration of ERK1/2 phosphorylation induced by FGF or ocular media determines lens cell fate. *Differentiation*. 2007;75(7):662–668.
59. Lo WK, Biswas SK, Brako L, Shiels A, Gu S, Jiang JX. Aquaporin-0 targets interlocking domains to control the integrity and transparency of the eye lens. *Invest Ophthalmol Vis Sci*. 2014;55(3):1202–1212.
60. Khan SY, Hackett SF, Lee M-CW, Pourmand N, Talbot CC, Riazuddin SA. Transcriptome Profiling of Developing Murine Lens Through RNA Sequencing. *Invest Ophthalmol Vis Sci*. 2015;56(8):4919–4926.
61. Hoang TV, Kumar PKR, Sutharzan S, Tsonis PA, Liang C, Robinson ML. Comparative transcriptome analysis of epithelial and fiber cells in newborn mouse lenses with RNA sequencing. *Molecular Vision*. 2014;20:1491–1517.
62. Cavalheiro GR, Matos-Rodrigues GE, Zhao Y, et al. N-myc regulates growth and fiber cell differentiation in lens development. *Dev Biol*. 2017;429(1):105–117.
63. Audette DS, Anand D, So T, et al. Prox1 and fibroblast growth factor receptors form a novel regulatory loop controlling lens fiber differentiation and gene expression. *Development*. 2016;143(2):318–328.
64. Kakrana A, Yang A, Anand D, et al. ISyTE 2.0: A database for expression-based gene discovery in the eye. *Nucleic Acids Res*. 2018;46(D1):D875–D885.
65. Arikawa E, Sun Y, Wang J, et al. Cross-platform comparison of SYBR Green real-time PCR with TaqMan PCR, microarrays and other gene expression measurement technologies evaluated in the MicroArray Quality Control (MAQC) study. *BMC Genomics*. 2008;9(1):328.
66. García B, García-Suárez O, Merayo-Llves J, et al. Differential Expression of Proteoglycans by Corneal Stromal Cells in Keratoconus. *Invest Ophthalmol Vis Sci*. 2016;57(6):2618–2628.
67. Hay ED, Meier S. Glycosaminoglycan synthesis by embryonic inducers: neural tube, notochord, and lens. *The Journal of Cell Biology*. 1974;62(3):889–898.
68. Mohan PS, Spiro RG. Characterization of heparan sulfate proteoglycan from calf lens capsule and proteoglycans synthesized by cultured lens epithelial cells. Comparison with other basement membrane proteoglycans. *Journal of Biological Chemistry*. 1991;266(13):8567–8575.
69. Maeda N, Ishii M, Nishimura K, Kamimura K. Functions of chondroitin sulfate and heparan sulfate in the developing brain. *Neurochemical Research*. 2011;36(7):1228–1240.
70. Vallet SD, Clerc O, Ricard-Blum S. Glycosaminoglycan-Protein Interactions: The First Draft of the Glycosaminoglycan Interactome. *J Histochem Cytochem*. 2020;21(7):22155420946403.
71. Clark SJ, Keenan TDL, Fielder HL, et al. Mapping the differential distribution of glycosaminoglycans in the adult human retina, choroid, and sclera. *Invest Ophthalmol Vis Sci*. 2011;52(9):6511–6521.
72. Smetsters TFCM, van de Westerlo EMA, Dam Ten GB, et al. Localization and characterization of melanoma-associated glycosaminoglycans: differential expression of chondroitin and heparan sulfate epitopes in melanoma. *Cancer Res*. 2003;63(11):2965–2970.
73. Karamanos NK, Piperigkou Z, Theocharis AD, et al. Proteoglycan chemical diversity drives multifunctional cell regulation and therapeutics. *Chem Rev*. 2018;118(18):9152–9232.
74. Schulz MW, Chamberlain CG, de Iongh RU, McAvoy JW. Acidic and basic FGF in ocular media and lens: implications for lens polarity and growth patterns. *Development*. 1993;118(1):117–126.
75. McAvoy JW. Binding of FGF-1 and FGF-2 to heparan sulphate proteoglycans of the mammalian lens capsule. *Growth Factors*. 1997;14(1):1–13.
76. Heathcote JG, Bruns RR, Orkin RW. Biosynthesis of Sulphated Macromolecules by Rabbit Lens Epithelium. II. Relationship to Basement Membrane Formation. *The Journal of Cell Biology*. 1984;99(3):861–869.
77. Koyama Y, Naruo H, Yoshitomi Y, et al. Matrix metalloproteinase-9 associated with heparan sulphate chains of GPI-anchored cell surface proteoglycans mediates motility of murine colon adenocarcinoma cells. *J of Biochem*. 2008;143(5):581–592.
78. Jang B, Jung H, Chung H, Moon B-I, Oh E-S. Syndecan-2 enhances E-cadherin shedding and fibroblast-like morphological changes by inducing MMP-7 expression in colon cancer cells. *Biochem Biophys Res Commun*. 2016;477(1):47–53.
79. Tholozan FMD, Gribbon C, Li Z, et al. FGF-2 release from the lens capsule by MMP-2 maintains lens epithelial cell viability. Omary MB, ed. *Molecular Biology of the Cell*. 2007;18(11):4222–4231.
80. Zhao Y, Zheng D, Cvekl A. A comprehensive spatial-temporal transcriptomic analysis of differentiating nascent mouse lens epithelial and fiber cells. *Exp Eye Res*. 2018;175:56–72.
81. Zhao G, Bailey CG, Feng Y, Rasko J, Lovicu FJ. Negative regulation of lens fiber cell differentiation by RTK antagonists Spry and Spred. *Exp Eye Res*. 2018;170:148–159.
82. Uechi G, Sun Z, Schreiber EM, Halfter W, Balasubramani M. Proteomic View of Basement Membranes from Human Retinal Blood Vessels, Inner Limiting Membranes, and Lens Capsules. *J Proteome Res*. 2014;13(8):3693–3705.
83. Groffen AJ, Ruegg MA, Dijkman H, et al. Agrin is a major heparan sulfate proteoglycan in the human glomerular basement membrane. *Journal of Histochemistry & Cytochemistry*. 1998;46(1):19–27.
84. Neill T, Schaefer L, Iozzo RV. Decoding the Matrix: Instructive Roles of Proteoglycan Receptors. *Biochemistry*. 2015;54(30):4583–4598.
85. Qin Y, Zhu Y, Luo F, Chen C, Chen X, Wu M. Killing two birds with one stone: dual blockade of integrin and FGF signaling through targeting syndecan-4 in postoperative capsular opacification. *Cell Death Dis*. 2017;8(7):e2920.
86. Elenius K, Salmivirta M, Inki P, Mali M, Jalkanen M. Binding of human syndecan to extracellular matrix proteins. *J Biol Chem*. 1990;265(29):17837–17843.
87. Kusano Y, Oguri K, Nagayasu Y, et al. Participation of Syndecan 2 in the Induction of Stress Fiber Formation in Cooperation with Integrin $\alpha 5 \beta 1$: Structural Characteristics of Heparan Sulfate Chains with Avidity to COOH-Terminal Heparin-Binding Domain of Fibronectin. *Exp Cell Res*. 2000;256(2):434–444.
88. Tumova S, Woods A, Couchman JR. Heparan sulfate chains from glypican and syndecans bind the Hep II domain of fibronectin similarly despite minor structural differences. *Journal of Biological Chemistry*. 2000;275(13):9410–9417.
89. Olivero DK, Furcht LT. Type IV collagen, laminin, and fibronectin promote the adhesion and migration of rabbit

- lens epithelial cells in vitro. *Invest Ophthalmol Vis Sci*. 1993;34(10):2825–2834.
90. Parmigiani CM, McAvoy JW. The roles of laminin and fibronectin in the development of the lens capsule. *Current Eye Res*. 2009;10(6):501–511.
 91. Filla MS, David G, Weinreb RN, Kaufman PL, Peters DM. Distribution of syndecans 1–4 within the anterior segment of the human eye: Expression of a variant syndecan-3 and matrix-associated syndecan-2. *Experimental Eye Research*. 2004;79(1):61–74.
 92. Esko JD, Selleck SB. *Order Out of Chaos: Assembly of Ligand Binding Sites in Heparan Sulfate*. Vol. 71. Palo Alto, CA: Annual Reviews; 2002:435–471.
 93. Carulli S, Beck K, Dayan G, Boulesteix S, Lortat-Jacob H, Rousselle P. Cell surface proteoglycans syndecan-1 and -4 bind overlapping but distinct sites in laminin $\alpha 3$ LG45 protein domain. *J Biol Chem*. 2012;287(15):12204–12216.
 94. Utani A. The Laminin $\alpha 3$ LG4 Domain Promotes Cell Adhesion through Syndecans. *Connective Tissue*. 2003;35(4):187–192.
 95. Kolset SO, Prydz K, Pejler G. Intracellular proteoglycans. *Biochem J*. 2004;379(Pt 2):217–227.
 96. Zhu Z, Friess H, Kleeff J, et al. Glypican-3 expression is markedly decreased in human gastric cancer but not in esophageal cancer. *Am J Surg*. 2002;184(1):78–83.
 97. Miyake M, Lawton A, Dai Y, et al. Clinical implications in the shift of syndecan-1 expression from the cell membrane to the cytoplasm in bladder cancer. *BMC Cancer*. 2014;14(1):885–887.
 98. Granés F, Ureña JM, Rocamora N, Vilaró S, Ezrin links syndecan-2 to the cytoskeleton. *J Cell Sci*. 2000;113(7):1267–1276.
 99. Stigliano I, Puricelli L, Filmus J, Sogayar MC, Bal De Kier Joffé E, Peters MG. Glypican-3 regulates migration, adhesion and actin cytoskeleton organization in mammary tumor cells through Wnt signaling modulation. *Breast Cancer Res Treat*. 2009;114(2):251–262.
 100. Chen L, Sanderson RD. Heparanase regulates levels of syndecan-1 in the nucleus. Chakravarti S, ed. *PLoS One*. 2009;4(3):e4947.
 101. Brockstedt U, Dobra K, Nurminen M, Hjerpe A. Immunoreactivity to cell surface syndecans in cytoplasm and nucleus: tubulin-dependent rearrangements. *Exp Cell Res*. 2002;274(2):235–245.
 102. Hakim SA, Abd Raboh NM. Immunohistochemical expression of glypican 3 in endometrial carcinoma and correlation with prognostic parameters. *Int J Clin Exp Pathol*. 2015;8(10):13225–13232.
 103. Li N, Wei L, Liu X, et al. A Frizzled-Like Cysteine-Rich Domain in Glypican-3 Mediates Wnt Binding and Regulates Hepatocellular Carcinoma Tumor Growth in Mice. *Hepatology*. 2019;70(4):1231–1245.
 104. Akutsu N, Yamamoto H, Sasaki S, et al. Association of glypican-3 expression with growth signaling molecules in hepatocellular carcinoma. *World J Gastroenterol*. 2010;16(28):3521–3528.
 105. Capurro M, Martin T, Shi W, Filmus J. Glypican-3 binds to frizzled and plays a direct role in the stimulation of canonical Wnt signaling. *J Cell Sci*. 2014;127(7):jcs.140871–jcs.141575.
 106. Kolluri A, Ho M. The Role of Glypican-3 in Regulating Wnt, YAP, and Hedgehog in Liver Cancer. *Front Oncol*. 2019;9:708.
 107. Dawes LJ, Sugiyama Y, Tanedo AS, Lovicu FJ, McAvoy JW. Wnt-frizzled signaling is part of an FGF-induced cascade that promotes lens fiber differentiation. *Invest Ophthalmol Vis Sci*. 2013;54(3):1582–1590.
 108. Chen Y, Stump RJW, Lovicu FJ, Shimono A, McAvoy JW. Wnt signaling is required for organization of the lens fiber cell cytoskeleton and development of lens three-dimensional architecture. *Dev Biol*. 2008;324(1):161–176.
 109. Mertens G, Van der Schueren B, Van den Berghe H, David G. Heparan sulfate expression in polarized epithelial cells: the apical sorting of glypican (GPI-anchored proteoglycan) is inversely related to its heparan sulfate content. *The Journal of Cell Biology*. 1996;132(3):487–497.
 110. Maday S, Anderson E, Chang HC, et al. A PDZ-binding motif controls basolateral targeting of syndecan-1 along the biosynthetic pathway in polarized epithelial cells. *Traffic*. 2008;9(11):1915–1924.
 111. Cheng C, Nowak RB, Fowler VM. The lens actin filament cytoskeleton: Diverse structures for complex functions. *Exp Eye Res*. 2017;156:58–71.
 112. Weber GF, Menko AS. Actin filament organization regulates the induction of lens cell differentiation and survival. *Dev Biol*. 2006;295(2):714–729.
 113. Khurana RN, Maddala RL, Shimokawa H, Samuel Zigler J, Epstein DL, Vasantha Rao P. Inhibition of Rho-kinase induces α B-crystallin expression in lens epithelial cells. *Biochem Biophys Res Commun*. 2002;294(5):981–987.
 114. Maddala R, Reddy VN, Epstein DL, Vis VRM. Growth factor induced activation of Rho and Rac GTPases and actin cytoskeletal reorganization in human lens epithelial cells. *Molecular Vision*. 2003;9(46):329–336.
 115. Oh ES, Woods A, Couchman JR. Syndecan-4 proteoglycan regulates the distribution and activity of protein kinase C. *J Biol Chem*. 1997;272(13):8133–8136.
 116. Horowitz A, Simons M. Phosphorylation of the cytoplasmic tail of syndecan-4 regulates activation of protein kinase Calpha. *J Biol Chem*. 1998;273(40):25548–25551.
 117. Echtermeyer F, Baciuc PC, Saoncella S, Ge Y, Goetinck PF. Syndecan-4 core protein is sufficient for the assembly of focal adhesions and actin stress fibers. *J Cell Sci*. 1999;112(20):3433–3441.
 118. Walker J, Menko AS. Integrins in lens development and disease. *Exp Eye Res*. 2009;88(2):216–225.
 119. Filla MS, Woods A, Kaufman PL, Peters DM. $\beta 1$ and $\beta 3$ integrins cooperate to induce syndecan-4-containing cross-linked actin networks in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci*. 2006;47(5):1956–1967.
 120. Couchman JR, Woods A. Syndecan-4 and integrins: combinatorial signaling in cell adhesion. *J Cell Sci*. 1999;112(20):3415–3420.
 121. Morgan MR, Humphries MJ, Bass MD. Synergistic control of cell adhesion by integrins and syndecans. *Nat Rev Mol Cell Biol*. 2007;8(12):957–969.
 122. Simirskii VN, Duncan MK, Paltsev MA, Suchkov SV. $\beta 1$ integrin as the integrating component in cell-cell cooperation for maintenance of lens transparency. *Doklady Biochemistry and Biophysics*. 2013;453(1):297–299.
 123. Bernfield M. Biology of the Syndecans: A Family of Transmembrane Heparan Sulfate Proteoglycans. *Annual Review of Cell and Developmental Biology*. 1992;8(1):365–393.
 124. Stewart MD, Sanderson RD. Heparan sulfate in the nucleus and its control of cellular functions. *Matrix Biology*. 2014;35:56–59.
 125. Kovalszky I, Hjerpe A, Dobra K. Nuclear translocation of heparan sulfate proteoglycans and their functional significance. *Biochim Biophys Acta*. 2014;1840(8):2491–2497.
 126. Maher PA. Nuclear Translocation of fibroblast growth factor (FGF) receptors in response to FGF-2. *The Journal of Cell Biology*. 1996;134(2):529–536.
 127. Amalric F, Bouche G, Bonnet H, et al. Fibroblast growth factor-2 (FGF-2) in the nucleus: Translocation process and targets. *Biochemical Pharmacology*. 1994;47(1):111–115.

128. Zong F, Fthenou E, Wolmer N, et al. Syndecan-1 and FGF-2, but Not FGF Receptor-1, Share a Common Transport Route and Co-Localize with Heparanase in the Nuclei of Mesenchymal Tumor Cells. Wölfl S, ed. *PLoS One*. 2009;4(10):e7346.
129. David G, Bai XM, Van der Schueren B, Cassiman JJ, Van den Berghe H. Developmental changes in heparan sulfate expression: in situ detection with mAbs. *The Journal of Cell Biology*. 1992;119(4):961–975.
130. Fedarko NS, Ishihara M, Conrad HE. Control of cell division in hepatoma cells by exogenous heparan sulfate proteoglycan. *J Cell Physiol*. 1989;139(2):287–294.
131. Szatmári T, Mundt F, Kumar-Singh A, et al. Molecular targets and signaling pathways regulated by nuclear translocation of syndecan-1. *BMC Cell Biol*. 2017;18(1):34.
132. Fedarko NS, Conrad HE. A Unique Heparan Sulfate in the Nuclei of Hepatocytes: Structural Changes with the Growth State of the Cells. *The Journal of Cell Biology*. 1986;102(2):587–599.
133. Jang E, Albadawi H, Watkins MT, Edelman ER, Baker AB. Syndecan-4 proteoliposomes enhance fibroblast growth factor-2 (FGF-2)-induced proliferation, migration, and neovascularization of ischemic muscle. *Proc Natl Acad Sci USA*. 2012;109(5):1679–1684.
134. Ito Y, Hikino M, Yajima Y, et al. Structural characterization of the epitopes of the monoclonal antibodies 473HD, CS-56, and MO-225 specific for chondroitin sulfate D-type using the oligosaccharide library. *Glycobiology*. 2005;15(6):593–603.
135. Rønning SB, Carlson CR, Stang E, Kolset SO, Hollung K, Pedersen ME. Syndecan-4 regulates muscle differentiation and is internalized from the plasma membrane during myogenesis. Asakura A, ed. *PLoS One*. 2015;10(6):e0129288.
136. Schrage YM, Hameetman L, Szuhai K, et al. Aberrant heparan sulfate proteoglycan localization, despite normal exostosin, in central chondrosarcoma. *Am J Pathol*. 2009;174(3):979–988.
137. Leadbeater WE, Gonzalez A-M, Logaras N, Berry M, Turnbull JE, Logan A. Intracellular trafficking in neurones and glia of fibroblast growth factor-2, fibroblast growth factor receptor 1 and heparan sulphate proteoglycans in the injured adult rat cerebral cortex. *J Neurochem*. 2006;96(4):1189–1200.
138. Smith SM-L, West LA, Govindraj P, Zhang X, Ornitz DM, Hassell JR. Heparan and chondroitin sulfate on growth plate perlecan mediate binding and delivery of FGF-2 to FGF receptors. *Matrix Biol*. 2007;26(3):175–184.
139. Garcia CM, Yu K, Zhao H, et al. Signaling through FGF receptor-2 is required for lens cell survival and for withdrawal from the cell cycle during lens fiber cell differentiation. *Dev Dyn*. 2005;233(2):516–527.
140. Chow RL, Roux GD, Roghani M, et al. FGF suppresses apoptosis and induces differentiation of fibre cells in the mouse lens. *Development*. 1995;121(12):4383–4393.
141. Lovicu FJ, McAvoy JW. Structural analysis of lens epithelial explants induced to differentiate into fibres by fibroblast growth factor (FGF). *Exp Eye Res*. 1989;49(3):479–494.
142. Richardson NA, McAvoy JW, Chamberlain CG. Age of rats affects response of lens epithelial explants to fibroblast growth factor. *Exp Eye Res*. 1992;55(5):649–656.
143. Peek R, McAvoy JW, Lubsen NH, Schoenmakers JGG. Rise and fall of crystallin gene messenger levels during fibroblast growth factor induced terminal differentiation of lens cells. *Dev Biol*. 1992;152(1):152–160.
144. Golestaneh N, Fan J, Zelenka P, Chepelinsky AB. PKC putative phosphorylation site Ser235 is required for MIP/AQP0 translocation to the plasma membrane. *Molec Vis*. 2008;14:1006–1014.
145. Golestaneh N, Fan J, Fariss RN, Lo WK, Zelenka PS, Chepelinsky AB. Lens major intrinsic protein (MIP)/aquaporin 0 expression in rat lens epithelia explants requires fibroblast growth factor-induced ERK and JNK signaling. *Journal of Biological Chemistry*. 2004;279(30):31813–31822.



Heparan sulfate proteoglycans (HSPGs) of the ocular lens

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ABSTRACT

Heparan sulfate proteoglycans (HSPGs) reside in most cells; on their surface, in the pericellular milieu and/or extracellular matrix. In the eye, HSPGs can orchestrate the activity of key signalling molecules found in the ocular environment that promote its development and homeostasis. To date, our understanding of the specific roles played by individual HSPG family members, and the heterogeneity of their associated sulfated HS chains, is in its infancy. The crystalline lens is a relatively simple and well characterised ocular tissue that provides an ideal stage to showcase and model the expression and unique roles of individual HSPGs. Individual HSPG core proteins are differentially localised to eye tissues in a temporal and spatial developmental- and cell-type specific manner, and their loss or functional disruption results in unique phenotypic outcomes for the lens, and other ocular tissues. More recent work has found that different HS sulfation enzymes are also presented in a cell- and tissue-specific manner, and that disruption of these different sulfation patterns affects specific HS-protein interactions. Not surprisingly, these sulfated HS chains have also been reported to be required for lens and eye development, with dysregulation of HS chain structure and function leading to pathogenesis and eye-related phenotypes. In the lens, HSPGs undergo significant and specific changes in expression and function that can drive pathology, or in some cases, promote tissue repair. As master signalling regulators, HSPGs may one day serve as valuable biomarkers, and even as putative targets for the development of novel therapeutics, not only for the eye but for many other systemic pathologies.

1. Introduction

The ocular lens is a well-established model for studying growth factor regulation of cell behaviour (Wormstone and Wride, 2011). The lens is a relatively simple tissue, composed of two distinct cell types: a monolayer of cuboidal epithelial cells that overlies the anterior surface of elongate and precisely aligned fibre cells that make up its mass. The highly ordered spatial arrangement of the lens is established during embryogenesis and is maintained as the lens grows throughout life. The normal architecture, and hence transparency of the lens, depends on maintaining this distinctive pattern of growth. Postnatally, lens growth is dependent on the proliferation of epithelial cells above the lens equator, in the germinative zone, and their subsequent differentiation into fibre cells at the transitional zone (Lovicu et al., 2014; Mochizuki et al., 2014; Shi et al., 2014). Fibre differentiation is characterised by distinct molecular and morphologic changes, such as exit from the cell cycle, cell elongation, loss of cytoplasmic organelles including nuclei, as well as the accumulation of fibre-specific crystallin proteins (Wride, 2011). The organisation and maintenance of the vertebrate lens throughout life is orchestrated by the interplay of a complex network of signalling molecules within the native ocular environment, the aqueous and vitreous humours, that bathe the lens. Decades of research have

identified key ocular molecules and characterised their differential and complementary roles in the coordination of specific morphogenetic events and cell behaviours. For instance, *in vitro* studies have shown that growth factors native to aqueous humour promote the mitogenic activity of the anterior lens epithelial cells (Iyengar et al., 2009), while posterior to the lens equator, factors in the vitreous humour induce the terminal differentiation of lens epithelial cells to secondary fibre cells (Lovicu and McAvoy, 2005; Wang et al., 2010). These diffusible native factors are secreted primarily from the retina and ciliary body, and include: fibroblast growth factor (FGF) (Lovicu and McAvoy, 2005), insulin-like growth factor (IGF) (Chamberlain et al., 1991; Shirke et al., 2001), epidermal growth factor (EGF) (Gospodarowicz et al., 1978; Majima, 1995), platelet-derived growth factor (PDGF) (Brewitt and Clark, 1988; Kok et al., 2002; Li et al., 2019a), hepatocyte growth factor (HGF) (Choi et al., 2004; Tian, 2014), vascular endothelial growth factor (VEGF) (Saint-Geniez et al., 2009), ephrins (Cheng and Gong, 2011), as well as other non-receptor tyrosine kinase (RTK)-binding morphogens, including bone morphogenetic proteins (BMPs) (Boswell et al., 2008), transforming growth factor beta (TGFβ) (de Jongh et al., 2001; Dawes et al., 2009) and Wnts (Lyu and Joo, 2004; Dawes et al., 2013). These factors traverse the ocular milieu to reach their target cells and act in concert to potentiate and regulate lens cell behaviour (i.e.

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differentiation, proliferation, migration and cell death), sometimes, in a concentration dependent manner, as reported for FGF (Schulz et al., 1993; Lovicu and McAvoy, 2001; Iyengar et al., 2006, 2007; Wang et al., 2009). During embryogenesis, the tightly controlled spatial and temporal modulation of the expression of these growth factors regulates the induction, and antero-posterior patterning of the developing lens (Reneker and Overbeek, 1996; Faber et al., 2002). Although the many distinct and overlapping effects of these factors on lens cell behaviour are well-characterised, the mechanisms underlying how these growth factors travel through the extracellular space, arrive precisely at target cells, and transmit their signalling within cells at the appropriate level, remain elusive. Studies in mice and other model animals have revealed the importance of heparan sulfate proteoglycans (HSPGs) for the signalling and extracellular distribution of many of these different growth factors.

HSPGs are complex glycoconjugates composed of a core protein “trunk” decorated with covalently attached linear anionic polysaccharide heparan sulfate (HS) glycosaminoglycan (GAG) chains (“branches”; Fig. 1A). HSPGs are a component of all known cells, and

can be localised to the cell surface, intracellularly in the cytoplasm and nucleus, or secreted by cells into the extracellular space where they form a major component of basement membranes and the extracellular matrix (ECM) (Sarrazin et al., 2011). Until relatively recently, our understanding of HSPGs was limited to their role as essential matrix components, lending structural stability to basement membranes. As our understanding of proteoglycans has expanded, the exciting discovery was made that HSPGs can bind to and regulate a variety of proteins, including growth factors, chemokines, matrix proteins and intracellular components (e.g. FGFs, integrins, laminin, fibronectin, collagens) (Bishop et al., 2007; Vallet et al., 2021). The negatively charged sulfate groups of HS chains electrostatically interact with positively charged lysine/arginine-rich regions of many proteins, influencing their structure and activity (Billings and Pacifici, 2015). Importantly, the interactions of growth factors with native HSPGs influences their receptor binding and signalling activation (Park et al., 2000; Forsten-Williams et al., 2008; Yu et al., 2018), gradient formation (Yan and Lin, 2009; Matsuo and Kimura-Yoshida, 2014), potentiates their signalling activity (Dreyfuss et al., 2009) and protects them from degradation (Sun et al.,

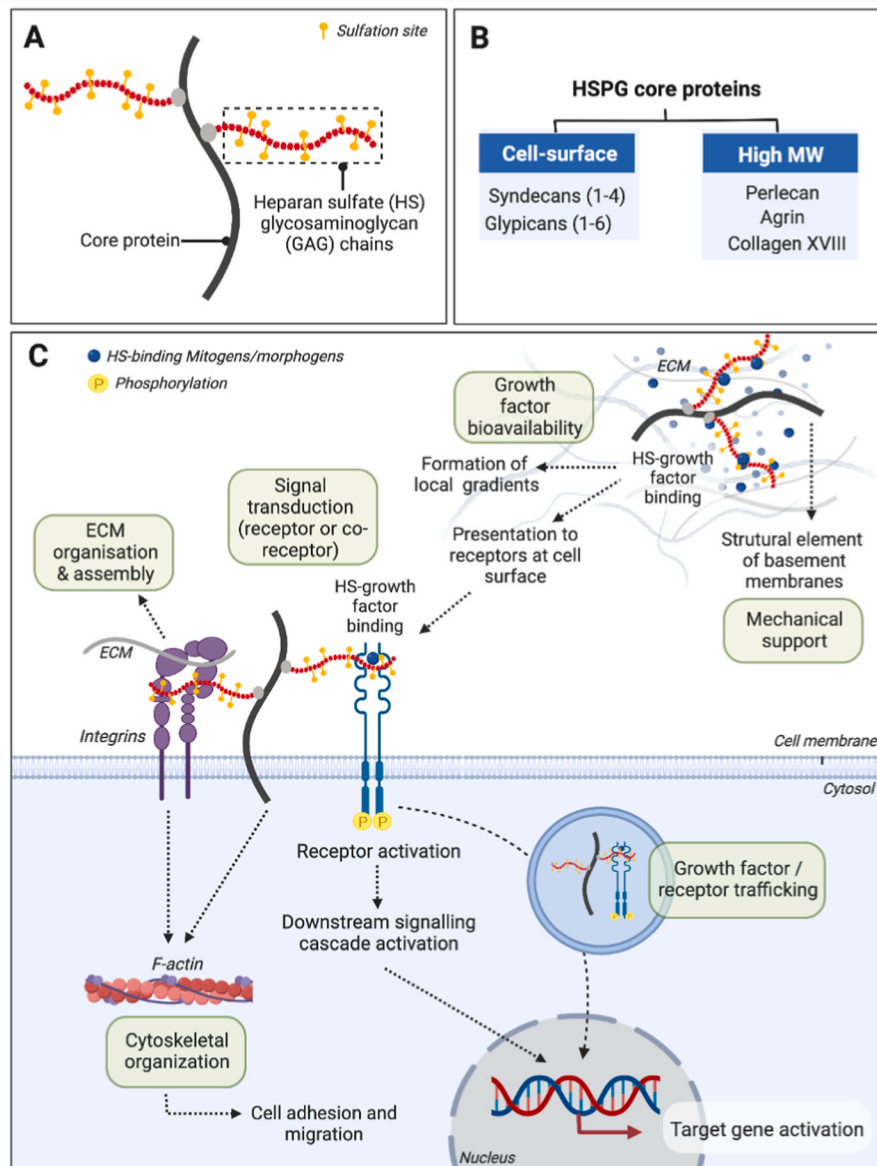


Fig. 1. Heparan sulfate proteoglycans. A) Heparan sulfate proteoglycan (HSPG) structure and B) core protein families. C) Summary of HSPG cell functions. Abbreviations: heparan sulfate (HS), glycosaminoglycan (GAG), molecular weight (MW), extracellular matrix (ECM).

2019) (Fig. 1C).

The thirteen 'full-time' HSPGs are divided into three major families (syndecans, glypicans and high-molecular weight HSPGs; perlecan, agrin and collagen XVIII) based on similarities in their core-protein structure (Fig. 1B) (Esko et al., 2017). For noting, 'part-time' HSPGs including betaglycan (*TGFBR3*), epican (HS/CS-carrying form of CD44), testicans (*SPOCK1-3*) and serglycin (*SRGN*, carrying heparin/CS) are also expressed in the lens (iSyte, Kakrana et al., 2018), and will not be discussed further. The core protein of a HSPG determines its localization (i.e. cell-surface, matrix, vesicles etc.), linking the HS-protein interactions of its associated GAG chains to specific regions of a cell (Sarrazin et al., 2011). For instance, the transmembrane syndecans (syndecans 1–4), and glycosylphosphatidylinositol (GPI)-anchored glypicans (glypicans 1–6), are primarily localised to the cell surface, while the high molecular weight HSPGs (perlecan, agrin and collagen XVIII) are mostly secreted by cells and deposited into the ECM and basement membranes (Couchman and Pataki, 2012). Although these are commonly accepted generalisations, it is important to note that our understanding of HSPG localization is expanding constantly, and there are indeed exceptions: for instance, the most common form of syndecan-2 in the eye is associated with the ECM (Filla et al., 2004), and for the two isoforms of agrin reported in the eye, one is cell-associated and the other found within the ECM (Fuerst et al., 2007). HSPG core proteins are also associated with numerous intracellular structures including the nucleus (Stewart and Sanderson, 2014), cytoskeleton (Rapraeger et al., 1986), Golgi apparatus (Li and Kusche-Gullberg, 2016) and exosomes (Christianson et al., 2013). Different HSPG core proteins display cell- and tissue-specific patterns of expression that are regulated in a spatiotemporally distinct manner during development (David et al., 1992; Bernfield et al., 1993). Moreover, the number, localization, and sulfation patterns of their GAG chains can modify its protein interactions, and hence its function (Poulain and Yost, 2015). For instance, HSPG core proteins identified in the vertebrate eye have been found to have functional roles unique to the tissue in which they are expressed. For example, HS-linked forms of perlecan are required for structural integrity of the lens capsule, but not required for the glomerular basement membrane (Rossi, 2003), and overexpression of agrin in transgenic mice causes a dysgenic phenotype that is specific to the eye (Fuerst et al., 2007).

The localization of HSPG core proteins can provide valuable information about their functional roles in a specific tissue. In the eye, HSPGs are differentially expressed in different tissues (e.g. the retina and cornea), where they serve both overlapping and unique roles in each tissue (Inatani and Tanihara, 2002; Tanihara et al., 2002; Massoudi et al., 2016). Immunolabelling 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 neural precursor cells, identified these HSPGs as key regulators of mitogens in early brain development (Ford-Perriss et al., 2003). The specificity of these HSPG-protein interactions not only depends on the core protein type (e.g. syndecan, glypican), and their localization (e.g. the cell surface or ECM), but also on the fine structure (i.e. sulfation) of the HS chains that associate with HSPGs.

At the cell surface, HSPGs bind growth factors and their cognate receptors to enhance formation of receptor-signalling complexes, as well as immobilise and regulate the turnover of ligands that act at the cell surface (Bishop et al., 2007). The pleiotropic modulation of growth factor receptors by HSPGs can control different outcomes from the same growth factor-signalling interaction, by altering ligand density, period of activation etc. HSPGs can also directly and indirectly influence intracellular signalling cascades, leading to the activation of key effector genes, changes in cell behaviour as well as cell phenotype (Bishop et al., 2007). Within the ECM, HSPGs can bind to factors to regulate their bioavailability at the cell surface and stabilise dynamic mitogen and morphogen gradients locally at the tissue level that is critical for organogenesis and the maintenance of tissue homeostasis. HSPGs also

regulate the binding and activation of several proteins required for cell-matrix interactions (e.g. integrins, laminin, collagens), thus regulating cell attachment, migration, transdifferentiation and polarity (Iozzo, 2005). Through modulation of the binding and activation of these key proteins, HSPGs are positioned as master regulators of the physiology, development and pathophysiology of many tissues (Bishop et al., 2007), not least in the eye (Puri et al., 2020).

Our understanding of the functional roles of HSPGs in the eye, and the differential roles that specific core proteins and HS-fine structural elements play in the regulation of the development, physiology and pathology of different ocular tissues, are in their infancy. The lens is uniquely positioned to provide a model on which the properties of HSPGs can be investigated, and their individual roles elucidated. This review aims to use our current understanding of HSPGs in the lens to help better define the major roles they play.

2. Expression and localization of HSPGs

HS-GAGs are present in every ocular tissue in cell-, tissue- and developmental stage-specific patterns (Sarrazin et al., 2011). Pioneering sulfate-radiolabelling studies revealed that ocular cells incorporate highly anionic-GAG species in prominent ocular basement membranes including the lens capsule (Young and Ocumpaugh, 1966), Bruch's membrane (Hewitt et al., 1989) and Descemet's membrane (Davies et al., 1999), later expanding to many other ocular cell types (see Table 1).

As increasing evidence suggests that HSPG core proteins play important roles in lens signalling and architecture, the unique profile of HSPGs expressed in the murine lens was recently characterised in detail (Wishart and Lovicu, 2021). In this study they reported that HSPGs are differentially localised to distinct functional regions of the lens, and that their expression is regulated in a distinct spatiotemporal manner through lens development (Wishart and Lovicu, manuscript in preparation). An interesting finding of this work was the significantly divergent expression patterns of the closely related members of one HSPG family, the syndecans, that suggested that these different HSPG core proteins may be differentially regulating different lens growth factors or specific biological events (e.g. lens proliferation, fibre differentiation, fibre elongation etc). Most HSPGs are expressed in the lens, consistent with other published postnatal HSPG transcript expression profiles from mouse lens datasets (Hoang et al., 2014; Khan et al., 2015; Audette et al., 2016; Cavalheiro et al., 2017; Kakrana et al., 2018).

2.1. HSPG-associated glycosaminoglycans

HSPGs are predominantly HS-GAGs; however, certain species such as syndecans-1/-3 (Kokenyesi and Bernfield, 1994; Gould et al., 1995), agrin (Winzen et al., 2003) and collagen XVIII (Dong et al., 2003) may also carry chondroitin sulfate (CS)-GAGs. Lens cells produce proteoglycans carrying both HS and CS, although HS is the predominant sulfated lens GAG (Heathcote and Orkin, 1984; Mohan and Spiro, 1991; Wishart and Lovicu, 2021), strongly labelling in analogous regions of lenses from different vertebrate species (Fig. 2). When directly compared, Heathcote and colleagues reported that cultured rabbit lens epithelial cells synthesise primarily HS-GAG (72% HS-GAG) with 28% being CS-GAGs (Heathcote and Orkin, 1984). Despite differences in their levels, regions of the eye labelling for HS-GAG and CS-GAG do not always overlap (Wishart and Lovicu, 2021), suggesting that chondroitin sulfate proteoglycans (CSPGs) may play a role in ocular biology that is distinct from HSPGs (see Tao et al., 2022). In the newly differentiating secondary lens fibres, there is an overlapping, yet divergent pattern of HS and CS, highlighting their putative joint contribution to this differentiation process (Fig. 3; Wishart and Lovicu, 2021). While other lens cellular processes may be influenced by the predominant antero-posterior HS gradient in the lens capsule (Fig. 3 insets, Lovicu and McAvoy, 1993; Wu et al., 2014), we see divergent functional roles

Table 1

Localization of HSPG core proteins in the mammalian eye.

Core protein	Localization (*mRNA expression reported only)
Syndecan-1 (Syndecan/CD138)	Cornea: epithelium (Stepp et al., 2002; Koso et al., 2008; Hayashida et al., 2015; Jinno et al., 2020; Zhou et al., 2006*); stroma (Koso et al., 2008; García et al., 2016*), conjunctiva (Heimann et al., 2001); limbal epithelium (Zhou et al., 2006*) Lens: capsule, tunica vasculosa lentis (Wishart and Lovicu, 2021) Iris: sphincter pupillae muscle (Koso et al., 2008); stroma (Wishart and Lovicu, 2021) Ciliary body: non-pigmented epithelium (Koso et al., 2008; Wishart and Lovicu, 2021); stroma (Koso et al., 2008); ciliary muscle cells (Filla et al., 2004) Retina: choroid (Wishart and Lovicu, 2021; Wistow et al., 2002*); retinal pigmented epithelium (Wistow et al., 2002*) Other: Trabecular meshwork (Filla et al., 2004; Peterson et al., 2005); lacrimal glands (Wistow et al., 2002*); Schlemm's canal cells (Filla et al., 2004)
Syndecan-2 (Fibroglycan/CD362)	Cornea: stroma (García et al., 2016*; Wishart and Lovicu, 2021); limbal epithelial progenitor cells (Polisetti et al., 2016*) Lens: capsule, fibre cells (Wishart and Lovicu, 2021) Iris: stroma (Wishart and Lovicu, 2021) Ciliary body: non-pigmented epithelium (Wishart and Lovicu, 2021); ciliary muscle cells (Filla et al., 2004) Retina: (Inatani et al., 2002*; Wishart and Lovicu, 2021) Other: Schlemm's canal cells (Filla et al., 2004)
Syndecan-3 (N-syndecan/Neuroglycan)	Cornea: stroma (García et al., 2016*; Wishart and Lovicu, 2021) Lens: epithelium, fibre cells (Wishart and Lovicu, 2021) Iris: stroma, iridial basement membranes (Wishart and Lovicu, 2021) Ciliary body: stroma, limiting basement membranes, ciliary epithelial bilayer (Wishart and Lovicu, 2021); ciliary muscle cells (Filla et al., 2004) Retina: (Inatani et al., 2002*; Wishart and Lovicu, 2021) Other: Trabecular meshwork cells; Schlemm's canal cells (Filla et al., 2004)
Syndecan-4 (Ryudocan/Amphiglycan)	Cornea: epithelium (Hayashida et al., 2015; Jinno et al., 2020); stroma (García et al., 2016*; Wishart and Lovicu, 2021); endothelium (Jinno et al., 2020); limbal epithelium (GDS2433) Lens: capsule (Qin et al., 2017; Wishart and Lovicu, 2021); epithelium (Qin et al., 2017; Wishart and Lovicu, 2021); fibre cells (Qin et al., 2017; Wishart and Lovicu, 2021) Iris: stroma, iridial basement membranes, sphincter/dilator pupillae muscles (Wishart and Lovicu, 2021) Ciliary body: (Qin et al., 2017); stroma, non-pigmented epithelium (Wishart and Lovicu, 2021); ciliary muscle cells (Filla et al., 2004) Retina: (Inatani et al., 2002*; Qin et al., 2017; Wishart and Lovicu, 2021) Other: Trabecular meshwork (Filla et al., 2004; Peterson et al., 2005); Schlemm's canal cells (Filla et al., 2004)
Glypican-1 (Glypican)	Cornea: stroma (García et al., 2016*) Lens: epithelium, fibre cells (Wishart and Lovicu, 2021)
Glypican-2 (Cerebroglycan)	Cornea: stroma (García et al., 2016*; Wishart and Lovicu, 2021) Lens: capsule; epithelium, fibre cells (Wishart and Lovicu, 2021) Iris: stroma (Wishart and Lovicu, 2021) Ciliary body: ciliary epithelial bilayer, stroma, endothelial layer (Wishart and Lovicu, 2021) Retina: (Wishart and Lovicu, 2021)
Glypican-3 (OCI-5)	Cornea: limbal epithelial progenitor cells (Polisetti et al., 2016*) Lens: epithelium, fibre cells (Wishart and Lovicu, 2021)
Glypican-4 (K-glypican)	Cornea: stroma (García et al., 2016*; Wishart and Lovicu, 2021); limbal epithelial progenitor cells (Polisetti et al., 2016*) Lens: capsule, epithelium (Wishart and Lovicu, 2021) Iris: stroma, iridial basement membranes (Wishart and Lovicu, 2021) Ciliary body: stroma, ciliary epithelial bilayer (Wishart and Lovicu, 2021)
Glypican-5	Cornea: stroma (García et al., 2016*) Lens: epithelium (Wishart and Lovicu, 2021*)
Glypican-6	Cornea: stroma (García et al., 2016*); epithelium, endothelium (Wishart and Lovicu, 2021) Lens: epithelium, fibre cells (Wishart and Lovicu, 2021)
Perlecan (HSPG2)	Cornea: stroma (García et al., 2016*; Wishart and Lovicu, 2021); Bowman's layer (Chen and Hazlett, 2000) Lens: capsule (Wu et al., 2014; Wishart and Lovicu, 2021); epithelial-capsule interface (Wu et al., 2014); epithelium, fibre cells (Wishart and Lovicu, 2021) Iris: stroma, iridial basement membranes (Wishart and Lovicu, 2021) Ciliary body: stroma (Wishart and Lovicu, 2021)
Aggrin (Neurocan)	Cornea: stroma (García et al., 2016*; Wishart and Lovicu, 2021), Bowman's layer (Fuerst et al., 2007); Descemet's membrane (Fuerst et al., 2007) Lens: capsule (Fuerst et al., 2007; Wishart and Lovicu, 2021); epithelium (Wishart and Lovicu, 2021) Iris: (Fuerst et al., 2007); stroma, iridial basement membranes (Wishart and Lovicu, 2021) Ciliary body: ciliary epithelial bilayer, stroma, endothelial layer (Wishart and Lovicu, 2021) Retina: (Mann and Kröger, 1996; Fuerst et al., 2007); optic nerve (Fuerst et al., 2007)
Collagen XVIII/endostatin	Cornea: stroma (García et al., 2016*; Wishart and Lovicu, 2021); Bowman's membrane (Fukai et al., 2002; Ohlmann et al., 2012) Lens: capsule (Fukai et al., 2002; Ohlmann et al., 2012; Wishart and Lovicu, 2021); epithelium (Ohlmann et al., 2012; Wishart and Lovicu, 2021) Iris: stroma (Fukai et al., 2002; Wishart and Lovicu, 2021), iridial basement membranes (Ohlmann et al., 2012; Wishart and Lovicu, 2021) Ciliary body: stroma (Wishart and Lovicu, 2021); non-pigmented epithelium (Ohlmann et al., 2012) Retina: inner limiting membrane (Fukai et al., 2002; Ohlmann et al., 2012); retinal blood vessels (Ohlmann et al., 2012) Other: Trabecular meshwork (Ohlmann et al., 2012)

for HS and CS in other tissues, such as in the retina (Tao et al., 2022). Here, CS has been reported to be important for the stabilisation of synaptic connections, while HS primarily functions to stimulate new synapse formation (Maeda et al., 2011). The distinct functional roles of HS and CS (Karamanos et al., 2018) align with their ability to each interact selectively with different proteins in a structure-dependent manner (Vallet et al., 2021), possibly also accounting for their differential tissue localization (Smetsers et al., 2003; Clark et al., 2011; Maeda et al., 2011). These regional differences in the patterns of sulfated GAGs usually correlate with specific regions of cellular activity and

transformation. For example, the sulfated HS-GAG species deposited in the lens capsule have been shown to be enriched specifically at the equatorial region of the lens (Young and Ocumpaugh, 1966), supported by more recent findings of increased sulfated HS-GAG in the transitional and germinative zones of the rat lens, specifically in actively dividing lens epithelial cells (Wishart and Lovicu, 2021). To note, in the posterior lens capsule, this HS-labelling is restricted to defined laminae suggesting differential incorporation of HS into the capsule during development (Young and Ocumpaugh, 1966; Wishart and Lovicu, 2021). This labelling is further elaborated when examining the different HSPGs in the

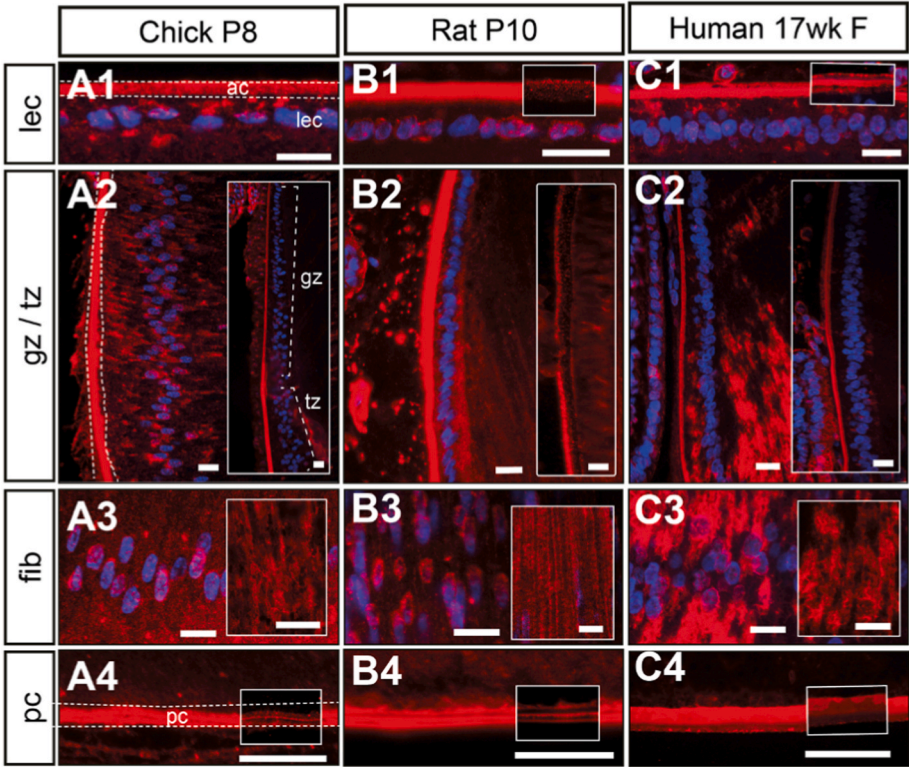


Fig. 2. Heparan sulfate glycosaminoglycan (HS-10E4 antibody) immunolabelling in the lens across different vertebrate species. Mid-sagittal eye sections from 8-day-old chick (A1-4), 10-day-old Wistar rat (B1-4) and 17-wk-old human foetal eyes (C1-4) were labelled for sulfated heparan sulfate glycosaminoglycan (HS-10E4 antibody) in the lens. Insets represent images that were underexposed to show further detail of the HS-10E4 Ab label in the anterior (B1, C1), equatorial (A2, B2, C2) and posterior (A4, B4, C4) lens capsules. Dotted white lines delineate the lens capsule. Nuclei were counterstained with Hoechst dye (blue). Abbreviations: central anterior epithelium (lec) and capsule (ac), germinative zone (gz), transitional zone (tz), lens fibre cells (fib), posterior lens capsule (pc). Post-mortem human foetal eyes (17-weeks gestation) were obtained from the Lions NSW Eye Bank, with informed consent, and approval from the University of Sydney Human Research Ethics Committee. All methods applied in the study were performed in accordance with the approved Human Research Ethics guidelines. Scale bars = 25 μ m (B1-4).

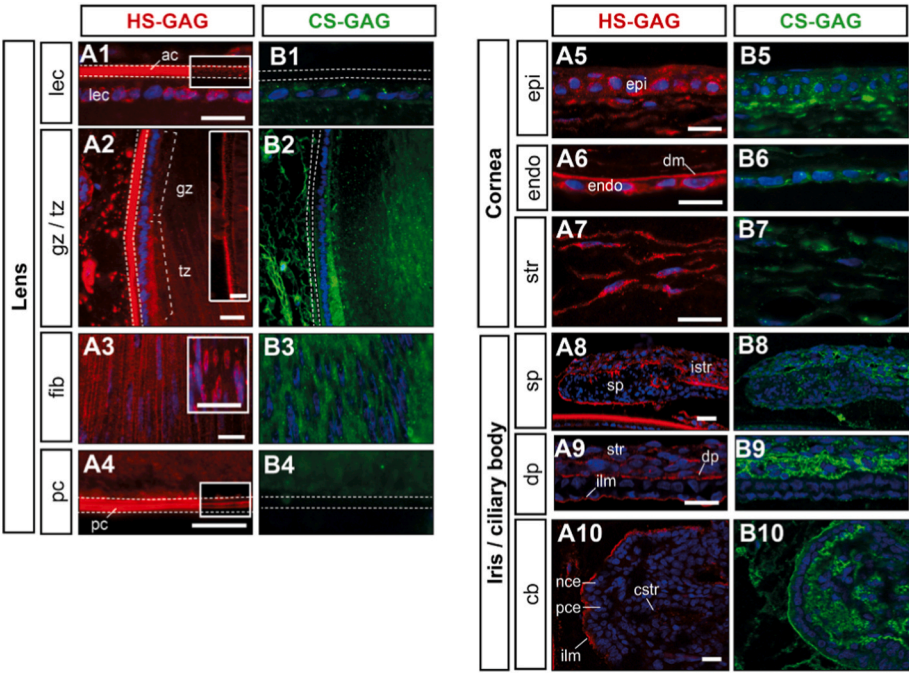


Fig. 3. Comparative immunolabelling of sulfated glycosaminoglycans in the anterior segment of the 10-day-old Wistar rat eye. Mid-sagittal eye sections immunolabel the highly sulfated domains of heparan sulfate (HS-GAG, HS-10E4 antibody; A1-10 red) or chondroitin sulfate (CS-GAG, CS-56 antibody; B1-10 green) glycosaminoglycan (GAG) chains in the lens (A/B1-4), cornea (A/B5-7), iris (A/B8-9), and ciliary body (A/B10). Dotted white lines demarcate the outline of the lens capsule (B1, B2, B4). Insets in A1, A2 and A4 represent images that were underexposed to show further detail of the HS-10E4 Ab label in the anterior, equatorial and posterior lens capsule, respectively. Nuclei were counterstained with Hoechst dye (blue). Abbreviations: Lens; central anterior epithelium (lec) and capsule (ac), germinative zone (gz), transitional zone (tz), lens fibre cells (fib), posterior lens capsule (pc). Cornea; epithelium (epi), stroma (str), endothelium (endo), Descemet's membrane (dm). Iris; anterior iris/sphincter pupillae (sp), iridial stroma (istr), posterior iris/dilator pupillae (dp), iridial inner limiting membrane (ilm). Ciliary body; non-pigmented ciliary epithelium (nce), pigmented ciliary epithelium (pce), ciliary inner limiting membrane (ilm), ciliary stroma (cstr). Scale bars = 25 μ m. (Adapted from Wishart and Lovicu, 2021).

ECM of the lens capsule.

2.2. ECM and basement membrane HSPGs

The lens capsule is a specialized basement membrane encasing the lens (reviewed in Danysh and Duncan, 2009; DeDreu et al., 2021). While providing structural support, the lens capsule also provides a reservoir for lens growth factors, that promote the proliferation, migration, and

differentiation of lens cells (Danysh and Duncan, 2009). Cell-matrix interactions involving integrins, HSPGs and other major structural proteins of the lens capsule, such as collagen IV, laminin, and nidogen (Mohan and Spiro, 1986) also contribute to these processes associated with lens development, homeostasis, and pathology. The lens capsule can also act as a selectively permeable sieve, passively filtering metabolic waste from the lens, and proteins from the surrounding ocular media by size and charge (Danysh and Duncan, 2009). HSPGs play a

central role in almost every aspect of these key functions of the lens capsule.

HSPGs initially isolated from anterior calf lens capsules were found to be immunologically related to those produced by bovine lens epithelial cells *in vitro*, and when isolated were revealed to be 400 kDa (akin to perlecan) and only carrying HS chains (Mohan and Spiro, 1991; Onodera 1991). Mohan and Spiro (1986) also reported a difference in the size of the predominant HSPG in the anterior (400 kDa) and posterior (>500 kDa) lens capsule. Soon after this, a predominant lens capsule HSPG (now recognized as perlecan) was observed to localise to defined laminae in the intact rat capsule (Lovicu and McAvoy, 1993), and more recently in embryonic chick (DeDreu et al., 2021).

HSPGs are also expressed in the basal laminae of different ocular tissues, including retinal vessels (Chai and Morris, 2009), and the inner limiting (Chai and Morris, 2009), and Bruch's membranes (Call and Hollyfield, 1990). To date, the predominant HSPGs in the lens capsule are recognized as perlecan (Uechi et al., 2014; Wu et al., 2014; Wishart and Lovicu, 2021; DeDreu et al., 2021), collagen XVIII (Uechi et al., 2014; Wishart and Lovicu, 2021), and agrin (Fuerst et al., 2007; Wishart and Lovicu, 2021). Recent studies have found other minor HSPG types in the lens capsule that are more commonly cell-associated, such as syndecans-1/-2/-4 and glypicans-2/-4 (Wishart and Lovicu, 2021). While this is the first time these have been reported in the lens capsule, with the exception of syndecan-4 (Qin et al., 2017), this is not overly surprising given syndecans have binding domains for different ECM molecules found in the lens capsule such as collagen IV (Esko and Selleck, 2002; Filla et al., 2004), laminin (Parmigiani and McAvoy, 1984, 1991; Utani, 2003; Carulli et al., 2012) and fibronectin (Parmigiani and McAvoy, 1984; Elenius et al., 1990; Kusano et al., 2000; Tumova et al., 2000), all of which can co-localise with syndecans (e.g. syndecan-2) (Filla et al., 2004). This association is consistent with the well-established roles that syndecans play as regulators of ECM assembly (Klass et al., 2000), cell-matrix interactions (Xian, 2010) and focal adhesion (Echtermeyer et al., 1999). Other syndecans (e.g. syndecan-4) have been reported to not only promote cell adhesion through binding lens capsule proteins (Uechi et al., 2014), including collagen IV (Esko and Selleck, 2002; Esko et al., 2017) and laminin (Utani, 2003; Carulli et al., 2012), but also play a role in integrin signalling-dependent migration of lens epithelia (Qin et al., 2017). While the specificity of cell-matrix interactions for cell adhesion is normally associated with integrin binding, given that major ECM molecules (e.g. collagen I and IV, fibronectin and laminin) also contain sites that interact with the glycosaminoglycan component of proteoglycans in the matrix, or on the cell surface, this broadens the functionality of cell surface HSPGs as modulators for cell-cell and cell-ECM interactions. It has been shown that the presence of HS chains promotes cell adhesion and that their absence can promote migration and invasion of malignant cells (Nagarajan et al., 2018).

HSPGs play essential roles in the structural architecture and assembly of the lens capsule. Perlecan-associated HS confers a structural role in the lens, as mice expressing perlecan deficient in HS have a compromised lens capsule (Rossi, 2003). Despite other HSPGs in the lens capsule, this highlights the individual importance of specific core proteins in lens maintenance.

HS is predominant in the lens capsule, with CS not detected (Dyksterhuis et al., 2011; Wishart and Lovicu, 2021). HSPGs are associated with the whole lens capsule; however, sulfated HS is unevenly distributed throughout the lens capsule. Highly sulfated HS-GAGs are concentrated in specific laminae of the lens capsule (Wishart and Lovicu, 2021), with the highest abundance localized to the inner- and outer-most laminae in young human (Fig. 2; Winkler et al., 2001), mouse (Webster et al., 1987), rat (Fig. 2; Wishart and Lovicu, 2021), rabbit (Landemore et al., 1999), and chick (Fig. 2; DeDreu et al., 2021) lenses. This uneven distribution of sulfated HSPGs seems to become less prominent with age (Webster et al., 1987; Winkler et al., 2001). DeDreu et al. (2021) describe the distribution of perlecan in two distinct laminae

of the equatorial chick lens capsule, the inner- and outer-most laminae, partially co-localised with laminin, bordering a central region rich in nidogen and collagen IV. A similar pattern is observed for sulfated HS (HS-10E4) in chick lenses (Wishart and Lovicu, unpublished data). Sulfated HSPGs have also been reported to increase in abundance in an antero-posterior gradient from the pre-equatorial proliferative zone of the lens to the post-equatorial transitional zone (Wishart and Lovicu, 2021) like that reported for perlecan and HS-bound FGFs (Lovicu and McAvoy, 1993; Schulz et al., 1997; Wu et al., 2014).

The uneven distribution of HSPGs in the lens capsule may suggest a role in regulating the diffusion and interactions of charged molecules within the lens capsule. Anionic sulfate groups within the lens capsule influence the binding and diffusion rates of charged proteins (Danysh et al., 2008), indicating that lens capsule HSPGs likely impart its negative charge, thus playing an important role in the passive perm selectivity of this membrane. Which HSPG(s) are important for this has yet to be entirely elucidated. For instance, similarly to the lens capsule, both perlecan and agrin are major components of the glomerular basement membrane (GBM) and contribute significantly to its charge selectivity; however, studies in transgenic animals concluded that neither perlecan (Rossi, 2003) or agrin (Harvey et al., 2007) are required for establishment or maintenance of the GBM charge barrier or its structural architecture, highlighting the complex nature of HSPG regulation of charge permeability barriers. The anionic properties of HSPGs may also contribute to the structural integrity of the lens capsule. Transgenic mice expressing perlecan with mutated HS attachment sites (no HS) had smaller eyes, and 'leaky' collapsed lenses that lacked the substructural properties of a normal lens capsule (Rossi, 2003). In contrast, chemical digestion of HS chains from bovine lens capsules resulted in stiffer lens capsules that were more resistant to mechanical stress (Dyksterhuis et al., 2011).

The uneven distribution of sulfated HSPGs in the lens capsule may also highlight an important role for these HSPGs in regulating the proliferation, migration and differentiation of lens cells, likely by regulating the distribution and availability of local growth factors to lens cells. The HSPGs associated with ECM and basement membranes commonly form local gradients of native factors and regulate their bioavailability to adjacent cells (Neill et al., 2015). The HSPGs of the lens capsule are known to directly bind to many lens mitogens including FGFs, PDGFs, and HB-EGF (Billings and Pacifici, 2015). These HSPGs can also indirectly regulate the signalling of other lens mitogens from the matrix by altering the formation of certain binding complexes between growth factors and their associated binding proteins (e.g. IGF-I with IGF-binding proteins; Arai et al., 1994). Enzymes including matrix metalloproteinases (MMPs) (Tholozan et al., 2007; Koyama et al., 2008; Jang et al., 2016), endosulphatases (Lamanna et al., 2007) and heparanase (Zhang et al., 2010) may release these HS-bound growth factors (e.g. FGF-2) making them available to cells. For instance, Tholozan et al. (2007) demonstrated the importance of the lens capsule as a local reservoir for FGF-2, and that its release from the capsule by MMP-2 is essential for lens epithelial cell viability and survival.

Like many components of living cells, proteoglycans on the cell-surface are turned over constantly. Membrane-bound 6-O-endosulphatases sulfatases-1 and -2 (SULF-1/2) are expressed by lens cells (Khan et al., 2015; Budak et al., 2018) where they may cleave specific (6-O-) sulfated domains (Lamanna et al., 2007). HSPGs may also be "shed" from the cell surface by proteolytic cleavage of their membrane-bound core protein (Lamanna et al., 2007). This shed heparan sulfate and any attached ligands can remain active as it travels to influence other distant cells and tissues.

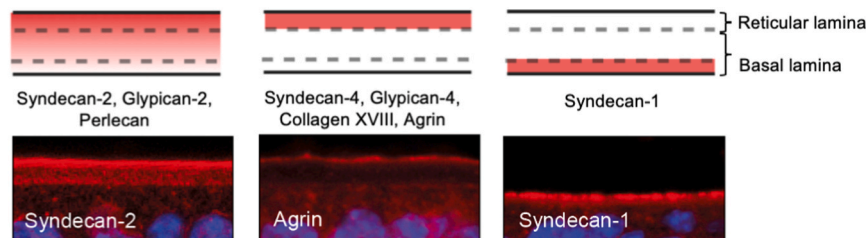
Extracellular heparanase enzymes may endoglycosidically cleave HS chains from their core proteins entirely, or partially at specific cleavage sites. Heparanase (heparan sulfate β -endoglucuronidase) activity may release HS-bound ligands, effectively freeing them from their sequestration, leaving them free to diffuse away from the cell or matrix-bound HSPG. Although heparanase plays a key role through this function in

many tissues, its role in lens biology is not well defined. While heparanase is reported to be expressed at low levels in the lens (Berk et al., 2004; Kakrana et al., 2018), it may play an indirect role in regulating lens cell behaviour through the shedding of HS and HS-bound growth factors from neighbouring tissues (i.e. the cornea and retina, see Berk et al., 2004). Its activity in the ocular media, as well as in ocular tissues that produce growth factors that act on the lens, may influence the formation of mitogenic and morphogenetic gradients controlling lens development and growth (Bishop et al., 2007). Another possible role for heparanase may be in lens pathology, given it is found to be expressed in the anterior lens capsule of human lenses from patients with systemic sclerosis (Gomes et al., 2021).

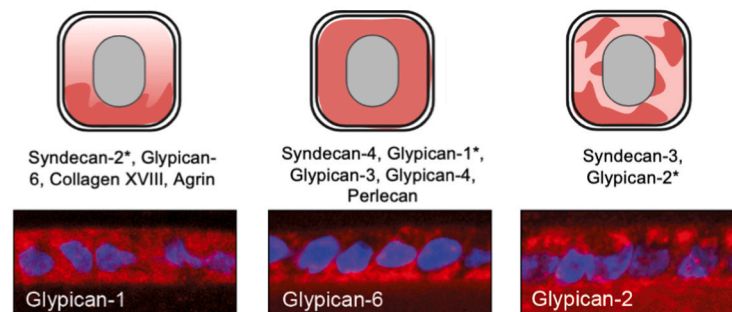
2.3. Cellular HSPGs

Aside from the normal association of HSPGs with cellular organelles involved in their biosynthesis and glycosylation (the endoplasmic reticulum and Golgi apparatus, respectively), HSPGs have been reported to localise to different cellular compartments (reviewed in Couchman and Pataki, 2012; Kolset et al., 2004), including the cytoplasm (Miyake et al., 2014; Sun et al., 2019), cytoskeleton (Granés et al., 2000; Stigliano et al., 2009), cell nuclei (Brockstedt et al., 2002; Chen and Sanderson, 2009) and endocytic vesicles (Kolset et al., 2004; Matsuo and Kimura-Yoshida, 2014). Lens epithelia express most of the different HSPGs (Wishart and Lovicu, 2021), and their labelling patterns and intensity vary in the different subcellular functional compartments and

A) Lens capsule



B) Lens epithelium



C) Lens fibres

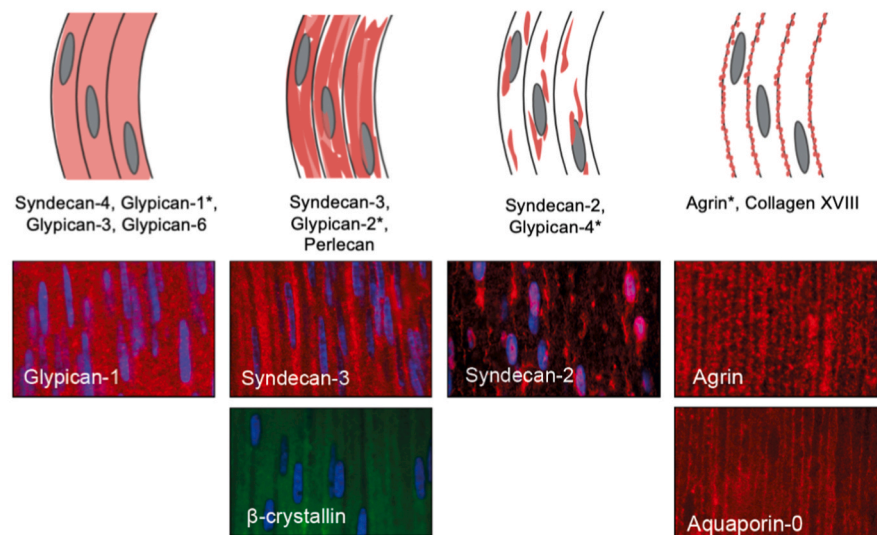


Fig. 4. Spatial localization patterns of HSPG core proteins in lens cells. Comparative schematic of immunolocalization patterns of HSPG core proteins in the capsule (A), anterior epithelium (B) and equatorial fibre cells (C) of the 10-day-old Wistar rat lens. * indicates nuclear labelling (Adapted from Wishart and Lovicu, 2021).

regions (anterior vs posterior) of these cells (see Fig. 4). While some HSPGs demonstrate a diffuse cytosolic label (syndecans-1/-4, glypicans-3/-4, perlecan), others (syndecans-2/-3, glypicans-1/-2/-6, collagen XVIII, agrin) are more polarised, with prominent labelling at the cell-cell interface (syndecan-3, glypican-3), and in (syndecan-2) or around cell nuclei (syndecan-4, glypicans-2/-6) (Wishart and Lovicu, 2021), suggestive of their differing functional roles in lens epithelia. For example, while syndecan-1 expression is low in most of the lens epithelium, its labelling is heightened in the spindle of mitotic cells, relative to those in interphase (Wishart and Lovicu, 2021) as previously reported, hypothesised to play a role in the stabilisation of the cellular mitotic machinery (Brockstedt et al., 2002).

There is a clear correlation of HSPG localization to function. Glypicans are present in the apical pole of epithelial cells (Wishart and Lovicu, 2021) (Fig. 4), and these HSPGs are known to regulate hedgehog (Liu et al., 2022; Capurro et al., 2017) and canonical Wnt-signalling (Capurro et al., 2014), that are involved in the maintenance and proliferation of the lens epithelium, as well as the regulation of fibre differentiation (Kerr et al., 2012; Fujimura, 2016). This is also seen with the diffuse cytosolic labelling for glypican-3 in liver cancer cells (Akutsu et al., 2010; Li et al., 2019b), where it too regulates canonical Wnt/ β -catenin signalling by modulating the subcellular localization of β -catenin to drive the proliferation of these cells (Capurro et al., 2014; Kolluri and Ho, 2019). Like glypicans, syndecans-2/-3 also localise to the apical pole of the lens epithelium, at the epithelial to fibre cell interface (Wishart and Lovicu, 2021) (Fig. 4), where there are also reports of labelling for β 1 integrin (Simirskii et al., 2007), and F-actin (Cheng et al., 2017); proteins that syndecans interact directly with, and/or regulate (Saoncella et al., 1999; Echtermeyer et al., 1999; Filla et al., 2006; Qin et al., 2017).

While a gradient of HS-labelling throughout the lens has previously been reported, the same labelling gradient is observed for many of the HSPG core proteins. This gradient may correlate with the heightened proliferation of lens epithelia in the equatorial germinative zone, where only the actively mitotic cells strongly label for syndecans-1/-4, glypicans-2, and collagen XVIII (Wishart and Lovicu, 2021). In the intact lens, prominent labelling of HSPGs in the nuclei of early differentiating fibre cells has been reported, a phenomenon also observed *in vitro*, where lens epithelia cultured with a fibre-differentiating dose of FGF, translocate HS to the nucleus of the elongating cells (Wishart and Lovicu, 2021). While HSPGs localise to the nuclear compartment of various cell-types, the function of nuclear HS/HSPGs is yet to be understood (Kovalszky et al., 2014; Stewart and Sanderson, 2014); however, their association with HS-binding proteins, including growth factors and their high affinity receptors, may implicate them in the regulation of gene expression and the control of cell behaviour (reviewed in Stewart and Sanderson, 2014). For example, FGF-2 and sometimes even its receptor, FGFR-1, have been detected in the nuclei of different cell types (Maher, 1996), including differentiating lens fibre cells (de Jongh et al., 1996), where they can co-localise with HSPGs, influencing cell proliferation and differentiation in a cell type-specific manner (Amalric et al., 1994), not always in a positive way (Fedarko and Conrad, 1986; Fedarko et al., 1989; Szatmári et al., 2017). Syndecan signalling is also required for nuclear localization of FGF-2 and subsequent proliferation of stromal fibroblasts in corneal injury (Hsia et al., 2003). In mesenchymal cancer cells, FGF-2 colocalizes with syndecan-1 in the nucleus, but not FGFR-1 (Zong et al., 2009). While it is not clear how these molecules co-localise, when HS chains are poorly synthesised, this reduces the levels of nuclear localised FGF-2, as shown in CHO cells (Hsia et al., 2003). It has been proposed that the nuclear translocation of syndecans and FGF is highly regulated through tubulin-mediated transport (Brockstedt et al., 2002), via a protein kinase Ca -dependent-signalling mechanism (Horowitz and Simons, 1998), with PI3K/Akt and ERK1/2 activation also thought to be involved in syndecan regulation of FGF-2-signalling (Qin et al., 2017). The internalisation and trafficking of cell surface syndecan-4 to cell nuclei, for example, is suggested to regulate the expression of genes

coding for integral proteins, such as β 1-integrin and actin in differentiating muscle (Rønning et al., 2015). In the lens, we see that syndecan-2 localises most strongly to cell nuclei (Wishart and Lovicu, 2021) despite it being one of the syndecans that lacks a juxta-membrane RMKKK (Arg-Met-Lys-Lys-Lys) nuclear localization sequence (Brockstedt et al., 2002), highlighting that it may be dependent on other binding partners (such as FGF) for this process. It has been reported to co-localise with FGF-2 in nuclei of neural cells (Leadbeater et al., 2006), and is also reported in chondrosarcoma cell nuclei (Schrage et al., 2009). Despite syndecan-2 having a structurally similar core protein to syndecan-4, the extracellular N-terminal domain of only syndecan-2, carrying N-sulfation-enriched HS chains, is reported to be necessary to induce formation of the VEGFA165-VEGFR2 complex and promote VEGFR2 activation (Corti et al., 2019).

3. Functional roles of different HSPGs

Not surprisingly given the extent of their expression, diverse roles for HSPGs have been described in the regulation of multiple key lens signalling molecules and developmental processes. By mediating the binding of a large number of extracellular ligands to their receptors, HSPGs trigger a cascade of reactions that subsequently regulate various cell processes including proliferation, differentiation, adhesion, migration, cell survival, and matrix assembly and organisation (Bishop et al., 2007). HSPGs can bind to and regulate the activity of PDGF-A, for example, that is essential for lens growth and transparency (Brewitt and Clark, 1988) through the regulation of lens epithelial cell proliferation (Iyengar et al., 2009) and fibre differentiation (Li et al., 2019a). With the diverse structure and overlapping temporal and spatial distribution of the different HSPGs in many tissues, it does not easily allow us to delineate their precise roles and activity. While mouse models with deficiencies in different HSPGs provide some insights (see Table 2), without compound knockouts to account for any redundancy, it is difficult to ascertain the precise role of any one specific HSPG core protein type. Here, we have examined different HSPG members in the context of what is currently known regarding their functional roles in the lens and its surrounding tissues.

3.1. Cellular HSPGs

3.1.1. Syndecans

The syndecans are a unique and highly versatile family of four HSPGs, syndecan-1 (syndecan, CD138), syndecan-2 (fibroglycan, CD362), syndecan-3 (N-syndecan, neuroglycan), and syndecan-4 (amphiglycan, ryudocan), primarily localised to the cell surface, mediating numerous cellular processes through signalling pathways that control cell proliferation, migration, mechano-transduction and endocytosis (Bernfield, 1992; Couchman et al., 2015; Afratis et al., 2017). Common to all syndecans is a highly conserved type-I transmembrane domain and extracellular domains, that function to target the syndecans to sites at the cell surface and pericellular matrix where they collaborate with other proteins to regulate growth factor signalling and cytoskeletal organisation (Rapraeger, 2001). The intracellular domain of the syndecan core protein comprises two “conserved” domains that flank a short “variable” region that binds cytosolic regulatory factors and uniquely defines the intracellular signalling interactions of each syndecan family member. While all syndecans have HS chains that mediate their interactions with extracellular signalling molecules, syndecans-1 and -3 may also carry CS (Rapraeger et al., 1985). While the functions of each syndecan family member are distinct, there is higher structural and functional similarity between syndecans-1 and -3 (Afratis et al., 2017), and similarly between syndecans-2 and -4 (Oh, 2004).

All syndecans have been identified as essential regulators of the signalling of several key ocular growth factors and morphogens, including FGFs (Zong et al., 2009; Jang et al., 2012; Qin et al., 2017), IGFs (Ruiz et al., 2012), EGFs (Dobra et al., 2003; Ibrahim et al., 2017),

Table 2
HSPG mutant phenotypes.

Core protein/ gene	Phenotype
Syndecan-1 <i>Sdc1</i>	<i>Sdc1</i> -deficiency causes defects in cell proliferation, migration, and regulation of integrin expression in corneal epithelial cells (Stepp et al., 2002), inflammation-mediated corneal angiogenesis (Götte et al., 2002), and resistance to corneal infection (Hayashida et al., 2011, 2015; Jinno et al., 2020).
Syndecan-2 <i>Sdc2</i>	<i>Sdc2</i> -deficiency causes (endothelial-cell specific) delayed retinal vascular development (inhibition of VEGFA ₁₆₅ -signalling and angiogenesis) (Corti et al., 2019). <i>Sdc2</i> ^{tm1b(KOMP)Wtsi} mice show abnormal lens morphology/cataract (Dickinson et al., 2016).
Syndecan-3 <i>Sdc3</i>	<i>Sdc3</i> -deficiency causes impaired locomotion; fibrosis, and hyperplasia, aberrant differentiation and increase in overall tyrosine phosphorylation in muscle cells (Cornelison et al., 2004); impaired hippocampus-dependent memory (Kaksonen et al., 2002).
Syndecan-4 <i>Sdc4</i>	<i>Sdc4</i> -deficiency causes increased ocular pathological angiogenesis and decreased retinal neovascularization. <i>Sdc4</i> ^{tm2b(KOMP)Wtsi} / <i>Sdc4</i> ⁺ mice show abnormal retinal blood vessel morphology, mydriasis (Dickinson et al., 2016), and tissues do not respond to VEGF-A with abnormal VE-Cadherin trafficking (De Rossi et al., 2021). <i>Sdc4</i> ^{−/−} mice have delayed wound healing associated with decreased cell proliferation/migration (Echtermeyer et al., 2001; Corti et al., 2013). <i>Sdc4</i> ^{−/−} mice show reduced capsular opacification after injury (Qin et al., 2017). Downregulation blocks FGF-signalling and integrin-mediated cell adhesion/migration (Qin et al., 2017).
Glypican-1 <i>Gpc1</i>	<i>Gpc1</i> ^{em1(IMPC)Mbp} / <i>Gpc1</i> ^{em1(IMPC)Mbp} mice have an abnormal eye phenotype (Dickinson et al., 2016). Overexpression of <i>Gpc1</i> in the chick trigeminal placodal ectoderm blocks proliferation and differentiation and can lead to the loss of the ophthalmic trigeminal ganglion (Jen et al., 2009).
Glypican-2 <i>Gpc2</i>	No reported eye phenotype for <i>Gpc2</i> deficiency (Dickinson et al., 2016)
Glypican-3 <i>Gpc3</i>	Despite Wnt and JNK signalling being altered in other <i>Gpc3</i> ^{−/−} tissues (Song et al., 2005), no eye phenotype has been reported (Cano-Gauci et al., 1999; Chiao et al., 2002)
Glypican-4 <i>Gpc4</i>	No reported eye phenotype associated with <i>Gpc4</i> -deficiency (Amor et al., 2019; Dowling and Allen 2018). GPC4 variants associated with Keipert Syndrome (Amor et al., 2019).
Glypican-5 <i>Gpc5</i>	There is no reported eye phenotype associated with a <i>Gpc5</i> -deficiency (Joslyn et al., 2011; Okamoto et al., 2015).
Glypican-6 <i>Gpc6</i>	<i>Gpc6</i> -deficiency causes neonatal lethality, with no reported eye phenotype (Campos-Xavier et al., 2009; Capurro et al., 2017); however, reduced GPC6 may be linked to sporadic retinoblastoma (Lau et al., 2010).
Perlecan <i>Hspg2</i>	<i>Hspg2</i> -deficiency is embryonic lethal and causes basement membrane malformations (Arikawa-Hirasawa et al., 1999); microphthalmia and thin corneal epithelium associated with decreased differentiation (Inomata et al., 2012). Pathological variants of <i>Hspg2</i> are linked to Schwartz-Jampel Syndrome (where Type 1 is associated with cataract) (Stum et al., 2006). HS-deficient perlecan (<i>Hspg2A3/A3</i>) results in an eye-specific phenotype with lens degeneration, corneal abnormalities, impaired corneal angiogenesis, delayed wound healing and microphthalmia (Rossi, 2003; Zhou et al., 2004).
Aggrin <i>Aggrn</i>	<i>Aggrn</i> deficiency in zebrafish causes microphthalmia, retinal mis-differentiation, abnormal retinal lamination, optic nerve hypoplasia, delayed regression of retinal blood vessels, lack of or abnormal outgrowth of retinal vessels, reduced size of the optic fibre layer, and disruption of retinal ganglion cell axon growth to the optic tectum (Kim et al., 2003, 2007; Liu et al., 2008). <i>Aggrn</i> overexpression causes anophthalmia, persistence of vitreal vessels, retinal mis-differentiation and fusion of anterior chamber structures (Fuerst et al., 2007).
Collagen XVIII <i>Col18a1</i>	<i>Col18a1</i> deficiency causes widespread anterior and posterior eye defects including lens subluxation, macular degeneration, ciliary body atrophy, iris fragility, delayed regression of retinal blood vessels, abnormal retinal vasculature (Fukai et al., 2002), attenuated visual function associated with impaired retinal pigmented epithelium function that worsens with age (Marneros and Olsen 2003; Marneros et al., 2004). Mutations of <i>Col18a1</i> are associated with Knobloch syndrome (severe myopia, vitreoretinal degeneration, anterior eye defects, occipital encephalocele) (Passos-Bueno et al., 2006; Khan et al., 2012). Overexpression of <i>Col18a1</i> (endostatin domain) can cause cataract (Elamaa et al., 2005; Aikio et al., 2013).

PDGFs (Das et al., 2016), HGF (Seidel et al., 2000), BMPs (Fisher et al., 2006), Wnts (Pataki, 2015) and Ephrins (Ethell et al., 2001). Moreover, syndecans can influence the downstream signalling pathways of these molecules, including the activation of MAPK/ERK (Bellin et al., 2009; Qin et al., 2017) and PI3/AKT/mammalian target of rapamycin (mTOR) (Partovian et al., 2008; Qin et al., 2017) signalling. In addition to their role as receptors and co-receptors, many other studies have suggested that signalling through the core protein of syndecans can regulate cytoskeletal organisation through their clustering, association with cytoskeletal structures, binding to cytoplasmic binding proteins, and intracellular phosphorylation (Yoneda and Couchman, 2003). For instance, the cytoplasmic domains of their core proteins can interact directly with cytoskeletal proteins (e.g. α -tubulin, cortactin, α -actinin), GTP-ases (RhoA, Rac1), kinases (protein kinase C- α , PKC α ; c-Src kinase; calcium/calmodulin-dependent serine protein kinase, CASK; focal-adhesion kinase, FAK), cytoplasmic proteins (e.g. ezrin/radixin/moesin; ERM) and other cell-surface receptors (integrins) to directly influence cell architecture, adhesion and motility (Afratis et al., 2017). All syndecans have been shown to interact with actin-associated proteins and evidence has been presented for signalling to the cytoskeleton (Morgan et al., 2007; Yoneda and Couchman, 2003). Syndecan-4 signals to the cytoskeleton primarily through PKC α that binds directly to the syndecan-4 core protein (Oh et al., 1997; Horowitz and Simons, 1998) and this phosphorylation of the syndecan-4 core protein is sufficient for the modulation of focal adhesions and actin stress fibres (Echtermeyer et al., 1999), linked to lens fibre cell differentiation (Weber and Menko, 2006).

Mutations in syndecans have revealed critical roles in development in lower vertebrates (Kuriyama and Mayor, 2009; Muñoz et al., 2006; Olivares et al., 2009), with similar roles described in mammals;

however, mutant mice with compromised syndecans-1, and -4, do not show any gross developmental abnormalities (Echtermeyer et al., 2001; Gougoula et al., 2019). Syndecan-3 null mice have been described to have some abnormalities in their memory (Kaksonen et al., 2002), locomotion (Cornelison et al., 2004) and metabolism (Dickinson et al., 2016). Following these reports, it was suggested that the syndecans may be functionally redundant in their regulatory roles; however, recent closer characterization of these syndecan-deficient animals has revealed abnormalities at the molecular level, suggesting distinct and separable roles for different syndecan family members. For instance, syndecan-3 deficiency impairs neural migration in the brain (Hienola et al., 2006), and explanted satellite cells from syndecan-3 null mice display aberrant differentiation, with a general increase in overall tyrosine phosphorylation (Cornelison et al., 2004). A role for syndecan-3 (SDC3) in lens fibre cells may relate to its association with SRC kinases and cortactin (Filla et al., 2004; Kinnunen et al., 1998) that regulate fibre differentiation in concert with Ephrin signalling (Cheng et al., 2013) that is required for the phosphorylation of the syndecan-3 core protein (Asundi and Carey, 1997). Mice deficient in syndecan-4 have delayed wound healing, angiogenesis and FGF-2/integrin signalling (Echtermeyer et al., 2001; Morgan et al., 2007; Qin et al., 2017). Recent labelling studies revealed novel expression patterns that implicate syndecan-4 in lens/eye development, where it appears to be induced in the placodal cells of the lens primordia and optic vesicles and increases in intensity with the invagination of the placodal cells to form the lens pit, later becoming abundant in the anterior lens epithelium, germinative zone and lens capsule (Wishart and Lovicu, manuscript in preparation). Moreover, its high expression at the anterior and posterior poles of the lens fibre tips (Wishart and Lovicu, 2021; see Fig. 4) is consistent with the expression of F-actin, FAK and β 1 integrin (Kokkinos et al.,

2007; Simirskii et al., 2007; Zhang et al., 2016a), that are all known to interact directly with, and/or are regulated by syndecan-4 (Morgan et al., 2007; Qin et al., 2017), bridging the extracellular matrix with the cytoskeleton (Rapraeger et al., 1986). In the lens, syndecan-4 is an essential regulator of FGF-2 and integrin signalling (Qin et al., 2017), acting as a FGFR co-receptor, strengthening the duration and intensity of downstream FGF-induced MAPK/ERK signalling, that has been shown to be required for lens fibre differentiation (Lovicu and McAvoy, 2001; Iyengar et al., 2007; Wang et al., 2010). Of greater interest, analysis of *Sdc2*^{-/-} (*Sdc2*^{tm1b(KOMP)Wtsi}) mice reported a high incidence of lens anomalies, including cataract (Dickinson et al., 2016), but these remain poorly characterised. It is not clear if this represents a lens-intrinsic- or secondary-phenotype, as syndecan-2 deficiency in ocular endothelial cells delays retinal vascular development and inhibits VEGFA₁₆₅-signalling and angiogenesis (Corti et al., 2019).

Integrin signalling is key in several lens processes in morphogenesis and for lens maintenance throughout life (Walker and Menko, 2009). In particular, β 1 integrin-signalling that has been shown to be regulated by syndecans-2 and -4 (Couchman and Woods, 1999; Filla et al., 2006; Kusano et al., 2000) and potentially other syndecans (Morgan et al., 2007), is essential for maintenance of the lens epithelium and for the structural maintenance and homeostasis of differentiating fibre cells (Simirskii et al., 2013). Activation of the canonical fibronectin receptor α 5 β 1-integrin requires a HSPG co-receptor such as syndecan and involves the activation of protein kinase C α . In syndecan-4-deficient murine lenses or with downregulation of syndecan-4 in human lens epithelial cells *in vitro*, this suppresses β 1 integrin-mediated activation and cell adhesion (Qin et al., 2017).

Other novel syndecan-associated molecules include equarin, which was first identified as a secreted molecule using a signal sequence trap screen in chick and shown to be specific to the lens (Mu et al., 2003), in both chick and mice (Song et al., 2012). Consistent with its co-expression with lens fibre-specific β -crystallin (Mu et al., 2003), it is thought to complex with syndecan-3 HS chains to regulate FGF-induced lens fibre differentiation and cell-adhesion, with its loss abrogating lens fibre cell markers (Song et al., 2013).

3.1.2. Glypicans

The glypican family is comprised of six HSPGs (glypicans-1 to -6) that form two subgroups; with glypicans-3/5 and glypicans-4/6 respectively sharing high homology (De Cat, 2001). Glypicans only carry HS-GAG chains, and each is linked to the cell surface via a GPI anchor (Sarrazin et al., 2011). With the exception of glypican-3, that has been extensively characterised in its role in the pathogenesis of hepatocellular carcinoma and other cancers (Zhou, 2018), the glypicans are arguably the least-studied of all the HSPGs, and their precise roles remain elusive.

In *Drosophila*, glypican homologues; division abnormally delayed (Dally) and dally-like, have been implicated as key orchestrators of cell polarity, morphogenesis, patterning, differentiation and growth during development, through their regulation of growth factor, cytokine, and morphogen signalling (Lin and Perrimon, 1999; Khare and Baumgartner, 2000; Williams et al., 2010; Zhang et al., 2013). Dally has been reported to be required for ordered cell division in the eye (Nakato et al., 1995), with *dally* mutants showing defects in progression through G2-M for specific sets of dividing cells. A number of studies have demonstrated that Dally serves as a regulator of growth factor signalling during development, affecting the activity of Wingless (Wg; Wnt homologue; Lin and Perrimon, 1999; Khare and Baumgartner, 2000; Waghmare et al., 2020), Decapentaplegic (Dpp; TGF β /BMP homologue; Jackson et al., 1997; Akiyama et al., 2008) and Hedgehog (Williams et al., 2010; Ayers et al., 2012) in a tissue-specific manner, consistent with Dally serving as a molecule that promotes the assembly of discrete signalling complexes on the cell surface, as we see for HSPGs. The interplay between secreted morphogens Wg, Dpp and Hh regulates important events in *Drosophila* eye development, controlling tissue specification, growth,

and patterning (Ma and Moses, 1995; Chang et al., 2001; Won et al., 2015). Similarly, in the vertebrate eye, their homologues Wnt (Fujimura, 2016), TGF β /BMP (Furuta and Hogan, 1998; Wawersik et al., 1999; Flügel-Koch et al., 2002) and Hh (Kerr et al., 2012) are major players in eye development.

Glypicans can negatively regulate Wnt signalling by competitively binding to secreted Wnt proteins, controlling their bioavailability to cell surface Frizzled receptors as a co-receptor (Sakane et al., 2012; Wang et al., 2016; Li et al., 2019b; Shi et al., 2020). Glypican-1 and -3 can act as both positive and negative regulators of canonical Wnt signalling (Stigliano et al., 2009; Shiao et al., 2010; Capurro et al., 2014). Expression of *GPC4* enhanced the Wnt3a-dependent β -catenin pathway and the Wnt5a-dependent β -catenin-independent pathway, while knockdown of *GPC4* suppressed both pathways (Sakane et al., 2012). *GPC3* expression is also correlated with nuclear/cytoplasmic localization of β -catenin (Akutsu et al., 2010); however, its HS chains were reported not to be required for its stimulatory activity on Wnt5a signalling and for the formation of glypican-3-Wnt5a complexes in mice (Song et al., 2005), highlighting HS-chain independent regulation. *Glypican-3* knockout mice exhibited alterations in Wnt signalling associated with the regulation of cell proliferation; the loss of glypican-3 inhibited non-canonical Wnt/JNK signalling, with concomitant activation of canonical Wnt/ β -catenin signalling and increased mitotic activity (Song et al., 2005). Contrary to these studies, glypican-3 can directly bind to Frizzled through its HS-GAG chains, consistent with Wnt binding at the cell membrane triggering the endocytosis of a complex that includes Wnt, Frizzled and glypican-3 (Capurro et al., 2014).

Glypicans also play an important role in orchestrating growth factor signalling to modulate proliferation and cell growth (Filmus and Capurro, 2008; Qiao et al., 2008; Cano-Gauci et al., 1999). For example, glypican-1 deficiency leads to reduced brain size associated with loss of developmental FGF signalling (Jen et al., 2009). Modulation of glypican-1 levels by expression of full-length *GPC1*, or a truncated form that is thought to act in a dominant-negative fashion, prevents trigeminal placodal differentiation or proper ganglion formation, respectively, with particularly strong effects on the ophthalmic lobe (Shiao et al., 2010). *GPC1* overexpression phenocopies Wnt inhibition in causing loss of ophthalmic placodal neurons, and increased Wnt activity can rescue the effects of *GPC1* overexpression (Shiao et al., 2010).

3.2. High-molecular weight HSPGs

3.2.1. Perlecan

Perlecan is a large multidomain HSPG that is a ubiquitous component of cellular basement membranes and extracellular matrices, dynamically regulated throughout vertebrate development (Handler et al., 1997). Perlecan plays an important role in controlling the bioavailability of growth factors at the cell surface, and in the formation and orchestration of mitogenic and morphogenetic gradients (Gubbiotti et al., 2017). It can bind to and influence the signalling activity of other mitogens and morphogens including FGFs (Knox et al., 2002), BMPs (Decarlo et al., 2012), Hh (Palma et al., 2011) and EGF (Zoeller et al., 2009). Perlecan and matrix-bound FGF-2 are co-expressed in the lens capsule in a complimentary antero-posterior gradient, with the levels of both, highest at the lens equator correlating with proliferating lens epithelial cells and phospho-ERK1/2 labelling (Wu et al., 2014).

In zebrafish, perlecan helps form a VEGF-A gradient, and the regulation of VEGF-VEGFR plays an important role in developmental angiogenesis (Zoeller et al., 2009), potentially explaining why perlecan (*Hspg2*)-deficient mice are embryonic lethal. In mice lacking exon 3 of perlecan (*Hspg2* Δ 3/ Δ 3), that results in loss of HS side chain attachment to its core protein (Rossi, 2003), mice are viable but present with microphthalmia. In these *Hspg2* Δ 3/ Δ 3 mutant mice, the lens capsule was compromised by cell remodelling that led to its rupture at birth, and subsequent lens degeneration by weaning age. As mentioned earlier, other perlecan-rich basement membranes in these mice, such as the

GBM were intact with normal kidney function (Rossi, 2003). The lens phenotype in *Hspg2Δ3/Δ3* mice was exacerbated with the added loss of collagen XVIII, with lens degeneration proceeding faster. This suggested that the HS chains of perlecan have a structural function in lens and can readily regulate incoming signals protecting the lens from its environment (Rossi, 2003).

3.2.2. Agrin

Agrin is a large HSPG core protein that can exist as either a membrane-bound or secreted HSPG (Tsen et al., 1995). It is associated with the cell bodies and processes of retinal neurons and is concentrated at retinal synapses (Fuerst et al., 2007), as it is at neuromuscular junctions where it was first identified as essential for clustering acetylcholine receptors (Bezakova and Lomo, 2001). The functions of agrin also encompass other important roles in the central nervous system such as regulation of brain development, plasticity, and maintenance of the blood-brain barrier (reviewed in Kröger and Pfister, 2009 and Daniels, 2012). Not surprisingly then, systemic knockout of agrin is embryonic lethal (Gautam et al., 1996); however, surprisingly, its overexpression led to a phenotype specific to the eye (Fuerst et al., 2007). Ocular dysgenesis was correlated with the amount of agrin expressed, with microphthalmia and anophthalmia resulting from fusion of anterior chamber structures, and persistent hyperplastic primary vitreous associated with a failure of foetal fissure closure, coloboma and aberrant formation of the optic stalk (Fuerst et al., 2007).

3.2.3. Collagen XVIII

Collagen XVIII is a large HSPG, predominantly found in the ECM and basement membranes. Variations of the N-terminal domain of Collagen XVIII produce three distinct isoforms (short, medium/intermediate, and long) with distinct activity and tissue distribution (Muragaki et al., 1995; Rehn and Pihlajaniemi, 1995; Saarela et al., 1998). In the eye, the short isoform is localised to the lens capsule and inner limiting membrane of the retina, while the medium/long isoforms are found in the ciliary and iris epithelia, and Bruch's membrane. All isoforms are also present in the tunica vasculosa lentis (Ohlmann et al., 2012; Aikio et al., 2013). The C-terminus of all Collagen XVIII proteins contains an anti-angiogenic endostatin domain, and all isoforms have conserved HS-GAG attachment sites (Dong et al., 2003).

Given its varied ocular expression, Collagen XVIII plays a key role in eye formation and homeostasis (Heljasvaara et al., 2017). Mutations of *Col18a1*, (the gene encoding the Collagen XVIII core protein), are linked to Knobloch syndrome, characterised by severe and diverse ophthalmic defects in both anterior and posterior segments (Passos-Bueno et al., 2006; Khan et al., 2012). Similarly, *Col18a1*^{-/-} mice display widespread ocular dysgenesis and malformation of several eye tissues (Fukai et al., 2002; Ylikärppä et al., 2003; Marneros et al., 2004). Moreover, overexpression of the endostatin domain of Collagen XVIII leads to cataract and ultrastructural abnormalities in basement membranes (Elamaa et al., 2005; Aikio et al., 2013).

Collagen XVIII binds to and regulates the activity of many proteins directly through interactions with its core protein (Rychkova et al., 2005), and via its associated HS-GAG chains (Dong et al., 2003), as reported in the regulation of integrin and Wnt signalling. Collagen XVIII also binds to and interacts with several ECM proteins (e.g. laminin, collagens, nidogen), and plays an important role in maintaining the structural integrity of ocular basement membranes. Collagen XVIII likely plays a distinct role from other major basement membrane HSPGs in ocular basement membrane maintenance; as mentioned earlier lens degeneration due to impaired HS activity of perlecan is exacerbated when Collagen XVIII is also compromised (Rossi, 2003).

4. HS-GAG chain structure and sulfation

HSPGs have been shown to directly or indirectly regulate most lens mitogens and morphogens, primarily through binding to sulfated

domains of their associated HS chains (Dreyfuss et al., 2009). Therefore, it is not surprising that HS sulfation is required for normal lens development and molecular functions (Pan et al., 2006). To elucidate the biological significance of HS-GAG chains, animal models together with chemical approaches have been employed to alter or inhibit their biosynthesis or function. As ocular morphogenesis is difficult to evaluate in constitutive mutant animals deficient for HS or HS sulfation, due to defective gastrulation or death at an early stage of embryonic development (Bullock et al., 1998; Lin et al., 1999) or at birth (Fan et al., 2000; Ringvall and Kjellén, 2010), the generation of structurally-defined HS oligosaccharides (Jemth et al., 2003) and phenotypic analyses of animal models with conditional deletions of genes encoding different HS sulfotransferases (Forsberg and Kjellén, 2001) have proven invaluable in the elucidation of relationships between HS structure and molecular phenotype.

Once specific core proteins are synthesised, GAG chains, including HS are synthesised from cytoplasmic nucleotide sugars and assembled onto core proteins by HS-polymerases, glycosyltransferases, epimerases, and sulfotransferases in the Golgi apparatus (Li and Kusche-Gullberg, 2016) (Fig. 5). There is significant heterogeneity in HS chain-link, structure, modification sites, extent of sulfation, electrostatic charge distribution, and epimerization. The “sugar code” hypothesis proposes that the biological activity of HS is encoded in its sulfation patterns (Poulain and Yost, 2015). The heterogeneity of HS chains is due to a series of sulfation modifications of its chains that occur after the initial synthesis of the HS chain precursor, through a non-template driven process that does not necessarily go to completion (Li and Kusche-Gullberg, 2016). The patterning and degree of HS chain sulfation is not predetermined but a result of the isoforms of HS sulfation enzymes expressed by a given cell. This results in modifications of variable size along the chain resulting in highly heterogeneous products with variable sulfation. HS sulfotransferase modifications occur with high specificity (Zhang et al., 2016b) in a cell-type and developmental stage specific manner (David et al., 1992; Maccarana et al., 1996; Poulain and Yost, 2015) resulting in highly heterogeneous HS chains interspersed with un-sulfated and highly sulfated regions, with differential levels of 2-O-, 6-O- and 3-O-sulfation (Fig. 5). Furthermore, the specific HS sulfation pattern requirements for ligand interactions vary with the ligand. This is important as variations in the sulfation patterns of HS chains can confer functionally important differences in HS-ligand binding interactions and therefore differential regulation of biological functions (Kato et al., 1994; Pratt et al., 2006). Phenotypes resulting from the conditional and selective depletion of specific HS biosynthesis and sulfation enzymes, or combinations of HS biosynthesis and sulfation enzymes from the developing eye tissues, exemplifies their importance and provides clues that point to the distinct and complementary roles of different HS sulfation and fine structure in the regulation of developmental and biological events.

4.1. HS chain polymerisation (EXTs)

HS chains are composed of repeating units of glucuronic acid (GlcA)-N-Acetylglucosamine (GlcNAc) synthesised by HS-polymerases Exostosin-1 and -2 (EXT1/EXT2) (Busse et al., 2007). This step is referred to as “chain extension” and determines the length of the GAG chain (Li and Kusche-Gullberg, 2016). The number of disaccharide repeats, and therefore the length of the chain, are variable, and clearly important for growth factor signalling. For example, HS-deficient Ext2 null mutant mouse embryos fail to respond to FGF (Shimokawa et al., 2011).

Ext1 depletion (lacking all HS chains) in the pericocular mesenchyme during embryogenesis leads to microphthalmia with reduced axial lengths, lens diameters, and vitreous volume (Iwao et al., 2010). Similarly, *Ext1* deletion in neural crest cells of the developing eye causes microphthalmos, dysgenesis in the primitive iris and ciliary body, the failure of lens-cornea separation and optic nerve head agenesis (Iwao et al., 2006). Given our understanding of the wide array of protein

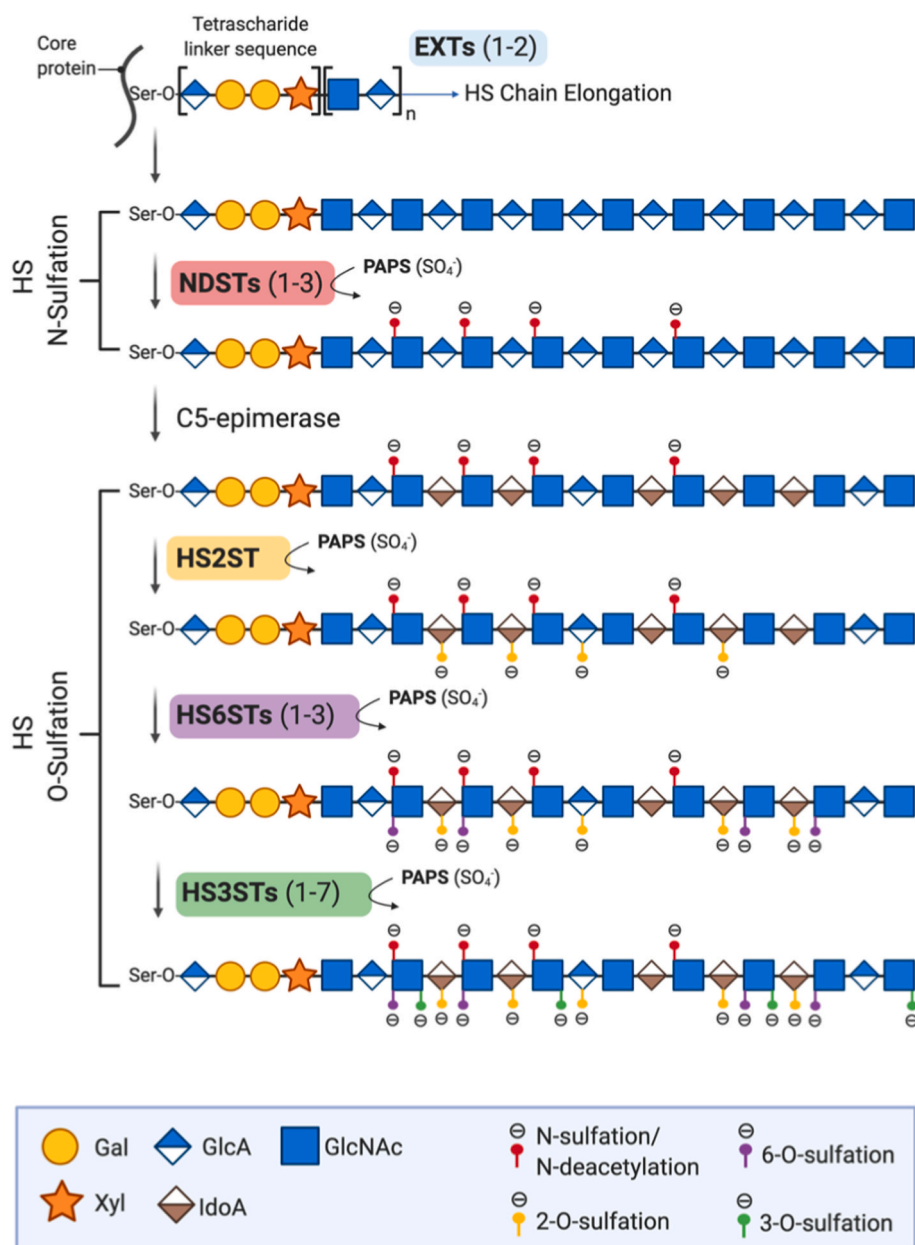


Fig. 5. Schematic representation of heparan sulfate proteoglycan biosynthesis and sulfation. Heparan sulfate (HS) glycosaminoglycan chain synthesis begins in the Golgi apparatus when Exostosin (EXT1/2) polymerises a series of repeating disaccharide units of glucuronic acid (GlcA)-N-Acetylglucosamine (GlcNAc) to the HS core-protein-associated tetrasaccharide linker sequence. Following HS-chain elongation, HS sulfotransferases transfer sulfate from the sulfate-donor molecule, 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to specific positions on the HS chain. The N-sulfated domains of the HS-chains are followed by 2-O-sulfation of selected iduronic acid (IdoA) residues by HS 2-O-sulfotransferase (HS2ST). Next, HS 6-O-sulfotransferases (HS6STs 1–3) transfer sulfate to the sixth position of N-Acetylglucosamine (GlcNAc) or N-sulfated GlcNAc (GlcNAS) residues. Finally, HS 3-O-sulfotransferases (HS3STs 1–6) transfer sulfate to the GlcNAS residues.

interactions orchestrated by HSPGs, it is perhaps not surprising that complete loss of HS-GAG would have detrimental effects on development. More complex and elusive are the divergent functional roles of the different HS sulfotransferases.

4.2. PAPS/PAPS synthetases

Following HS-chain elongation by EXT enzymes, HS sulfotransferases transfer sulfate from the sulfate-donor molecule, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), synthesised by cellular PAPS synthetases (PAPSS1/2) to specific positions on the HS chain. The two isoforms of PAPS synthase, PAPSS1 and PAPSS2 are localised primarily to the nucleus and cytoplasm of cells, respectively (Schröder et al., 2012). Transcriptome data identifies both PAPS synthetase isoforms in the lens (Kakrana et al., 2018); with immunolocalization studies demonstrating strong labelling for PAPSS2 in the developing murine and postnatal rat eye, showing high specificity to lens in both prenatal and postnatal ages (Fig. 6).

Of all the chemical inhibitors of HS activity, sodium chlorate

(NaClO₃) is the best characterised. Sodium chlorate inhibits the formation of PAPS, the sulfate donor, by competitively binding to PAPS synthetases thereby blocking the production of PAPS, the essential rate-limiting sulfate (SO₄⁻) donor required for all cellular sulfation events in the Golgi, including HS-GAG sulfation. Sodium chlorate treatment inhibits different types of HS-GAG sulfation in a concentration-dependent manner (Safaiyan et al., 1999) without compromising HS-GAG chain length or structural properties (Safaiyan et al., 1999; Conrad, 2001). This effectively reduces the sulfation of all sulfated GAGs (i.e. both CS- and HS-GAG), with the production of under-sulfated proteoglycans whose secretion is partially prevented by the quality control mechanisms of the cell (Fannon and Nugent, 1996). As a result, sodium chlorate-supplemented media has been widely used in various cellular systems to define the biological functions of sulfated GAG chains/HSPGs; however, a relatively high concentration of chlorate (up to 30 mM) is required to completely block all HS-GAG sulfation (Safaiyan et al., 1999; Conrad, 2001). Such high concentrations are not suitable for *in situ* studies in model organisms (Humphries and Silbert, 1988). High concentrations of chlorate (20–50 mM) have been shown to

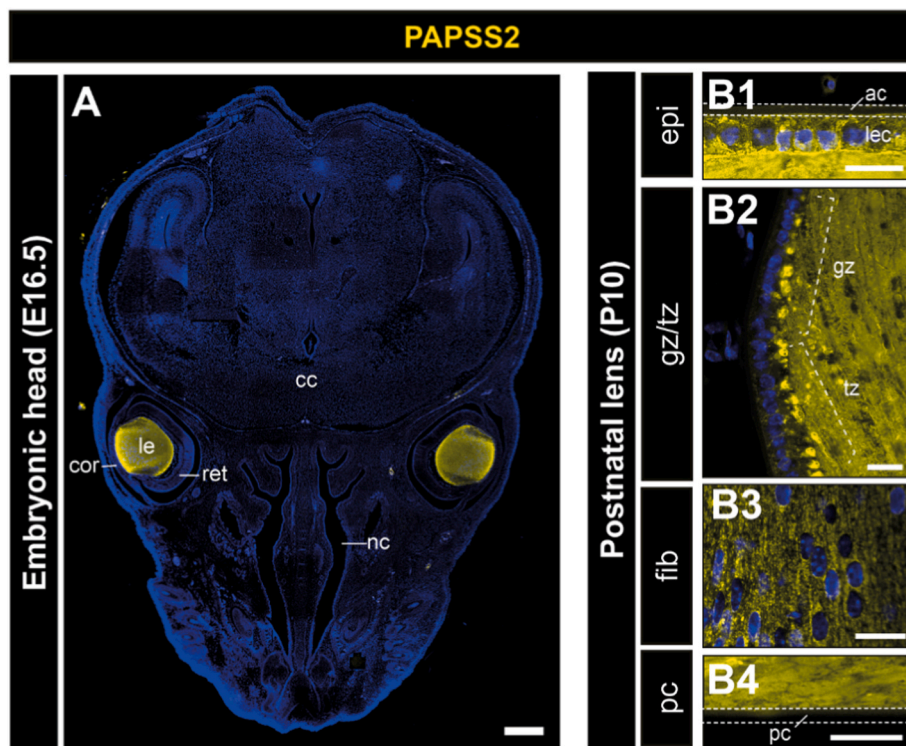


Fig. 6. Immunolocalization of PAPS synthase 2 (PAPSS2). A) Immunolocalization of PAPSS2 in the 16.5-day old embryonic (E16.5) mouse eye (A), and 10-day-old (P10) Wistar rat lens (B1-4). Dotted white lines delineate lens capsule. Nuclei were counterstained with Hoechst dye (blue). Abbreviations: cornea (cor), lens (le), retina (ret), cerebral cortex (cc), central anterior epithelium (lec) and capsule (ac), germinative zone (gz), transitional zone (tz), lens fibre cells (fib), posterior lens capsule (pc). Scale bars = 200 μ m (A), 25 μ m (B1-4).

block the signalling activity of aqueous humour (Iyengar and Lovicu, 2017; Wishart and Lovicu, 2021) and vitreous humour (Wishart and Lovicu, 2021) on primary rat lens epithelial cells in explants (West-Mays et al., 2010), as well as the activity of several ocular mitogens and morphogens including FGF-2 (Iyengar and Lovicu, 2017; Wishart and Lovicu, 2021), and VEGF (Ashikari-Hada et al., 2005). FGF- and aqueous-induced ERK1/2 signalling in lens epithelia was completely disrupted in the presence of sodium chlorate (Iyengar and Lovicu, 2017; Wishart and Lovicu, 2021), while aqueous-induced Akt phosphorylation was not (Iyengar and Lovicu, 2017). The effects of sodium chlorate can be replicated by digestion of cell-surface HS with heparitinase and rescued by simultaneous addition of sodium sulfate (restores HS sulfation; Iyengar and Lovicu, 2017) or porcine heparin (exogenous sulfated GAGs), indicating that the observed effects of sodium chlorate treatment in these studies are likely primarily attributable to the de-sulfation and transient loss of HS-GAG activity.

Despite sodium chlorate's ability to block the sulfation of all GAGs (both CS- and HS-GAG), our more recent studies showed similar results to sodium chlorate application in lens cells, when only selectively blocking HS-sulfation. Using Surfen hydrate, a small molecule antagonist of HS-protein interactions (Schuksz et al., 2008), we were able to effectively block only sulfation of HS, leading to impaired FGF-induced lens epithelial cell proliferation and fibre differentiation, as well as the corresponding activation of intracellular ERK1/2-signalling (Fig. 7; Wishart and Lovicu, 2021). In addition to its essential role for lens cell survival (Chamberlain and McAvoy, 1989; Chow et al., 1995; Garcia et al., 2005), FGF-induced lens fibre differentiation promotes cell elongation (Lovicu and McAvoy, 1989) and the accumulation of fibre-specific β -crystallin (Chamberlain and McAvoy, 1989; Peek et al., 1992) and aquaporin-0 (AQP0/MIP) (Golestaneh et al., 2008). Interestingly, FGF-induced ERK1/2-signalling is required for the elongation of fibre cells (Lovicu and McAvoy, 2001; Le and Musil, 2001) and expression of filensin and AQP0/MIP (Lovicu and McAvoy, 2001; Golestaneh et al., 2008), but not for β - and γ -crystallins (Lovicu and McAvoy, 2001; Wang et al., 2009), that may be under the control of alternate signalling pathways, such as PI3K/Akt (Wang et al., 2009). As

Surfen is able to block FGF-induced β -crystallin accumulation (Wishart and Lovicu, 2021), this suggests that these alternative pathways regulating crystallin expression are also dependent on HS sulfation, consistent with HSPGs role as an upstream co-receptor for FGFR-signalling in lens. As FGFs are the only class of growth factors to induce lens fibre differentiation in mammals (Lovicu and McAvoy, 2005), Surfen blocking studies signify HSPGs as important regulators of lens cell fate (Wishart and Lovicu, 2021).

The Surfen blocking studies support that the functional roles of HSPGs in the lens are predominantly through HS sulfation and not via other sulfated GAGs (e.g. CS-GAG). Despite the fact that both HS-GAG and CS-GAG are able to bind to FGF, they do not necessarily both need to contribute to FGFR-signalling, with binding favouring HS-GAG over CS-GAG. For example, a form of perlecan carrying both HS-GAG and CS-GAG chains is reported to regulate FGF-2-mediated chondrocyte proliferation. If the CS-GAG chains are enzymatically digested, the HS-carrying perlecan is not only sufficient, but can augment FGF2-FGFR binding and chondrocyte proliferation (Smith et al., 2007). In fact, HS-GAG and CS-GAG often display opposing effects on cell activity, with their respective structural motifs providing unique binding sites for specific ligands, with CS-GAG-protein binding interactions being more limited relative to HS-GAG (Vallet et al., 2021).

These experimental blocking studies *in vitro* focus attention on the different modalities of HSPG function, as co-receptors for matrix molecules and their ability to serve as independent receptors for heparin-binding growth factors. HSPGs also indirectly influence the intracellular signalling (e.g. MAPK/ERK, PI3K/AKT/mTOR, and the Rho family of GTPases of these molecules) by regulating receptor binding interactions, activation and trafficking (Bishop et al., 2007).

4.3. HS N-sulfation (NDSTs)

HS sulfation proceeds in a stepwise manner, beginning with the N-sulfation of specific glucosamine (GlcNAc) residues of the HS chain by N-deacetylase/N-sulfotransferase (Ndsts1-3) enzymes (Poulain and Yost, 2015). These enzymes play important roles in eye development

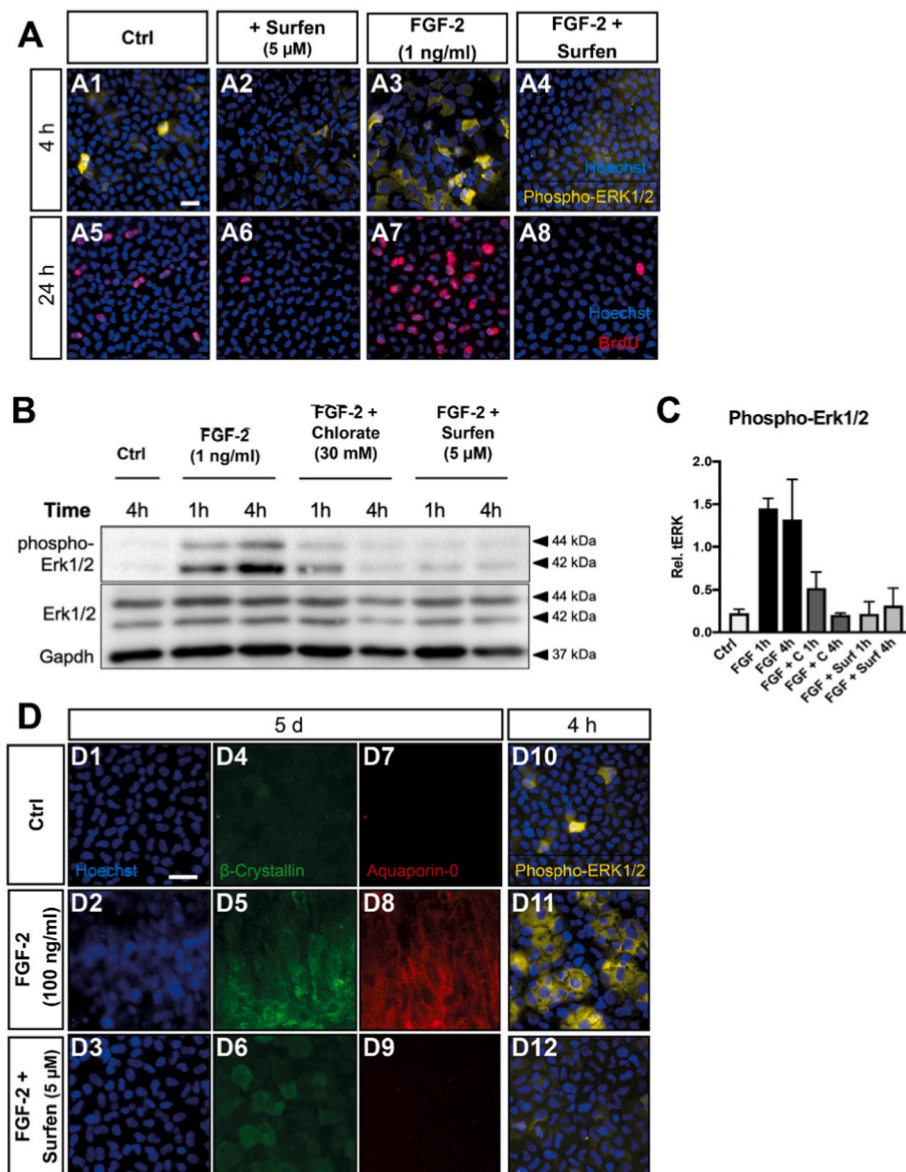


Fig. 7. Heparan sulfate is essential for FGF-2-induced lens epithelial cell proliferation and fiber differentiation in rat lens epithelial cells. **A)** 10-day-old rat lens epithelial explants treated with a proliferating dose of FGF-2 (1 ng/ml) immunolabeled for phosphorylated-ERK1/2 (4 h, A1-4) show increased proliferation (BrdU-incorporation; 24 h, A5-8). Co-treatment with Surfen hydrate (5 μ M), a small-molecule inhibitor of HS-protein interactions, blocked FGF-induced ERK1/2 phosphorylation (A4) and lens epithelial cell proliferation (A8). **B)** Western blotting for phosphorylated-ERK1/2 and total-ERK1/2 protein in explants treated with FGF-2 (1 ng/ml), with or without (NT) sodium chlorate (Chlorate, 30 mM) or Surfen (5 μ M) at 1 h and 4 h. **C)** Quantification of phospho-ERK1/2 (p-ERK1/2) relative to total-ERK1/2 (t-ERK1/2) and Gapdh protein, with representative western blot. Data are the mean $\pm$ SEM from at least three independent experiments. **D)** 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 μ M). Relative to controls (D1, D4, D7), explants treated with FGF-2 alone for 5 days labelled for fibre protein markers, β -crystallin (D5) and Aquaporin-0 (D8). FGF-2-treated explants showed increased phosphorylated-ERK1/2 labelling at 4 h (D11) relative to controls (D10). Co-treatment with Surfen blocked FGF-induced fibre elongation, accumulation of fibre markers (D6, D9), and ERK1/2 phosphorylation (D12). Scale bars = 25 μ m. (Adapted from Wishart and Lovicu, 2021).

with mutations in *Ndst1/2* displaying highly variable dysgenic eye defects, ranging from bilateral anophthalmia, microphthalmia, and/or aphakia, and to a lesser degree unilateral aphakia in embryos (Qu et al., 2011b) (see Table 2). N-sulfation of HS by *Ndst1* has been reported to be required for lens induction and development, with *Ndst1*^{-/-} mice displaying reduced lens cell proliferation and delayed or failed induction and vesicle formation of the lens (Pan et al., 2006). This was accompanied by severely disrupted expression of key lens proteins and transcription factors, including AP2 α , α A-crystallin, Pax6 and Prox1 (Pan et al., 2006). Interestingly, depletion of *Ndst1* caused the dysregulation of FGF-signalling in early lens development, but not other important pathways such as BMP- or Wnt-signalling (Pan et al., 2006). Conditional ablation of *Ndst1* and *Ndst2* (lacking HS N-sulfation) after the lens induction stage caused reduced lens cell proliferation, increased cell death and defective lens fibre differentiation in later lens development (Qu et al., 2011b). Thus, it is clear that HS sulfation is important in lens morphogenesis and postnatal growth, and that there is specificity in the binding interactions of growth factors and specific sulfated residues (e.g. N-sulfated) of HS. HS sulfation is also critical for normal growth factor signalling and development of other ocular tissues, such as the lacrimal gland (Qu et al., 2012) with N-sulfation of HS required for FGF-induced

induction events in lacrimal gland development (Pan et al., 2008), as seen in lens (Pan et al., 2006).

4.4. HS O-sulfation (HS-OSTs)

The N-sulfated domains of the HS-chains are followed by 2-O-sulfation of selected iduronic acid (IdoA) residues by HS 2-O-sulfotransferase (Hs2st). Next, HS 6-O-sulfotransferases (Hs6st1-3) transfer sulfate to the sixth position of GlcNAc or N-sulfated GlcNAc (GlcNAS) residues. Finally, HS 3-O-sulfotransferases (Hs3st1-6) transfer sulfate to the GlcNAS residues (Li and Kusche-Gullberg, 2016) (see Fig. 5). HSPGs carrying differentially O-sulfated HS have distinct and complementary roles in their regulation of developmental and biological events (Tillo et al., 2016). The interactions of HS with FGF-2 and its cognate receptors (FGFRs) have been characterised in detail. HS chains strongly facilitate the formation of ternary complexes with FGF and FGFR (Escobar Galvis et al., 2007), serving to lower the activation energy required for the conformational change of the FGFR and initiation of intracellular signalling, prolonging the duration of FGFR activation (Forsten-Williams et al., 2008). Distinct sulfate patterns can modulate different FGF-10-mediated gland developmental processes, such as proliferation,

duct elongation, bud expansion and differentiation (Pan et al., 2008; Patel et al., 2008).

Mice deficient in *Hs2st* (lacking HS 2-O-sulfation) display eye defects, including the formation of cataracts, abnormal pigmented retinal epithelium and bilateral coloboma of the iris (Bullock et al., 1998). The disruption of the mouse gene encoding d-glucuronyl C5-epimerase (the precursor for HS 2-O-sulfation) also causes frequent association with iris coloboma (Li et al., 2003). Despite binding of HS to HGF and PDGF-A being dependent on 6-O-sulfation, mice with systemic inactivation of *Hs6st1* (lacking HS 6-O-sulfation) develop without any major gross abnormalities, with the exception of errors in axonal guidance at the optic chiasm (Pratt et al., 2006). In early adulthood they develop growth retardation, body weight loss and lung defects leading to late postnatal mortality (Izvolosky et al., 2008). Few studies have investigated the interactions of HS 3-O-sulfation due to its rarity (De Agostini et al., 1990). Further investigations revealed unique and differential roles for 2-O- and 6-O- sulfation of HS in the regulation of later stages of lacrimal gland development. *Hs6st*^{-/-} mutants exhibited lacrimal gland hypoplasia and disrupted FGF signalling, while *Hs2st*^{-/-} mutants had a less severe phenotype. In *Hs2st*^{-/-}/*Hs6st*^{-/-} double mutants, lacrimal gland development and associated FGF-induced downstream ERK-signalling activation were completely abolished (Qu et al., 2011a). While not directly reported in this study, the developing lens of these mutants was also much smaller (Xin Zhang, personal communication).

Compared with 2-O- and 6-O-sulfation, 3-O-sulfation of HS is relatively rare, and typically comprises less than 1% of overall HS sulfation (De Agostini et al., 1990). *Hs3sts* are also expressed by fewer cell types (De Agostini et al., 1990). The lens expresses at least one isoform of each type of sulfotransferase, including several isoforms of 3-O-sulfotransferase (Fig. 8). Transcripts of genes encoding these HS-O-sulfotransferases are differentially regulated throughout lens development in mice (Fig. 8A). Interestingly, despite its rarity in other tissues (De Agostini et al., 1990), *Hs3st1* is selectively enriched in the lens relative to the rest of the eye throughout development, and postnatally (Fig. 8B). Of the seven HS 3-O-sulfotransferase genes present in the mouse, transcripts for *Hs3st1* represent close to two thirds of all the HS 3-O-sulfotransferase transcripts in the newborn lens (Hoang et al., 2014). The *Hs3st1*^{-/-} genotype is early postnatal lethal on a C57BL/6 inbred background, where homozygous embryos exhibit intrauterine growth retardation (HajMohammadi et al., 2003); however, F1 hybrids are relatively normal, but have been shown to have an enhanced sensitivity to

pro-inflammatory molecules, such as tumour necrosis factor (TNF, Smits et al., 2017).

5. Coordination of HSPGs in lens pathology

HSPGs have also been reported to play a key role in the damage and repair processes of diseased ocular tissues. These processes are often linked to significant changes in specific HSPGs (Heimann et al., 2001; Ibrahim et al., 2017) or HS sulfation enzymes (Saravanan et al., 2019) that can either support these pathogenic processes, as seen in cataract (the opacification of the lens) (Qin et al., 2017), or can promote healing (reviewed in Park and Shukla, 2013). These changes can affect both specific core proteins (i.e. cleavage of specific cell surface HSPGs, up-/down-regulation of specific core proteins, changes in tissue expression patterns) and/or HS chain structure (i.e. cleavage of HS chains, up-/down-regulation of specific HS biosynthesis/sulfation enzymes).

Cataract is the leading cause of blindness worldwide (Foster and Resnikoff, 2005). HSPGs have been implicated in the pathology of congenital cataract with a study by Sulochana and colleagues reporting that 65.9% of children with congenital cataracts also presented with elevated levels of HS and CS-GAGs in the blood, urine and lens. The authors speculated that the additional anionic GAG chains may accumulate in the lens and induce the extracellular accumulation of Na⁺ ions and water, causing cataracts in a similar manner to the pathogenesis of adult cortical cataract (Sulochana et al., 1997). Other forms of cataract, such as fibrotic subcapsular cataracts (anterior or posterior) result from aberrant lens epithelial differentiation processes. In anterior subcapsular cataract or capsular opacification (ASC/ACO), LECs are thought to transdifferentiate into myofibroblasts under the influence of TGFβ-2, secreting abundant ECM containing fibronectin and collagen I, as they adhere to the anterior lens capsule to create a fibrotic plaque (Fig. 9A and B) (de Jongh et al., 2005). Posterior capsular opacification (PCO) is a complication that may occur after routine cataract surgery (Awasthi, 2009), resembling the aberrant cellular processes associated with ASC/ACO. In PCO, LECs left behind after surgical phacoemulsification of most lens cells respond to aberrant, elevated levels of active TGFβ-2, that stimulates them to undergo an epithelial to mesenchymal transition (EMT) into myofibroblastic cells (Shu and Lovicu, 2017; Jiang et al., 2018). These myofibroblasts migrate to the posterior lens pole driven by FGF-2 and integrin signalling (Qin et al., 2017), where they

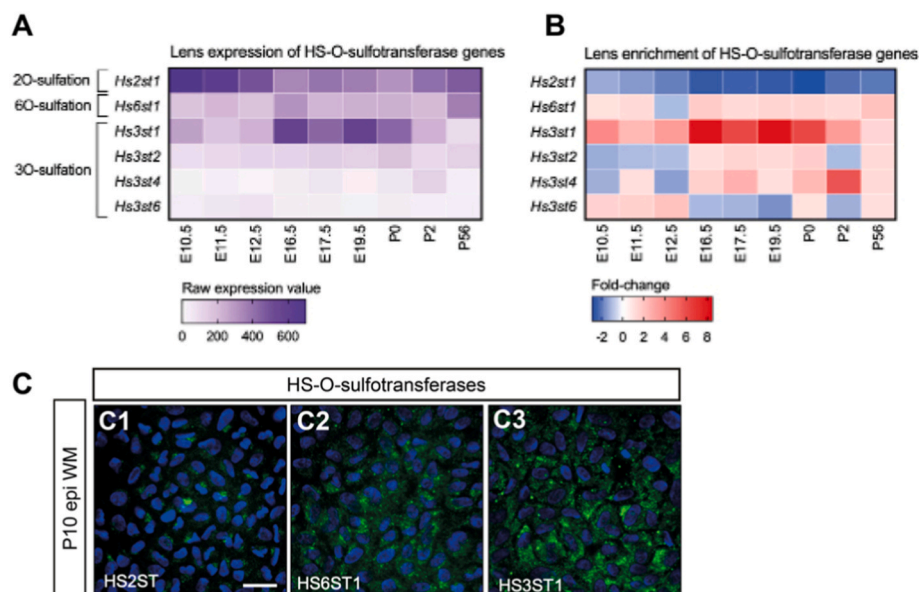


Fig. 8. Heparan sulfate O-sulfotransferase expression in the murine and rat lens. Transcript expression (A) and lens-enrichment (B) profiles of genes encoding heparan sulfate- 2O- (*Hs2st1*), 6O- (*Hs6st1*) and 3O- (*Hs3st1*, *Hs3st2*, *Hs3st4*, *Hs3st6*) sulfotransferase genes in Affymetrix microarrays from mouse lenses at embryonic stages 10.5 (E10.5), E11.5, E12.5, E16.5, E17.5 and E19.5; and postnatal days 0 (P0), P2 and P56. Lens expression and enriched expression data represent processed normalised microarray data from Affymetrix Mouse 430 2.0 platform sourced from the iSyTE (Integrated Systems Tool for Eye gene discovery) web-tool (Kakrana et al., 2018). A) Heat map intensity (purple) represents high to low gene expression. B) Lens-enrichment heat map reflects high (red) to low (blue) fold-change in gene expression relative to age-matched aphakic whole embryo control (in silico subtraction). C) Localization of heparan sulfate O-sulfotransferases heparan sulfate 2O-sulfotransferase (*HS2ST*; C1), heparan sulfate 6O-sulfotransferase-1 (*HS6ST1*; C2), and heparan sulfate 3O-sulfotransferase-1 (*HS3ST1*; C3) in 10-day-old (P10) Wistar rat lens epithelial (epi) wholemounts (WM). Nuclei were counterstained with Hoechst dye (blue). Abbreviations: Scale bar = 25 μm (C1-3).

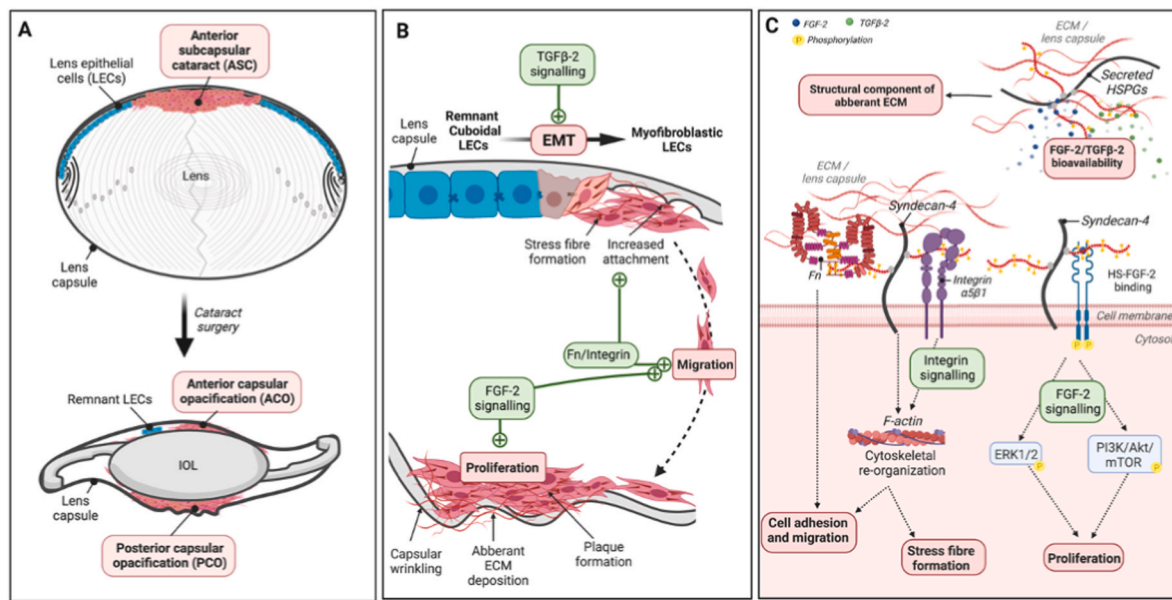


Fig. 9. Schematic diagram of the aetiology and pathogenesis of fibrotic cataracts, and the role of HSPGs in the regulation of key cellular events. A) Fibrotic cataracts may form at the anterior or posterior pole of the lens, resulting in a plaque and opacification of the lens, such as in primary anterior subcapsular cataract (ASC), or secondary cataracts, such as anterior capsular opacification (ACO) or posterior capsular opacification (PCO). These secondary cataracts arise from LECs left behind during cataract surgery that undergo a wound healing response, causing them to transdifferentiate into myofibroblasts, where they form a plaque. B) The pathogenesis of PCO involves three main steps: i) LECs undergo an epithelial-to-mesenchymal transition (EMT) under the influence of TGF β -2 signalling to become motile myofibroblastic cells. These cells undergo cytoskeletal reorganisation (e.g. stress fibre formation) and secrete abundant aberrant extracellular matrix containing fibronectin (Fn), as they adhere to the lens capsule. ii) The myofibroblastic LECs migrate to the posterior capsule driven by FGF-2 and integrin signalling. iii) The myofibroblastic LECs undergo FGF-2 driven proliferation to form a plaque, adhering to and modulating the posterior lens capsule. C) Heparan sulfate proteoglycans (HSPGs) can contribute to the fibrotic events underpinning ASC/ACO/PCO pathogenesis in several ways: i) Secreted HSPGs form an integral part of the new secreted matrix, where they can sequester TGF β -2 and FGF-2, controlling their bioavailability to the myofibroblastic cells; ii) through interactions with matrix proteins (e.g. α 5 β 1-integrin/fibronectin, Fn) HSPGs (e.g. syndecan-4) can regulate cell adhesion and migration, and stress-fibre formation; iii) HSPGs (e.g. syndecan-4) are essential co-receptors for FGF-2. FGF receptor (FGFR)-FGF-2-HS signalling complexing and activation results in ERK1/2 and PI3K/Akt/mTOR signalling activation required for FGF-2 driven proliferation of myofibroblastic LECs.

proliferate to form a subcapsular plaque, associated with posterior capsular wrinkling and a secondary opacification, compromising the functionality of the intraocular lens (Fig. 9A and B) (Neumayer et al., 2017). These cells deposit a fibronectin matrix substrate that promotes their migration, and has also been shown to promote lens epithelial cell migration, both *in vivo* and *in vitro* (VanSlyke et al., 2018). The TGF β -mediated wound healing response is regulated by growth factors (e.g. FGF-2) and ECM components (i.e. α 5 β 1-integrin/fibronectin) (reviewed by Wormstone, 2002 and Liu and Kao, 2015). HSPGs have been reported to directly bind to and regulate the distribution and signalling activity of both FGF-2 and TGF β in fibrosis (Chen et al., 2004; Qin et al., 2017), and have also been shown to be involved in the activity of key regulators of myofibroblast migration, including fibronectin (Kusano et al., 2000; Stepp et al., 2010), integrins (Couchman and Woods, 1999; Filla et al., 2006; Morgan et al., 2007; Qin et al., 2017), and focal adhesion kinase (Wilcox-Adelman et al., 2002; Shiha et al., 2020) (Fig. 9C). HS/HSPGs have been shown to bind to TGF β -2 (Lyon et al., 1997; Rider and Mulloy, 2017) and are important for TGF β -2 signalling in some systems. For instance, disruption of HS synthesis (*Ext1* knockdown) in mouse neural crest cells resulted in disrupted TGF β -2 signalling (Iwao et al., 2009). HS-GAG also binds to protein antagonists of TGF β -2, such as gremlin, that has recently been reported to drive the development of PCO by promoting LEC proliferation and activation of TGF β -2/Smad-signalling pathways (Ma et al., 2019). The direct effects of specifically disrupting HS-GAG associated with TGF β -2 signalling in the lens has yet to be investigated, and the subject of future studies.

Limited studies to date have thoroughly investigated the roles of specific HSPGs in lens pathology; however, a recent body of work has extensively characterised the role of Syndecan-4 in the pathogenesis of fibrotic cataract and demonstrated its potential as a therapeutic target

for fibrotic lens pathologies such as fibrotic forms of cataract (i.e. ACO/PCO) (Qin et al., 2017). In this landmark study, syndecan-4 was reported to regulate FGF-2 and α 5 β 1-integrin signalling, with both FGF-driven proliferation and integrin-mediated adhesion and migration of LECs involved in the development of ACO/PCO requiring HSPG co-receptor activity. Moreover, mice lacking syndecan-4 have significantly reduced occurrence of lens opacification in an injury-induced model of fibrotic wound healing (Qin et al., 2017). RNA interference knockdown of syndecan-4 in cultured human LECs also blocked FGF-induced proliferation and associated ERK1/2 and PI3K/Akt/mTOR signalling pathway activation (Qin et al., 2017), with the same study reporting increased syndecan-4 expression in the LECs of age-related cataract patients (Qin et al., 2017). Taken together, this data highlights HSPGs, namely, syndecan-4 as a key regulator of the pathogenesis of fibrotic cataract. The findings of this study align with earlier work that identified increased HSPGs localised to the basal lamina of lens epithelial cells in PCO plaques (Ishibashi et al., 1995). Thus, Syndecan-4 and possibly also other unidentified HSPGs may be ideal therapeutic targets for the prevention and treatment of fibrotic forms of cataract.

6. Conclusions

Advances in the field of glycobiology have revealed a critical role for HSPGs in the activation and regulation of growth factors, matrix proteins and signalling molecules. This places HSPGs at the forefront of master regulators of homeostatic processes including cell proliferation, differentiation, and survival. The broad expression of HSPGs throughout the different ocular cells and tissues regulate many key signalling molecules to promote ocular development and homeostasis. The crystalline lens is a relatively simple and well characterised tissue that presents an ideal model to study the unique roles of individual HSPGs. By further

investigating the functional properties of these different sulfated proteins, we hope to better understand how key lens HSPGs regulate different growth factor-mediated cellular processes.

It is important to note that functional glycobiology is a rapidly expanding but still newly developing area of research (Yates et al., 2019). As such, relative to other systems, limited research has been directed toward the study of HSPGs and their regulatory networks in relation to lens. For example, in this review we have not managed to delve into other areas of interest in lens, including the relevance of HS-GAG/HSPG-protein interactions in regulating lens water content and transport (Comper and Williams, 1987), responses to oxidative stress (Fuchs and Schiller, 2014; Reynolds-Peterson et al., 2020), interactions between HS and lens intrinsic metal ions (Remko et al., 2013; Farrell et al., 2018), and some key lens signalling networks (e.g. regulation of protein phosphatases) by lens HSPGs (Aricescu et al., 2002; Umeda et al., 2004; Katagiri et al., 2018). What we have done though, for the first time in any one specific tissue, is showcase the detailed spatial expression profile of all known HSPG core proteins in the ocular lens. These findings are supported by many studies in animal models showing that the loss or overexpression of specific HSPG core proteins results in unique phenotypic outcomes for the lens, as well as in other ocular tissues. Much of this may be brought on by the loss or dysregulation of their HS chain structure and function leading to ocular pathogenesis. Indeed, similar to HSPG core proteins, HS sulfation enzymes are expressed in a cell- and tissue-specific manner and the disruption of different sulfation patterns impacts growth factors, perturbing cellular function. The fine structure of HS-GAGs, characterized by their sulfation pattern is an important determinant of the specificity of HS-protein interactions. Although HSPG sulfation clearly plays a critical role in the lens, the specific HS chain sulfation patterns required for HSPG interactions with ocular growth factors remain unknown. Evaluation of interactions between lens growth factors and structurally defined HS oligosaccharides, and the phenotypic analyses of animal models with lens-specific knockdown of different HS-sulfotransferases would be valuable to further elucidate these relationships. Determining how HS fine structure regulates HSPG interaction with the different lens regulatory molecules, will expand our understanding of the contribution of different sulfation patterns to ligand binding kinetics and receptor dimerization, as well as the regulation of lens epithelial cell proliferation, fibre differentiation and EMT, along with their respective downstream signalling pathways. In many eye tissues, including the lens, significant changes in HSPG expression and function can either promote repair or drive pathogenesis. Often, this comes down to changes and regulatory activity of only a handful of specific HSPGs that are unique to different pathological processes. Given the importance of RTK-signalling in development and disease, and the known regulation of these RTKs by HSPGs, it is surprising that a comprehensive developmental evaluation of tissues lacking different HSPG components is lacking for any one tissue. Further characterisation of lenses of HSPG-mutant animals, and the generation of lens-specific knockdown mutants, together with a comprehensive evaluation of how the established HSPG profile of the lens changes throughout lens development, or in disease (e.g. cataract) would be a valuable step towards better understanding the specific HSPG functional modulation pathways in the lens. Once identified, these key HSPGs may serve as valuable biomarkers and targets for the development of novel therapeutics, as has been done for other ocular and systemic diseases, including cancer.

Author contribution

Taylor F. L. Wishart: Conceptualization, Data curation; Formal analysis; Project administration Validation; Visualisation; Writing-original draft; Writing-review & editing. Frank J. Lovicu: Funding acquisition, Supervision, Resources, Project administration, Writing-review & editing.

Declaration of competing interest

The authors have declared that no conflict of interest exists and have no financial disclosures.

Data availability

No data was used for the research described in the article.

References

- Afratis, N.A., Nikitovic, D., Mulhaupt, H.A.B., Theocharis, A.D., Couchman, J.R., Karamanos, N.K., 2017. Syndecans – key regulators of cell signaling and biological functions. *FEBS J.* 284, 27–41.
- Aikio, M., Hurskainen, M., Brideau, G., Hägg, P., Sormunen, R., Heljasvaara, R., Gould, D.B., Pihlajaniemi, T., 2013. Collagen XVIII short isoform is critical for retinal vascularization, and overexpression of the Tsp-1 domain affects eye growth and cataract formation. *Investig. Ophthalmol. Vis. Sci.* 54, 7450–7462.
- Akiyama, T., Kamimura, K., Firkus, C., Takeo, S., Shimmi, O., Nakato, H., 2008. Dally regulates Dpp morphogen gradient formation by stabilizing Dpp on the cell surface. *Dev. Biol.* 313, 408–419.
- Akutsu, N., Yamamoto, H., Sasaki, S., Taniguchi, H., Arimura, Y., Imai, K., Shinomura, Y., 2010. Association of glypican-3 expression with growth signaling molecules in hepatocellular carcinoma. *World J. Gastroenterol.* 16, 3521–3528.
- Amalric, F., Bouche, G., Bonnet, H., Brethenou, P., Roman, A.M., Truchet, L., Quarto, N., 1994. Fibroblast growth factor-2 (FGF-2) in the nucleus: translocation process and targets. *Biochem. Pharmacol.* 47, 111–115.
- Amor, D.J., Stephenson, S.E.M., Mustapha, M., Mensah, M.A., Ockeloen, C.W., Lee, W.S., Tankard, R.M., Phelan, D.G., Shinawi, M., de Brouwer, A.P.M., Pfundt, R., Dowling, C., Toler, T.L., Sutton, V.R., Agolini, E., Rinelli, M., Capolino, R., Martinelli, D., Zampino, G., Dumić, M., Reardon, W., Shaw-Smith, C., Leventer, R.J., Delatycki, M.B., Kleefstra, T., Mundlos, S., Mortier, G., Bahlo, M., Allen, N.J., Lockhart, P.J., 2019. Pathogenic variants in GPC4 cause Keipert syndrome. *Am. J. Hum. Genet.* 104, 914–924.
- Arai, T., Parker, A., Busby Jr., W., Clemmons, D.R., 1994. Heparin, heparan sulfate, and dermatan sulfate regulate formation of the insulin-like growth factor-I and insulin-like growth factor-binding protein complexes. *J. Biol. Chem.* 269, 20388–20393.
- Aricescu, A.R., McKinnell, I.W., Halfter, W., Stoker, A.W., 2002. Heparan sulfate proteoglycans are ligands for receptor protein tyrosine phosphatase α . *Mol. Cell Biol.* 22, 1881–1892.
- Arikawa-Hirasawa, E., Watanabe, H., Takami, H., Hassell, J.R., Yamada, Y., 1999. Perlecan is essential for cartilage and cephalic development. *Nat. Genet.* 23, 354–358.
- Ashikari-Hada, S., Habuchi, H., Kariya, Y., Kimata, K., 2005. Heparin regulates vascular endothelial growth factor165-dependent mitogenic activity, tube formation, and its receptor phosphorylation of human endothelial cells. Comparison of the effects of heparin and modified heparins. *J. Biol. Chem.* 280, 31508–31515.
- Asundi, V.K., Carey, D.J., 1997. Phosphorylation of recombinant N-syndecan (syndecan 3) core protein. *Biochem. Biophys. Res. Commun.* 240, 502–506.
- Audette, D.S., Anand, D., So, T., Rubenstein, T.B., Lachke, S.A., Lovicu, F.J., Duncan, M. K., 2016. Prox1 and fibroblast growth factor receptors form a novel regulatory loop controlling lens fiber differentiation and gene expression. *Development* 143, 318–328.
- Awasthi, N., 2009. Posterior capsular opacification. *Arch. Ophthalmol.* 127, 555.
- Ayers, K.L., Mteirek, R., Cervantes, A., Lavenant-Staccini, L., Théron, P.P., Gallet, A., 2012. Dally and notum regulate the switch between low and high level hedgehog pathway signalling. *Development* 139, 3168–3179.
- Bellin, R.M., Kubicek, J.D., Frigault, M.J., Kamien, A.J., Steward, R.L., Barnes, H.M., DiGiacomo, M.B., Duncan, L.J., Edgerly, C.K., Morse, E.M., Park, C.Y., Fredberg, J.J., Cheng, C.-M., LeDuc, P.R., 2009. Defining the role of syndecan-4 in mechanotransduction using surface-modification approaches. *Proc. Natl. Acad. Sci. U.S.A.* 106, 22102–22107.
- Berk, R.S., Dong, Z., Alousi, S., Kosir, M.A., Wang, Y., Vlodavsky, I., 2004. Murine ocular heparanase expression before and during infection with *Pseudomonas aeruginosa*. *Investig. Ophthalmol. Vis. Sci.* 45, 1182–1187.
- Bernfield, M., 1992. Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans. *Annu. Rev. Cell Dev. Biol.* 8, 365–393.
- Bernfield, M., Hinkes, M.T., Gallo, R.L., 1993. Developmental expression of the syndecans: possible function and regulation. *Development* 119, 205–212.
- Bezakova, G., Lomo, T., 2001. Muscle activity and muscle agrin regulate the organization of cytoskeletal proteins and attached acetylcholine receptor (AChR) aggregates in skeletal muscle fibers. *J. Cell Biol.* 153, 1453–1463.
- Billings, P.C., Pacifici, M., 2015. Interactions of signaling proteins, growth factors and other proteins with heparan sulfate: mechanisms and mysteries. *Connect. Tissue Res.* 56, 272–280.
- Bishop, J.R., Schuksz, M., Esko, J.D., 2007. Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature* 446, 1030–1037.
- Boswell, B.A., Overbeek, P.A., Musil, L.S., 2008. Essential role of BMPs in FGF-induced secondary lens fiber differentiation. *Dev. Biol.* 324, 202–212.
- Brewitt, B., Clark, J.I., 1988. Growth and transparency in the lens, an epithelial tissue, stimulated by pulses of PDGF. *Science* 242, 777–779.

- Brockstedt, U., Dobra, K., Nurminen, M., Hjerpe, A., 2002. Immunoreactivity to cell surface syndecans in cytoplasm and nucleus: tubulin-dependent rearrangements. *Exp. Cell Res.* 274, 235–245.
- Budak, G., Dash, S., Srivastava, R., Lachke, S.A., Janga, S.C., 2018. Express: a database of transcriptome profiles encompassing known and novel transcripts across multiple development stages in eye tissues. *Exp. Eye Res.* 168, 57–68.
- Bullock, S.L., Fletcher, J.M., Beddington, R.S., Wilson, V.A., 1998. Renal agenesis in mice homozygous for a gene trap mutation in the gene encoding heparan sulfate 2-sulfotransferase. *Genes Dev.* 12, 1894–1906.
- Busse, M., Feta, A., Presto, J., Wilén, M., Grønning, M., Kjellén, L., Kusche-Gullberg, M., 2007. Contribution of EXT1, EXT2, and EXT3 to heparan sulfate chain elongation. *J. Biol. Chem.* 282, 32802–32810.
- Call, T.W., Hollyfield, J.G., 1990. Sulfated proteoglycans in Bruch's membrane of the human eye: localization and characterization using cupromeronic blue. *Exp. Eye Res.* 51, 451–462.
- Campos-Xavier, A.B., Martinet, D., Bateman, J., Belluocchio, D., Rowley, L., Tan, T.Y., Baxová, A., Gustavson, K.H., Borochoy, Z.U., Innes, A.M., Unger, S., 2009. Mutations in the heparan-sulfate proteoglycan glypican 6 (GPC6) impair endochondral ossification and cause recessive omdysplasia. *Am. J. Hum. Genet.* 84, 760–770.
- Cano-Gauci, D.F., Song, H.H., Yang, H., McKelvie, C., Choo, B., Shi, W., Pullano, R., Piscione, T.D., Grisaru, S., Soon, S., Sedlackova, L., Tanswell, A.K., Mak, T.W., Yeger, H., Lockwood, G.A., Rosenblum, N.D., Filmus, J., 1999. Glypican-3-deficient mice exhibit developmental overgrowth and some of the abnormalities typical of Simpson-Golabi-Behmel syndrome. *J. Cell Biol.* 146, 255–264.
- Capurro, M., Martin, T., Shi, W., Filmus, J., 2014. Glypican-3 binds to frizzled and plays a direct role in the stimulation of canonical Wnt signaling. *J. Cell Sci.* 127, 140871–141575.
- Capurro, M., Izumikawa, T., Suarez, P., Shi, W., Cydzik, M., Kaneiwa, T., Garipey, J., Bonafe, L., Filmus, J., 2017. Glypican-6 promotes the growth of developing long bones by stimulating Hedgehog signaling. *J. Cell Biol.* 216, 2911–2926.
- Carulli, S., Beck, K., Dayan, G., Boulesteix, S., Lortat-Jacob, H., Rousselle, P., 2012. Cell surface proteoglycans syndecan-1 and -4 bind overlapping but distinct sites in laminin $\alpha 3$ LG45 protein domain. *J. Biol. Chem.* 287, 12204–12216.
- Cavalheiro, G.R., Matos-Rodrigues, G.E., Zhao, Y., Gomes, A.L., Anand, D., Predes, D., de Lima, S., Abreu, J.G., Zheng, D., Lachke, S.A., Cvekl, A., Martins, R.A.P., 2017. N-myc regulates growth and fiber cell differentiation in lens development. *Dev. Biol.* 429, 105–117.
- Chai, L., Morris, J.E., 2009. Distribution of heparan sulfate proteoglycans in embryonic chicken neural retina and isolated inner limiting membrane. *Curr. Eye Res.* 13, 669–677.
- Chamberlain, C.G., McAvoy, J.W., 1989. Induction of Lens fibre differentiation by acidic and basic fibroblast growth factor (FGF). *Growth Factors* 1, 125–134.
- Chamberlain, C.G., McAvoy, J.W., Richardson, N.A., 1991. The effects of insulin and basic fibroblast growth factor on fibre differentiation in rat Lens epithelial explants. *Growth Factors* 4, 183–188.
- Chang, T., Mazotta, J., Dumstrei, K., Dumitrescu, A., Hartenstein, V., 2001. Dpp and Hh signaling in the Drosophila embryonic eye field. *Development* 128, 4691–4704.
- Chen, L.D., Hazlett, L.D., 2000. Perlecan in the basement membrane of corneal epithelium serves as a site for P. aeruginosa binding. *Curr. Eye Res.* 20, 260–267.
- Chen, L., Klass, C., Woods, A., 2004. Syndecan-2 regulates transforming growth factor- β signaling. *J. Biol. Chem.* 279, 15715–15718.
- Chen, L., Sanderson, R.D., 2009. Heparanase regulates levels of syndecan-1 in the nucleus. *PLoS One* 4, e4947.
- Cheng, C., Gong, X., 2011. Diverse roles of Eph/ephrin signaling in the mouse lens. *PLoS One* 6, e28147.
- Cheng, C., Ansari, M.M., Cooper, J.A., Gong, X., 2013. EphA2 and Src regulate equatorial cell morphogenesis during lens development. *Development* 140, 4237–4245.
- Cheng, C., Nowak, R.B., Fowler, V.M., 2017. The lens actin filament cytoskeleton: diverse structures for complex functions. *Exp. Eye Res.* 156, 58–71.
- Chiao, E., Fisher, P., Crisponi, L., Deiana, M., Dragatsis, I., Schlessinger, D., Pilia, G., Efstratiadis, A., 2002. Overgrowth of a mouse model of the Simpson-Golabi-Behmel syndrome is independent of IGF signaling. *Dev. Biol.* 243, 185–206.
- Choi, J., Park, S.Y., Joo, C.K., 2004. Hepatocyte growth factor induces proliferation of lens epithelial cells through activation of ERK1/2 and JNK/SAPK. *Investig. Ophthalmol. Vis. Sci.* 45, 2696–2704.
- Chow, R.L., Roux, G.D., Roghani, M., Palmer, M.A., Rifkin, D.B., Moscatelli, D.A., Lang, R.A., 1995. FGF suppresses apoptosis and induces differentiation of fibre cells in the mouse lens. *Development* 121, 4383–4393.
- Christianson, H.C., Svensson, K.J., van Kuppevelt, T.H., Li, J.-P., Belting, M., 2013. Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. *Proc. Natl. Acad. Sci. U.S.A.* 110, 17380–17385.
- Clark, S.J., Keenan, T.D.L., Fielder, H.L., Collinson, L.J., Holley, R.J., Merry, C.L.R., van Kuppevelt, T.H., Day, A.J., Bishop, P.N., 2011. Mapping the differential distribution of glycosaminoglycans in the adult human retina, choroid, and sclera. *Investig. Ophthalmol. Vis. Sci.* 52, 6511–6521.
- Comper, W.D., Williams, R.P., 1987. Hydrodynamics of concentrated proteoglycan solutions. *J. Biol. Chem.* 262, 13464–13471.
- Conrad, H.E., 2001. Inhibition of heparan sulfate synthesis by chlorate. In: *Proteoglycan Protocols*. Humana Press, New Jersey, pp. 325–328.
- Cornelison, D.D.W., Wilcox-Adelman, S.A., Goetinck, P.F., Rauvala, H., Rapraeger, A.C., Olwin, B.B., 2004. Essential and separable roles for Syndecan-3 and Syndecan-4 in skeletal muscle development and regeneration. *Genes Dev.* 18, 2231–2236.
- Corti, F., Finetti, F., Ziche, M., Simons, M., 2013. The syndecan-4/protein kinase C α pathway mediates prostaglandin E2-induced extracellular regulated kinase (ERK) activation in endothelial cells and angiogenesis in vivo. *J. Biol. Chem.* 288, 12712–12721.
- Corti, F., Wang, Y., Rhodes, J.M., Atri, D., Archer-Hartmann, S., Zhang, J., Zhuang, Z.W., Chen, D., Wang, T., Wang, Z., Azadi, P., Simons, M., 2019. N-terminal syndecan-2 domain selectively enhances 6-O heparan sulfate chains sulfation and promotes VEGFA 165 -dependent neovascularization. *Nat. Commun.* 10, 1–14.
- Couchman, J.R., Woods, A., 1999. Syndecan-4 and integrins: combinatorial signaling in cell adhesion. *J. Cell Sci.* 112, 3415–3420.
- Couchman, J.R., Pataki, C.A., 2012. An introduction to proteoglycans and their localization. *J. Histochem. Cytochem.* 60, 885–897.
- Couchman, J.R., Gopal, S., Lim, H.C., Norgaard, S., Mulhaupt, H.A.B., 2015. Fell-Muir Lecture: syndecans: from peripheral coreceptors to mainstream regulators of cell behaviour. *Int. J. Exp. Pathol.* 96, 1–10.
- Daniels, M.P., 2012. The role of agrin in synaptic development, plasticity and signaling in the central nervous system. In: *Presented at the Neurochemistry International*, pp. 848–853.
- Danysh, B., Patel, T.P., Edwards, D.A., Czymmek, K.J., Duncan, M.K., 2008. Sulfate and carboxyl groups within the anterior lens capsule regulates diffusion and interactions of charged proteins. *Investig. Ophthalmol. Vis. Sci.* 49, 1524–1524.
- Danysh, B.P., Duncan, M.K., 2009. The lens capsule. *Exp. Eye Res.* 88, 151–164.
- Das, S., Majid, M., Baker, A.B., 2016. Syndecan-4 enhances PDGF-BB activity in diabetic wound healing. *Acta Biomater.* 42, 56–65.
- David, G., Bai, X.M., Van der Schueren, B., Cassiman, J.-J., van den Berghe, H., 1992. Developmental changes in heparan sulfate expression: in situ detection with mAbs. *J. Cell Biol.* 119, 961–975.
- Davies, Y., Lewis, D., Fullwood, N.J., Nieduszynski, I.A., Marcyniuk, B., Albon, J., Tullo, A., 1999. Proteoglycans on normal and migrating human corneal endothelium. *Exp. Eye Res.* 68, 303–311.
- Dawes, L.J., Sleeman, M.A., Anderson, L.K., Reddan, J.R., Wormstone, I.M., 2009. TGF β /Smad4-Dependent and -independent regulation of human lens epithelial cells. *Investig. Ophthalmol. Vis. Sci.* 50, 5318–5327.
- Dawes, L.J., Sugiyama, Y., Tanedo, A.S., Lovicu, F.J., McAvoy, J.W., 2013. Wnt-frizzled signaling is part of an FGF-induced cascade that promotes lens fiber differentiation. *Investig. Ophthalmol. Vis. Sci.* 54, 1582–1590.
- De Agostini, A.L., Lau, H.K., Leone, C., Youssoufian, H., Rosenberg, R.D., 1990. Cell mutants defective in synthesizing a heparan sulfate proteoglycan with regions of defined monosaccharide sequence. *Proc. Natl. Acad. Sci. U.S.A.* 87, 9784–9788.
- De Cat, B., 2001. Developmental roles of the glypicans. *Semin. Cell Dev. Biol.* 12, 117–125.
- Decarlo, A.A., Belousova, M., Ellis, A.L., Petersen, D., Grenett, H., Hardigan, P., O'Grady, R., Lord, M., Whitelock, J.M., 2012. Perlecan domain 1 recombinant proteoglycan augments BMP-2 activity and osteogenesis. *BMC Biotechnol.* 12, 60–16.
- DeDreu, J., Walker, J.L., Menko, A.S., 2021. Dynamics of the lens basement membrane capsule and its interaction with connective tissue-like extracapsular matrix proteins. *Matrix Biol.* 96, 18–46.
- de Jongh, R.U., Lovicu, F.J., Hanneken, A., Baird, A., McAvoy, J.W., 1996. FGF receptor-1 (flg) expression is correlated with fibre differentiation during rat lens morphogenesis and growth. *Dev. Dynam.* 206, 412–426.
- de Jongh, R.U., Gordon-Thomson, C., Chamberlain, C.G., Hales, A.M., McAvoy, J.W., 2001. TGF beta receptor expression in lens: implications for differentiation and cataractogenesis. *Exp. Eye Res.* 72, 649–659.
- de Jongh, R., Wederell, E., Lovicu, F., McAvoy, J., 2005. Transforming growth factor- β -induced epithelial-mesenchymal transition in the lens: a model for cataract formation. *Cells Tissues Organs* 179, 43–55.
- De Rossi, G., Vähätupa, M., Cristante, E., Arokiasamy, S., Liyanage, S.E., May, U., Pellinen, L., Uusitalo-Järvinen, H., Bainbridge, J.W., Järvinen, T.A., Whiteford, J.R., 2021. Pathological angiogenesis requires syndecan-4 for efficient VEGFA-induced VE-cadherin internalization. *Arterioscler. Thromb. Vasc. Biol.* 41, 1374–1389.
- Dickinson, M.E., Flenniken, A.M., Ji, X., Teboul, L., Wong, M.D., White, J.K., Meehan, T.F., Weninger, W.J., Westerberg, H., Adissu, H., Baker, C.N., Bower, L., Brown, J.M., Caddle, L.B., Chiani, F., Clary, D., Cleak, J., Daly, M.J., Denegre, J.M., Doe, B., Dolan, M.E., Edie, S.M., Fuchs, H., Gailus-Durner, V., Galli, A., Gambadoro, A., Gallegos, J., Guo, S., Horner, N.R., Hsu, C.-W., Johnson, S.J., Kalaga, S., Keith, L.C., Lanoue, L., Lawson, T.N., Lek, M., Mark, M., Marschall, S., Mason, J., McElwee, M.L., Newbigging, S., Nutter, L.M.J., Peterson, K.A., Ramirez-Solis, R., Rowland, D.J., Ryder, E., Samocha, K.E., Seavitt, J.R., Selloum, M., Szoke-Kovacs, Z., Tamura, M., Trainor, A.G., Tudose, I., Wakana, S., Warren, J., Wendling, O., West, D.B., Wong, L., Yoshiki, A., Wurst, W., MacArthur, D.G., Tocchini-Valentini, G.P., Gao, X., Flicek, P., Bradley, A., Skarnes, W.C., Justice, M.J., Parkinson, H.E., Moore, M., Wells, S., Braun, R.E., Svenson, K.L., de Angelis, M.H., Herault, Y., Mohun, T., Mallon, A.-M., Henkelman, R.M., Brown, S.D.M., Adams, D.J., Lloyd, K.C.K., McKelvie, C., Beaudet, A.L., Bučan, M., Murray, S.A., 2016. High-throughput discovery of novel developmental phenotypes. *Nature* 537, 508–514.
- Dobra, K., Nurminen, M., Hjerpe, A., 2003. Growth factors regulate the expression profile of their syndecan co-receptors and the differentiation of mesothelioma cells. *Anticancer Res.* 23, 2435–2444.
- Dong, S., Cole, G.J., Halfter, W., 2003. Expression of collagen XVIII and localization of its glycosaminoglycan attachment sites. *J. Biol. Chem.* 278, 1700–1707.
- Dowling, C., Allen, N.J., 2018. Mice lacking glypican 4 display juvenile hyperactivity and adult social interaction deficits. *Brain Plast.* 4, 197–209.
- Dreyfuss, J.L., Regatieri, C.V., Jarrouge, T.R., Cavalheiro, R.P., Sampaio, L.O., Nader, H. B., 2009. Heparan sulfate proteoglycans: structure, protein interactions and cell signaling. *An. Acad. Bras. Cienc.* 81, 409–429.
- Dyksterhuis, L.D., White, J.F., Hickey, M., Kirby, N., Mudie, S., Hawley, A., Vashi, A., Nigro, J., Werkmeister, J.A., Ramshaw, J.A.M., 2011. Impact of heparan sulfate

- chains and sulfur-mediated bonds on the mechanical properties of bovine lens capsule. *Biophys. J.* 100, 2077–2083.
- Echtermeyer, F., Baci, P.C., Saoncella, S., Ge, Y., Goetinck, P.F., 1999. Syndecan-4 core protein is sufficient for the assembly of focal adhesions and actin stress fibers. *J. Cell Sci.* 112, 3433–3441.
- Echtermeyer, F., Streit, M., Wilcox-Adelman, S., Saoncella, S., Denhez, F., Detmar, M., Goetinck, P.F., 2001. Delayed wound repair and impaired angiogenesis in mice lacking syndecan-4. *J. Clin. Invest.* 107, R9–R14.
- Elamaa, H., Sormunen, R., Rehn, M., Soininen, R., Pihlajaniemi, T., 2005. Endostatin overexpression specifically in the lens and skin leads to cataract and ultrastructural alterations in basement membranes. *Am. J. Pathol.* 166, 221–229.
- Elenius, K., Salmivirta, M., Inki, P., Mali, M., Jalkanen, M., 1990. Binding of human syndecan to extracellular matrix proteins. *J. Biol. Chem.* 265, 17837–17843.
- Escobar Galvis, M.L., Jia, J., Zhang, X., Jastrebova, N., Spillmann, D., Gottfridsson, E., van Kuppevelt, T.H., Zcharia, E., Vlodavsky, I., Lindahl, U., Li, J.-P., 2007. Transgenic or tumor-induced expression of heparanase upregulates sulfation of heparan sulfate. *Nat. Chem. Biol.* 3, 773–778.
- Esko, J.D., Selleck, S.B., 2002. Order out of chaos: assembly of ligand binding sites in heparan sulfate. *Annu. Rev. Biochem.* 71, 435–471.
- Esko, J.D., Prestegard, J.H., Linhardt, R.J., 2017. Proteins that bind sulfated glycosaminoglycans. In: Varki, A., Cummings, R.D., Esko, J.D., Stanley, P., Hart, G. W., Aebi, M., Darvill, A.G., Kinoshita, T., Packer, N.H., Prestegard, J.H., Schnaar, R. L., Seeberger, P.H. (Eds.), *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 493–503.
- Ethell, I.M., Irie, F., Kalo, M.S., Couchman, J.R., Pasquale, E.B., Yamaguchi, Y., 2001. EphB/Syndecan-2 signaling in dendritic spine morphogenesis. *Neuron* 31, 1001–1013.
- Faber, S.C., Robinson, M.L., Makarenkova, H.P., Lang, R.A., 2002. Bmp signaling is required for development of primary lens fiber cells. *Development* 129, 3727–3737.
- Fan, G., Xiao, L., Cheng, L., Wang, X., Sun, B., Hu, G., 2000. Targeted disruption of NDST-1 gene leads to pulmonary hypoplasia and neonatal respiratory distress in mice. *FEBS Lett.* 467, 7–11.
- Fannon, M., Nugent, M.A., 1996. Basic fibroblast growth factor binds its receptors, is internalized, and stimulates DNA synthesis in Balb/c3T3 cells in the absence of heparan sulfate. *J. Biol. Chem.* 271, 17949–17956.
- Farrell, N.P., Gorle, A.K., Peterson, E.J., Berners-Price, S.J., 2018. Metalloglycomics. *Metal Ions in Life Sci.* vol. 18.
- Fedarko, N.S., Conrad, H.E., 1986. A unique heparan sulfate in the nuclei of hepatocytes: structural changes with the growth state of the cells. *J. Cell Biol.* 102, 587–599.
- Fedarko, N.S., Ishihara, M., Conrad, H.E., 1989. Control of cell division in hepatoma cells by exogenous heparan sulfate proteoglycan. *J. Cell. Physiol.* 139, 287–294.
- Filla, M.S., David, G., Weinreb, R.N., Kaufman, P.L., Peters, D.M., 2004. Distribution of syndecans 1–4 within the anterior segment of the human eye: expression of a variant syndecan-3 and matrix-associated syndecan-2. *Exp. Eye Res.* 79, 61–74.
- Filla, M.S., Woods, A., Kaufman, P.L., Peters, D.M., 2006. $\beta 1$ and $\beta 3$ integrins cooperate to induce syndecan-4-containing cross-linked actin networks in human trabecular meshwork cells. *Investig. Ophthalmol. Vis. Sci.* 47, 1956–1967.
- Filmus, J., Capurro, M., 2008. The role of glypican-3 in the regulation of body size and cancer. *Cell Cycle* 7, 2787–2790.
- Fisher, M.C., Li, Y., Seghatoleslami, M.R., Dealy, C.N., Kosher, R.A., 2006. Heparan sulfate proteoglycans including syndecan-3 modulate BMP activity during limb cartilage differentiation. *Matrix Biol.* 25, 27–39.
- Flügel-Koch, C., Ohlmann, A., Piatigorsky, J., Tamm, E.R., 2002. Disruption of anterior segment development by TGF- $\beta 1$ overexpression in the eyes of transgenic mice. *Dev. Dynam.* 225, 111–125.
- Ford-Perriss, M., Turner, C., Guimond, S., Apedaile, A., Haubeck, H.D., Turnbull, J., Murphy, M., 2003. Localisation of specific heparan sulfate proteoglycans during the proliferative phase of brain development. *Dev. Dynam.* 227, 170–184.
- Forsberg, E., Kjellen, L., 2001. Heparan sulfate: lessons from knockout mice. *J. Clin. Invest.* 108, 175–180.
- Forsten-Williams, K., Chu, C.L., Fannon, M., Buczek-Thomas, J.A., Nugent, M.A., 2008. Control of growth factor networks by heparan sulfate proteoglycans. *Ann. Biomed. Eng.* 36, 2134–2148.
- Foster, A., Resnikoff, S., 2005. The impact of Vision 2020 on global blindness. *Eye* 19, 1133–1135.
- Fuchs, B., Schiller, J., 2014. Glycosaminoglycan degradation by selected reactive oxygen species. *Antioxidants Redox Signal.* 21, 1044–1062.
- Fuerst, P.G., Rauch, S.M., Burgess, R.W., 2007. Defects in eye development in transgenic mice overexpressing the heparan sulfate proteoglycan agrin. *Dev. Biol.* 303, 165–180.
- Fujimura, N., 2016. WNT/ β -Catenin signaling in vertebrate eye development. *Front. Cell Dev. Biol.* 4, 138.
- Fukai, N., Eklund, L., Marneros, A.G., Oh, S.P., Keene, D.R., Tamarkin, L., Niemelä, M., Ilves, M., Li, E., Pihlajaniemi, T., Olsen, B.R., 2002. Lack of collagen XVIII/endostatin results in eye abnormalities. *EMBO J.* 21, 1535–1544.
- Furuta, Y., Hogan, B.L., 1998. BMP4 is essential for lens induction in the mouse embryo. *Genes Dev.* 12, 3764–3775.
- García, C.M., Yu, K., Zhao, H., Ashery-Padan, R., Ornitz, D.M., Robinson, M.L., Beebe, D. C., 2005. Signaling through FGF receptor-2 is required for lens cell survival and for withdrawal from the cell cycle during lens fiber cell differentiation. *Dev. Dynam.* 233, 516–527.
- García, B., García-Suárez, O., Merayo-Llves, J., Alcalde, I., Alfonso, J.F., Fernández-Vega Cueto, L., Meana, Á., Vázquez, F., Quirós, L.M., 2016. Differential expression of proteoglycans by corneal stromal cells in Keratoconus. *Investig. Ophthalmol. Vis. Sci.* 57, 2618–2628.
- Gautam, M., Noakes, P.G., Moscoso, L., Rupp, F., Scheller, R.H., Merlie, J.P., Sanes, J.R., 1996. Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* 85, 525–535.
- Golestaneh, N., Fan, J., Zelenka, P., Chepelinsky, A.B., 2008. PKC putative phosphorylation site Ser235 is required for MIP/AQP0 translocation to the plasma membrane. *Mol. Vis.* 14, 1006–1014.
- Gomes, B.F., Crema, A.S., Santhiago, M.R., Costa Filho, A.A., Moraes Jr., H.V., Paiva, D. S., Miguel, N.C., 2021. Morphologic and molecular study on the lens anterior capsule in systemic sclerosis. *Arq. Bras. Oftalmol.* 84, 271–274.
- Gospodarowicz, D., Greenburg, G., Bialecki, H., Zetter, B.R., 1978. Factors involved in the modulation of cell proliferation in vivo and in vitro: the role of fibroblast and epidermal growth factors in the proliferative response of mammalian cells. *In Vitro* 14, 85–118.
- Götte, M., Jousen, A.M., Klein, C., Andre, P., Wagner, D.D., Hinkes, M.T., Kirchhof, B., Adamis, A.P., Bernfield, M., 2002. Role of syndecan-1 in leukocyte-endothelial interactions in the ocular vasculature. *Investig. Ophthalmol. Vis. Sci.* 43, 1135–1141.
- Gougoula, C., Bielfeld, A.P., Pour, S.J., Krüssel, J.-S., Götte, M., Benten, W.P.M., Baston-Büst, D.M., 2019. Physiological and anatomical aspects of the reproduction of mice with reduced Syndecan-1 expression. *Reprod. Biol. Endocrinol.* 17, 28–12.
- Gould, S.E., Upholt, W.B., Kosher, R.A., 1995. Characterization of chicken syndecan-3 as a heparan sulfate proteoglycan and its expression during embryogenesis. *Dev. Biol.* 168, 438–451.
- Granés, F., Ureña, J.M., Rocamora, N., Vilaró, S., 2000. Ezrin links syndecan-2 to the cytoskeleton. *J. Cell Sci.* 113, 1267–1276.
- Gubbiotti, M.A., Neill, T., Iozzo, R.V., 2017. A current view of perlecan in physiology and pathology: a mosaic of functions. *Matrix Biol.* 57 (58), 285–298.
- HajMohammadi, S., Enjyoji, K., Princivalle, M., Christ, P., Lech, M., Beeler, D., Rayburn, H., Schwartz, J.J., Barzegar, S., de Agostini, A.I., Post, M.J., 2003. Normal levels of anticoagulant heparan sulfate are not essential for normal hemostasis. *J. Clin. Invest.* 111, 989–999.
- Handler, M., Yurchenco, P.D., Iozzo, R.V., 1997. Developmental expression of perlecan during murine embryogenesis. *Dev. Dynam.* 210, 130–145.
- Harvey, S.J., Jarad, G., Cunningham, J., Rops, A.L., Van Der Vlag, J., Berden, J.H., Moeller, M.J., Holzman, L.B., Burgess, R.W., Miner, J.H., 2007. Disruption of glomerular basement membrane charge through podocyte-specific mutation of agrin does not alter glomerular permselectivity. *Am. J. Pathol.* 171, 139–152.
- Hayashida, A., Amano, S., Park, P.W., 2011. Syndecan-1 promotes Staphylococcus aureus corneal infection by counteracting neutrophil-mediated host defense. *J. Biol. Chem.* 286, 3288–3297.
- Hayashida, A., Amano, S., Gallo, R.L., Linhardt, R.J., Liu, J., Park, P.W., 2015. 2-O-Sulfated domains in syndecan-1 heparan sulfate inhibit neutrophil cathelicidin and promote Staphylococcus aureus corneal infection. *J. Biol. Chem.* 290, 16157–16167.
- Heathcote, J.G., Orkin, R.W., 1984. Biosynthesis of sulphated macromolecules by rabbit lens epithelium. I. Identification of the major macromolecules synthesized by lens epithelial cells in vitro. *J. Cell Biol.* 99, 852–860.
- Heimann, H., Coupland, S.E., Gochman, R., Hellmich, M., Foerster, M.H., 2001. Alterations in expression of mucin, tenascin-c and syndecan-1 in the conjunctiva following retinal surgery and plaque radiotherapy. *Graefes Arch. Clin. Exp. Ophthalmol.* 239, 488–495.
- Heljasvaara, R., Aikio, M., Ruotsalainen, H., Pihlajaniemi, T., 2017. Collagen XVIII in tissue homeostasis and dysregulation - lessons learned from model organisms and human patients. *Matrix Biol.* 57–58, 55–75.
- Hewitt, A.T., Nakazawa, K., Newsome, D.A., 1989. Analysis of newly synthesized Bruch's membrane proteoglycans. *Investig. Ophthalmol. Vis. Sci.* 30, 478–486.
- Hienola, A., Tumova, S., Kulleski, E., Rauvala, H., 2006. N-syndecan deficiency impairs neural migration in brain. *J. Cell Biol.* 174, 569–580.
- Hoang, T.V., Kumar, P.K.R., Sutharzan, S., Tsonis, P.A., Liang, C., Robinson, M.L., 2014. Comparative transcriptome analysis of epithelial and fiber cells in newborn mouse lenses with RNA sequencing. *Mol. Vis.* 20, 1491–1517.
- Horowitz, A., Simons, M., 1998. Phosphorylation of the cytoplasmic tail of syndecan-4 regulates activation of protein kinase C α . *J. Biol. Chem.* 273, 25548–25551.
- Hsia, E., Richardson, T.P., Nugent, M.A., 2003. Nuclear localization of basic fibroblast growth factor is mediated by heparan sulfate proteoglycans through protein kinase C signaling. *J. Cell. Biochem.* 88, 1214–1225.
- Humphries, D.E., Silbert, J.E., 1988. Chlorate: a reversible inhibitor of proteoglycan sulfation. *Biochem. Biophys. Res. Commun.* 154, 365–371.
- Ibrahim, S.A., Gadalla, R., El-Ghonaimy, E.A., Samir, O., Mohamed, H.T., Hassan, H., Greve, B., El-Shinawi, M., Mohamed, M.M., Götte, M., 2017. Syndecan-1 is a novel molecular marker for triple negative inflammatory breast cancer and modulates the cancer stem cell phenotype via the IL-6/STAT3, Notch and EGFR signaling pathways. *Mol. Cancer* 16, 57.
- Inatani, M., Tanihara, H., 2002. Proteoglycans in retina. *Prog. Retin. Eye Res.* 21, 429–447.
- Inatani, M., Honjo, M., Oohira, A., Kido, N., Otori, Y., Tano, Y., Honda, Y., Tanihara, H., 2002. Spatiotemporal expression patterns of N-syndecan, a transmembrane heparan sulfate proteoglycan, in developing retina. *Investig. Ophthalmol. Vis. Sci.* 43, 1616–1621.
- Inomata, T., Ebihara, N., Funaki, T., Matsuda, A., Watanabe, Y., Ning, L., Xu, Z., Murakami, A., Arikawa Hirasawa, E., 2012. Perlecan-deficient mutation impairs corneal epithelial structure. *Investig. Ophthalmol. Vis. Sci.* 53, 1277–1284.
- Iozzo, R.V., 2005. Basement membrane proteoglycans: from cellar to ceiling. *Nat. Rev. Mol. Cell Biol.* 6, 646–656.
- Ishibashi, T., Araki, H., Sugai, S., Tawara, A., Inomata, H., 1995. Detection of proteoglycans in human posterior capsule opacification. *Ophthalmic Res.* 27, 208–213.

- Iwao, K., Inatani, M., Tanihara, H., 2006. The deficiency of Heparan Sulfate in neural crest cells is associated with the anomalies in the anterior segment of the eye. *Invest. Ophthalmol. Vis. Sci.* 47, 5627–5627.
- Iwao, K., Inatani, M., Matsumoto, Y., Ogata-Iwao, M., Takihara, Y., Irie, F., Yamaguchi, Y., Okinami, S., Tanihara, H., 2009. Heparan sulfate deficiency leads to Peters anomaly in mice by disturbing neural crest TGF- β 2 signaling. *J. Clin. Invest.* 119, 1997–2008.
- Iwao, K., Inatani, M., Ogata-Iwao, M., Yamaguchi, Y., Okinami, S., Tanihara, H., 2010. Heparan sulfate deficiency in perocular mesenchyme causes microphthalmia and ciliary body dysgenesis. *Exp. Eye Res.* 90, 81–88.
- Iyengar, L., Patkunanathan, B., Lynch, O.T., McAvoy, J.W., Rasko, J.E.J., Lovicu, F.J., 2006. Aqueous humour- and growth factor-induced lens cell proliferation is dependent on MAPK/ERK1/2 and Akt/PI3-K signalling. *Exp. Eye Res.* 83, 667–678.
- Iyengar, L., Wang, Q., Rasko, J.E.J., McAvoy, J.W., Lovicu, F.J., 2007. Duration of ERK1/2 phosphorylation induced by FGF or ocular media determines lens cell fate. *Differentiation* 75, 662–668.
- Iyengar, L., Patkunanathan, B., McAvoy, J.W., Lovicu, F.J., 2009. Growth factors involved in aqueous humour-induced lens cell proliferation. *Growth Factors* 27, 50–62.
- Iyengar, L., Lovicu, F.J., 2017. Aqueous humour-induced lens epithelial cell proliferation requires FGF-signalling. *Growth Factors* 35, 131–143.
- Izvolosky, K.I., Lu, J., Martin, G., Albrecht, K.H., Cardoso, W.V., 2008. Systemic inactivation of Hs6st1 in mice is associated with late postnatal mortality without major defects in organogenesis. *Genesis* 46, 8–18.
- Jackson, S.M., Nakato, H., Sugiyama, M., Jannuzzi, A., Oakes, R., Kaluza, V., Golden, C., Selleck, S.B., 1997. dally, a Drosophila glypican, controls cellular responses to the TGF- β -related morphogen. *Dpp. Development* 124, 4113–4120.
- Jang, B., Jung, H., Chung, H., Moon, B.-I., Oh, E.-S., 2016. Syndecan-2 enhances E-cadherin shedding and fibroblast-like morphological changes by inducing MMP-7 expression in colon cancer cells. *Biochem. Biophys. Res. Commun.* 477, 47–53.
- Jang, E., Albadawi, H., Watkins, M.T., Edelman, E.R., Baker, A.B., 2012. Syndecan-4 proteoliposomes enhance fibroblast growth factor-2 (FGF-2)-induced proliferation, migration, and neovascularization of ischemic muscle. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1679–1684.
- Jemth, P., Smeds, E., Do, A.T., Habuchi, H., Kimata, K., Lindahl, U., Kusche-Gullberg, M., 2003. Oligosaccharide library-based assessment of heparan sulfate 6-O-sulfotransferase substrate specificity. *J. Biol. Chem.* 278, 24371–24376.
- Jen, Y.H.L., Musacchio, M., Lander, A.D., 2009. Glypican-1 controls brain size through regulation of fibroblast growth factor signaling in early neurogenesis. *Neural Dev.* 4, 33–19.
- Jiang, J., Shiha, M.H., Wang, Y., Duncan, M.K., 2018. Lens epithelial cells initiate an inflammatory response following cataract surgery. *Investig. Ophthalmol. Vis. Sci.* 59, 4986–4997.
- Jinno, A., Hayashida, A., Jenkinson, H.F., Park, P.W., 2020. Syndecan-1 promotes *Streptococcus pneumoniae* corneal infection by facilitating the assembly of adhesive fibronectin fibrils. *mBio* 11, e01907–e01920.
- Joslyn, G., Wolf, F.W., Brush, G., Wu, L., Schuckit, M., White, R.L., 2011. Glypican gene GPC5 participates in the behavioral response to ethanol: evidence from humans, mice, and fruit flies. *G3 (Bethesda)* 1, 627–635.
- Kakrana, A., Yang, A., Anand, D., Djordjevic, D., Ramachandruni, D., Singh, A., Huang, H., Ho, J.W.K., Lachke, S.A., 2018. ISyTE 2.0: a database for expression-based gene discovery in the eye. *Nucleic Acids Res.* 46, D875–D885.
- Kaksonen, M., Pavlov, I., Vöikar, V., Lauri, S.E., Hienola, A., Riekk, R., Lakso, M., Taira, T., Rauvala, H., 2002. Syndecan-3-deficient mice exhibit enhanced LTP and impaired hippocampus-dependent memory. *Mol. Cell. Neurosci.* 21, 158–172.
- Karamanos, N.K., Piperigkou, Z., Theocharis, A.D., Watanabe, H., Franchi, M., Baud, S., Brézillon, S., Götte, M., Passi, A., Vignetti, D., Ricard-Blum, S., Sanderson, R.D., Neill, T., Iozzo, R.V., 2018. Proteoglycan chemical diversity drives multifunctional cell regulation and therapeutics. *Chem. Rev.* 118, 9152–9232.
- Katagiri, Y., Morgan, A.A., Yu, P., Bangayan, N.J., Junka, R., Geller, H.M., 2018. Identification of novel binding sites for heparin in receptor protein-tyrosine phosphatase (RPTP): implications for proteoglycan signaling. *J. Biol. Chem.* 293, 11639–11647.
- Kato, M., Wang, H., Bernfield, M., Gallagher, J.T., Turnbull, J.E., 1994. Cell surface syndecan-1 on distinct cell types differs in fine structure and ligand binding of its heparan sulfate chains. *J. Biol. Chem.* 269, 18881–18890.
- Kerr, C.L., Huang, J., Williams, T., West-Mays, J.A., 2012. Activation of the hedgehog signaling pathway in the developing lens stimulates ectopic FoxE3 expression and disruption in fiber cell differentiation. *Investig. Ophthalmol. Vis. Sci.* 53, 3316–3330.
- Khan, A.O., Aldahmesh, M.A., Mohamed, J.Y., Al-Mesfer, S., Alkuraya, F.S., 2012. The distinct ophthalmic phenotype of Knobloch syndrome in children. *Br. J. Ophthalmol.* 96, 890–895.
- Khan, S.Y., Hackett, S.F., Lee, M.-C.W., Pourmand, N., Talbot, C.C., Riazuddin, S.A., 2015. Transcriptome profiling of developing murine lens through RNA sequencing. *Investig. Ophthalmol. Vis. Sci.* 56, 4919–4926.
- Khare, N., Baumgartner, S., 2000. Dally-like protein, a new Drosophila glypican with expression overlapping with wingless. *Mech. Dev.* 99, 199–202.
- Kim, M.J., Cotman, S.L., Halfter, W., Cole, G.J., 2003. The heparan sulfate proteoglycan agrin modulates neurite outgrowth mediated by FGF-2. *J. Neurobiol.* 55, 261–277.
- Kim, M.J., Liu, L.H., Song, Y., Lee, J.-A., Halfter, W., Balice-Gordon, R.J., Linney, E., Cole, G.J., 2007. Agrin is required for posterior development and motor axon outgrowth and branching in embryonic zebrafish. *Glycobiology* 17, 231–247.
- Kinnunen, T., Kaksonen, M., Saarinen, J., Kalkkinen, N., Peng, H.B., Rauvala, H., 1998. Cortactin-Src kinase signaling pathway is involved in N-syndecan-dependent neurite outgrowth. *J. Biol. Chem.* 273, 10702–10708.
- Klass, C.M., Couchman, J.R., Woods, A., 2000. Control of extracellular matrix assembly by syndecan-2 proteoglycan. *J. Cell Sci.* 113, 493–506.
- Knox, S., Merry, C., Stringer, S., Melrose, J., Whitelock, J., 2002. Not all perlecan are created equal: interactions with fibroblast growth factor (FGF) 2 and FGF receptors. *J. Biol. Chem.* 277, 14657–14665.
- Kok, A., Lovicu, F.J., Chamberlain, C.G., McAvoy, J.W., 2002. Influence of platelet-derived growth factor on lens epithelial cell proliferation and differentiation. *Growth Factors* 20, 27–34.
- Kokenyesi, R., Bernfield, M., 1994. Core protein structure and sequence determine the site and presence of heparan sulfate and chondroitin sulfate on syndecan-1. *J. Biol. Chem.* 269, 12304–12309.
- Kokkinos, M.I., Brown, H.J., de Jongh, R.U., 2007. Focal adhesion kinase (FAK) expression and activation during lens development. *Mol. Vis.* 13, 418–430.
- Kolluri, A., Ho, M., 2019. The role of glypican-3 in regulating Wnt, YAP, and hedgehog in liver cancer. *Front. Oncol.* 9, 708.
- Kolset, S.O., Prydz, K., Pejler, G., 2004. Intracellular proteoglycans. *Biochem. J.* 379, 217–227.
- Koso, H., Iida, A., Tabata, Y., Baba, Y., Satoh, S., Taketo, M.M., Watanabe, S., 2008. CD138/syndecan-1 and SSEA-1 mark distinct populations of developing ciliary epithelium that are regulated differentially by Wnt signal. *Stem Cell.* 26, 3162–3171.
- Kovalsky, I., Hjerpe, A., Dobrá, K., 2014. Nuclear translocation of heparan sulfate proteoglycans and their functional significance. *Biochim. Biophys. Acta* 1840, 2491–2497.
- Koyama, Y., Naruo, H., Yoshitomi, Y., Munesue, S., Kiyono, S., Kusano, Y., Hashimoto, K., Yokoi, T., Nakanishi, H., Shimizu, S., Okayama, M., Oguri, K., 2008. Matrix metalloproteinase-9 associated with heparan sulphate chains of GPI-anchored cell surface proteoglycans mediates motility of murine colon adenocarcinoma cells. *J. Biochem.* 143, 581–592.
- Kröger, S., Pfister, H., 2009. Agrin in the nervous system: synaptogenesis and beyond. *Future Neurol.* 4, 67–86.
- Kuriyama, S., Mayor, R., 2009. A role for Syndecan-4 in neural induction involving ERK- and PKC-dependent pathways. *Development* 136, 575–584.
- Kusano, Y., Oguri, K., Nagayasu, Y., Munesue, S., Ishihara, M., Saiki, I., Yonekura, H., Yamamoto, H., Okayama, M., 2000. Participation of syndecan 2 in the induction of stress fiber formation in cooperation with integrin $\alpha 5 \beta 1$: structural characteristics of heparan sulfate chains with avidity to COOH-terminal heparin-binding domain of fibronectin. *Exp. Cell Res.* 256, 434–444.
- Lamanna, W.C., Kalus, I., Padva, M., Baldwin, R.J., Merry, C.L., Dierks, T., 2007. The heparanome—the enigma of encoding and decoding heparan sulfate sulfation. *J. Biotechnol.* 129, 290–307.
- Landemore, G., Stefani, P., Quillec, M., Lecoq-Guilbert, P., Billotte, C., Izard, J., 1999. Uneven distribution and size of rabbit lens capsule proteoglycans. *Histochem. J.* 31, 161–167.
- Lau, C.S.L., Yu, C.B.O., Wong, H.K., Fan, D.S.P., Mak, H.T., Wong, K.W., Lam, D.S.C., Pang, C.P., Choy, K.W., 2010. Allelic imbalance at 13q31 is associated with reduced GPC6 in Chinese with sporadic retinoblastoma. *Br. J. Ophthalmol.* 94, 357–362.
- Le, A.C., Musil, L.S., 2001. A novel role for FGF and extracellular signal-regulated kinase in gap junction-mediated intercellular communication in the lens. *J. Cell Biol.* 154, 197–216.
- Leadbeater, W.E., Gonzalez, A.-M., Logaras, N., Berry, M., Turnbull, J.E., Logan, A., 2006. Intracellular trafficking in neurones and glia of fibroblast growth factor-2, fibroblast growth factor receptor 1 and heparan sulphate proteoglycans in the injured adult rat cerebral cortex. *J. Neurochem.* 96, 1189–1200.
- Li, J.-P., Gong, F., Hagner-McWhirter, A., Forsberg, E., Abrink, M., Kisilevsky, R., Zhang, X., Lindahl, U., 2003. Targeted disruption of a murine glucuronyl C5-epimerase gene results in heparan sulfate lacking L-iduronic acid and in neonatal lethality. *J. Biol. Chem.* 278, 28363–28366.
- Li, J.P., Kusche-Gullberg, M., 2016. Heparan sulfate: biosynthesis, structure, and function. *Int. Rev. Cell Mol. Biol.* 325, 215–273.
- Li, H., Mao, Y., Bouaziz, M., Yu, H., Qu, X., Wang, F., Feng, G.-S., Shawber, C., Zhang, X., 2019a. Lens differentiation is controlled by the balance between PDGF and FGF signaling. *PLoS Biol.* 17, e3000133.
- Li, N., Wei, L., Liu, X., Bai, H., Ye, Y., Li, D., Li, N., Baxa, U., Wang, Q., Lv, L., Chen, Y., Feng, M., Lee, B., Gao, W., Ho, M., 2019b. A frizzled-like cysteine-rich domain in glypican-3 mediates Wnt binding and regulates hepatocellular carcinoma tumor growth in mice. *Hepatology* 70, 1231–1245.
- Lin, X., Perrimon, N., 1999. Dally cooperates with Drosophila Frizzled 2 to transduce Wingless signalling. *2007 Nature* 446, 281–284, 7139 400.
- Lin, X., Buff, E.M., Perrimon, N., Michelson, A.M., 1999. Heparan sulfate proteoglycans are essential for FGF receptor signaling during Drosophila embryonic development. *Development* 126, 3715–3723.
- Liu, L.H., Zhang, C., Min, J.K., Cole, G.J., 2008. Retina development in zebrafish requires the heparan sulfate proteoglycan agrin. *Dev. Neurobiol.* 68, 877–898.
- Liu, C.-Y., Kao, W.W.-Y., 2015. Corneal epithelial wound healing. *Progress in Molecular Biology and Translational Science* 134, 61–71.
- Liu, Y.C., Wierbowski, B.M., Salic, A., 2022. Hedgehog pathway modulation by glypican 3-conjugated heparan sulfate. *J. Cell Sci.* 135.
- Lovicu, F.J., McAvoy, J.W., 1989. Structural analysis of lens epithelial explants induced to differentiate into fibres by fibroblast growth factor (FGF). *Exp. Eye Res.* 49, 479–494.
- Lovicu, F.J., McAvoy, J.W., 1993. Localization of acidic fibroblast growth factor, basic fibroblast growth factor, and heparan sulphate proteoglycan in rat lens: implications for lens polarity and growth patterns. *Investig. Ophthalmol. Vis. Sci.* 34, 3355–3365.
- Lovicu, F.J., McAvoy, J.W., 2001. FGF-induced lens cell proliferation and differentiation is dependent on MAPK (ERK1/2) signalling. *Development* 128, 5075–5084.

- Lovicu, F.J., McAvoy, J.W., 2005. Growth factor regulation of lens development. *Dev. Biol.* 280, 1–14.
- Lovicu, F.J., Iyengar, L., Dawes, L.J., McAvoy, J.W., 2014. Lens epithelial cell proliferation. In: *Lens Epithelium and Posterior Capsular Opacification*. Springer Japan, Tokyo, pp. 59–80.
- Lyon, M., Rushton, G., Gallagher, J.T., 1997. The interaction of the transforming growth factor- β s with heparin/heparan sulfate is isoform-specific. *J. Biol. Chem.* 272, 18000–18006.
- Lyu, J., Joo, C.K., 2004. Wnt signaling enhances GGF2-triggered lens fiber cell differentiation. *Development* 131, 1813–1824.
- Ma, C., Moses, K., 1995. Wingless and patched are negative regulators of the morphogenetic furrow and can affect tissue polarity in the developing *Drosophila* compound eye. *Development* 121, 2279–2289.
- Ma, B., Jing, R., Liu, J., Qi, T., Pei, C., 2019. Gremlin is a potential target for posterior capsular opacification. *Cell Cycle* 18, 1714–1726.
- Maccarana, M., Sakura, Y., Tawada, A., Yoshida, K., Lindahl, U., 1996. Domain structure of heparan sulfates from bovine organs. *J. Biol. Chem.* 271, 17804–17810.
- Maeda, N., Ishii, M., Nishimura, K., Kamimura, K., 2011. Functions of chondroitin sulfate and heparan sulfate in the developing brain. *Neurochem. Res.* 36, 1228–1240.
- Maher, P.A., 1996. Nuclear Translocation of fibroblast growth factor (FGF) receptors in response to FGF-2. *J. Cell Biol.* 134, 529–536.
- Majima, K., 1995. Human lens epithelial cells proliferate in response to exogenous EGF and have EGF and EGF receptor. *Ophthalmic Res.* 27, 356–365.
- Mann, S., Kröger, S., 1996. Agrin is synthesized by retinal cells and colocalizes with gephyrin. *Mol. Cell. Neurosci.* 8, 1–13.
- Marneros, A.G., Olsen, B.R., 2003. Age-dependent iris abnormalities in collagen XVIII/endostatin deficient mice with similarities to human pigment dispersion syndrome. *Investig. Ophthalmol. Vis. Sci.* 44, 2367–2372.
- Marneros, A.G., Keene, D.R., Hansen, U., Fukai, N., Moulton, K., Goletz, P.L., Moiseyev, G., Pawlyk, B.S., Halfter, W., Dong, S., Shibata, M., Li, T., Crouch, R.K., Bruckner, P., Olsen, B.R., 2004. Collagen XVIII/endostatin is essential for vision and retinal pigment epithelial function. *EMBO J.* 23, 89–99.
- Massoudi, D., Malecize, F., Galiacy, S.D., 2016. Collagens and proteoglycans of the cornea: importance in transparency and visual disorders. *Cell Tissue Res.* 363, 337–349.
- Matsuo, I., Kimura-Yoshida, C., 2014. Extracellular distribution of diffusible growth factors controlled by heparan sulfate proteoglycans during mammalian embryogenesis. *Phil. Trans.: Biol. Sci.* 369, 1–9.
- Miyake, M., Lawton, A., Dai, Y., Chang, M., Mengual, L., Alcaraz, A., Goodison, S., Rosser, C.J., 2014. Clinical implications in the shift of syndecan-1 expression from the cell membrane to the cytoplasm in bladder cancer. *BMC Cancer* 14, 885–887.
- Mochizuki, T., Suzuki, S., Masai, I., 2014. Spatial pattern of cell geometry and cell-division orientation in zebrafish lens epithelium. *Biol. Open* 3, 982–994.
- Mohan, P.S., Spiro, R.G., 1986. Macromolecular organization of basement membranes. Characterization and comparison of glomerular basement membrane and lens capsule components by immunochemical and lectin affinity procedures. *J. Biol. Chem.* 261, 4328–4336.
- Mohan, P.S., Spiro, R.G., 1991. Characterization of heparan sulfate proteoglycan from calf lens capsule and proteoglycans synthesized by cultured lens epithelial cells. Comparison with other basement membrane proteoglycans. *J. Biol. Chem.* 266, 8567–8575.
- Morgan, M.R., Humphries, M.J., Bass, M.D., 2007. Synergistic control of cell adhesion by integrins and syndecans. *Nat. Rev. Mol. Cell Biol.* 8, 957–969.
- Mu, H., Ohta, K., Kuriyama, S., Shimada, N., Tanihara, H., Yasuda, K., Tanaka, H., 2003. Equarin, a novel soluble molecule expressed with polarity at chick embryonic lens equator, is involved in eye formation. *Mech. Dev.* 120, 143–155.
- Muñoz, R., Moreno, M., Oliva, C., Orbenes, C., Larraín, J., 2006. Syndecan-4 regulates non-canonical Wnt signalling and is essential for convergent and extension movements in *Xenopus* embryos. *Nat. Cell Biol.* 8, 492–500.
- Muragaki, Y., Timmons, S., Griffith, C.M., Oh, S.P., Fadel, B., Quettermous, T., Olsen, B. R., 1995. Mouse Col18a1 is expressed in a tissue-specific manner as three alternative variants and is localized in basement membrane zones. *Proc. Natl. Acad. Sci. USA* 92, 8763–8767.
- Nagarajan, A., Malvi, P., Wajapeyee, N., 2018. Heparan sulfate and Heparan Sulfate Proteoglycans in cancer initiation and progression. *Front. Endocrinol.* 9, 646.
- Nakato, H., Futch, T.A., Selleck, S.B., 1995. The division abnormally delayed (dally) gene: a putative integral membrane proteoglycan required for cell division patterning during postembryonic development of the nervous system in *Drosophila*. *Development* 121, 3687–3702.
- Neill, T., Schaefer, L., Iozzo, R.V., 2015. Decoding the matrix: instructive roles of proteoglycan receptors. *Biochemistry* 54, 4583–4598.
- Neumayer, T., Hirschall, N., Georgopoulos, M., Findl, O., 2017. Natural course of posterior subcapsular cataract over a short time period. *Curr. Eye Res.* 42, 1604–1607.
- Oh, E., 2004. Syndecans-2 and -4; close cousins, but not identical twins. *Mol. Cell.* 17, 181–187.
- Oh, E.S., Woods, A., Couchman, J.R., 1997. Syndecan-4 proteoglycan regulates the distribution and activity of protein kinase C. *J. Biol. Chem.* 272, 8133–8136.
- Ohlmann, A.V., Ohlmann, A., Welge-Lüssen, U., May, C.A., 2012. Localization of collagen XVIII and endostatin in the human eye. *Curr. Eye Res.* 30, 27–34.
- Okamoto, K., Honda, K., Doi, K., Ishizu, T., Katagiri, D., Wada, T., Tomita, K., Ohtake, T., Kaneko, T., Kobayashi, S., Nangaku, M., Tokunaga, K., Noiri, E., 2015. Glypican-5 increases susceptibility to nephrotic damage in diabetic kidney. *Am. J. Pathol.* 185, 1889–1898.
- Olivares, G.H., Carrasco, H., Aroca, F., Carvallo, L., Segovia, F., Larraín, J., 2009. Syndecan-1 regulates BMP signaling and dorso-ventral patterning of the ectoderm during early *Xenopus* development. *Dev. Biol.* 329, 338–349.
- Onodera, S., 1991. Presence of the basement membrane component-heparan sulfate proteoglycan in bovine lens capsules. *Chem. Pharm. Bull.* 39, 1059–1061.
- Palma, V., Carrasco, H., Reinchi, G., Olivares, G., Faunes, F., Larraín, J., 2011. Shh activity and localization is regulated by perlecan. *Biol. Res.* 44, 63–67.
- Pan, Y., Woodbury, A., Esko, J.D., Grobe, K., Zhang, X., 2006. Heparan sulfate biosynthetic gene *Ndst1* is required for FGF signaling in early lens development. *Development* 133, 4933–4944.
- Pan, Y., Carbe, C., Powers, A., Zhang, E.E., Esko, J.D., Grobe, K., Feng, G.-S., Zhang, X., 2008. Bud specific N-sulfation of heparan sulfate regulates Shp2-dependent FGF signaling during lacrimal gland induction. *Development* 135, 301–310.
- Parmigiani, C., McAvoy, J., 1984. Localisation of laminin and fibronectin during rat lens morphogenesis. *Differentiation* 28, 53–61.
- Parmigiani, C.M., McAvoy, J.W., 1991. The roles of laminin and fibronectin in the development of the lens capsule. *Curr. Eye Res.* 10, 501–511.
- Park, P.J., Shukla, D., 2013. Role of heparan sulfate in ocular diseases. *Exp. Eye Res.* 110, 1–9.
- Park, P.W., Reizes, O., Bernfield, M., 2000. Cell surface heparan sulfate proteoglycans: selective regulators of ligand-receptor encounters. *J. Biol. Chem.* 275, 29923–29926.
- Partovian, C., Ju, R., Zhuang, Z.W., Martin, K.A., Simons, M., 2008. Syndecan-4 regulates subcellular localization of mTOR Complex2 and Akt activation in a PKC alpha-dependent manner in endothelial cells. *Mol. Cell* 32, 140–149.
- Passos-Bueno, M.R., Suzuki, O.T., Armelin-Correa, L.M., Sertié, A.L., Errera, F.I.V., Bagatini, K., Kok, F., Leite, K.R.M., 2006. Mutations in collagen 18A1 and their relevance to the human phenotype. *An. Acad. Bras. Cienc.* 78, 123–131.
- Pataki, C., 2015. Wnt signaling cascades and the roles of syndecan proteoglycans. *J. Histochem. Cytochem.* 63, 465–480.
- Patel, V.N., Likar, K.M., Zisman-Rozen, S., Cowherd, S.N., Lassiter, K.S., Sher, I., Yates, E. A., Turnbull, J.E., Ron, D., Hoffman, M.P., 2008. Specific heparan sulfate structures modulate FGF10-mediated submandibular gland epithelial morphogenesis and differentiation. *J. Biol. Chem.* 283, 9308–9317.
- Peek, R., McAvoy, J.W., Lubben, N.H., Schoenmakers, J.G.G., 1992. Rise and fall of crystallin gene messenger levels during fibroblast growth factor induced terminal differentiation of lens cells. *Dev. Biol.* 152, 152–160.
- Peterson, J.A., Sheibani, N., David, G., Garcia-Pardo, A., Peters, D.M., 2005. Heparin II domain of fibronectin uses $\alpha 4 \beta 1$ integrin to control focal adhesion and stress fiber formation, independent of syndecan-4. *J. Biol. Chem.* 280, 6915–6922.
- Polisetti, N., Zenkel, M., Menzel-Severing, J., Kruse, F.E., Schlötzer-Schrehardt, U., 2016. Cell adhesion molecules and stem cell-niche-interactions in the limbal stem cell niche. *Stem Cell.* 34, 203–219.
- Poulain, F.E., Yost, H.J., 2015. Heparan sulfate proteoglycans: a sugar code for vertebrate development? *Development* 142, 3456–3467.
- Pratt, T., Conway, C.D., Tian, N.M.-L., Price, D.J., Mason, J.O., 2006. Heparan sulphation patterns generated by specific heparan sulfotransferase enzymes direct distinct aspects of retinal axon guidance at the optic chiasm. *J. Neurosci.* 26, 6911–6923.
- Puri, S., Coulson-Thomas, Y.M., Gesteira, T.F., Coulson-Thomas, V.J., 2020. Distribution and function of glycosaminoglycans and proteoglycans in the development, homeostasis and pathology of the ocular surface. *Front. Cell Dev. Biol.* 8, 731.
- Qiao, D., Yang, X., Meyer, K., Friedl, A., 2008. Glypican-1 regulates anaphase promoting complex/cyclosome substrates and cell cycle progression in endothelial cells. *Mol. Biol. Cell* 19, 2789–2801.
- Qin, Y., Zhu, Y., Luo, F., Chen, C., Chen, X., Wu, M., 2017. Killing two birds with one stone: dual blockade of integrin and FGF signaling through targeting syndecan-4 in postoperative capsular opacification. *Cell Death Dis.* 8, e2920.
- Qu, X., Carbe, C., Tao, C., Powers, A., Lawrence, R., van Kuppevelt, T.H., Cardoso, W.V., Grobe, K., Esko, J.D., Zhang, X., 2011a. Lacrimal gland development and Fgf10-Fgfr2b signaling are controlled by 2-O- and 6-O-sulfated heparan sulfate. *J. Biol. Chem.* 286, 14435–14444.
- Qu, X., Hertzler, K., Pan, Y., Grobe, K., Robinson, M.L., Zhang, X., 2011b. Genetic epistasis between heparan sulfate and FGF-Ras signaling controls lens development. *Dev. Biol.* 355, 12–20.
- Qu, X., Pan, Y., Carbe, C., Powers, A., Grobe, K., Zhang, X., 2012. Glycosaminoglycan-dependent restriction of FGF diffusion is necessary for lacrimal gland development. *Development* 139, 2730–2739.
- Rapraeger, A., Jalkanen, M., Endo, E., Koda, J., Bernfield, M., 1985. The cell surface proteoglycan from mouse mammary epithelial cells bears chondroitin sulfate and heparan sulfate glycosaminoglycans. *J. Biol. Chem.* 260, 11046–11052.
- Rapraeger, A., Jalkanen, M., Bernfield, M., 1986. Cell surface proteoglycan associates with the cytoskeleton at the basolateral cell surface of mouse mammary epithelial cells. *J. Cell Biol.* 103, 2683–2696.
- Rapraeger, A.C., 2001. Molecular interactions of syndecans during development. *Semin. Cell Dev. Biol.* 12, 107–116.
- Rehn, M., Pihlajaniemi, T., 1995. Identification of three N-terminal ends of type XVIII collagen chains and tissue-specific differences in the expression of the corresponding transcripts. The longest form contains a novel motif homologous to rat and *Drosophila* frizzled proteins. *J. Biol. Chem.* 270, 4705–4711.
- Remko, M., Van Duijnen, P.T., Broer, R., 2013. Effect of metal ions (Li⁺, Na⁺, K⁺, Mg²⁺ and Ca²⁺) and water on the conformational changes of glycosidic bonds in heparin oligosaccharides. *RSC Adv.* 3, 9843–9853.
- Reneker, L.W., Overbeek, P.A., 1996. Lens-specific expression of PDGF-A alters lens growth and development. *Dev. Biol.* 180, 554–565.
- Reynolds-Peterson, C., Xu, J., Zhao, N., Cruise, C., Yonel, B., Trasorras, C., Toyoda, H., Kinoshita-Toyoda, A., Dobson, J., Schultheis, N., Jiang, M., 2020. Heparan sulfate structure affects autophagy, lifespan, responses to oxidative stress, and cell

- degeneration in *Drosophila* parkin mutants. *G3: Genes, Genomes, Genetics* 10, 129–141.
- Rider, C.C., Mulloy, B., 2017. Heparin, heparan sulphate and the TGF- β cytokine superfamily. *Molecules* 22, 713.
- Ringvall, M., Kjell  n, L., 2010. Mice deficient in heparan sulfate N-deacetylase/N-sulfotransferase 1. *Progress in Molecular Biology and Translational Science* 93, 35–58.
- R  nning, S.B., Carlson, C.R., Stang, E., Kolset, S.O., Hollung, K., Pedersen, M.E., 2015. Syndecan-4 regulates muscle differentiation and is internalized from the plasma membrane during myogenesis. *PLoS One* 10, e0129288.
- Rossi, M., 2003. Heparan sulfate chains of perlecan are indispensable in the lens capsule but not in the kidney. *EMBO J.* 22, 236–245.
- Ruiz, X.D., Mlakar, L.R., Yamaguchi, Y., Su, Y., Larregina, A.T., Pilewski, J.M., Feghali-Bostwick, C.A., 2012. Syndecan-2 is a novel target of insulin-like growth factor binding protein-3 and is over-expressed in fibrosis. *PLoS One* 7, e43049.
- Rychkova, N., Stahl, S., Gaetzer, S., Felbor, U., 2005. Non-heparan sulfate-binding interactions of endostatin/collagen XVIII in murine development. *Dev. Dynam.* 232, 399–407.
- Saarela, J., Rehn, M., Oikarinen, A., Autio-Harmainen, H., Pihlajaniemi, T., 1998. The short and long forms of type XVIII collagen show clear tissue specificities in their expression and location in basement membrane zones in humans. *Am. J. Pathol.* 153, 611–626.
- Safaian, F., Kolset, S.O., Prydz, K., Gottfridsson, E., Lindahl, U., Salmivirta, M., 1999. Selective effects of sodium chlorate treatment on the sulfation of heparan sulfate. *J. Biol. Chem.* 274, 36267–36273.
- Saint-Geniez, M., Kurihara, T., D'Amore, P.A., 2009. Role of cell and matrix-bound VEGF isoforms in lens development. *Investig. Ophthalmol. Vis. Sci.* 50, 311–321.
- Sakane, H., Yamamoto, H., Matsumoto, S., Sato, A., Kikuchi, A., 2012. Localization of glypican-4 in different membrane microdomains is involved in the regulation of Wnt signaling. *J. Cell Sci.* 125, 449–460.
- Saoncella, S., Echtermeier, F., Denhez, F., Nowlen, J.K., Mosher, D.F., Robinson, S.D., Hynes, R.O., Goetinck, P.F., 1999. Syndecan-4 signals cooperatively with integrins in a Rhodopent manner in the assembly of focal adhesions and actin stress fibers. *Proc. Natl. Acad. Sci. USA* 96, 2805–2810.
- Saravanan, C., Cao, Z., Head, S.R., Panjwani, N., 2019. Analysis of differential expression of glycosyltransferases in healing corneas by glycogene microarrays. *Glycobiology* 29, 188–189.
- Sarrazin, S., Lamanna, W.C., Esko, J.D., 2011. Heparan sulfate proteoglycans. *Cold Spring Harbor Perspect. Biol.* 3, 1–33.
- Schrage, Y.M., Hameetman, L., Suzhai, K., Cleton-Jansen, A.-M., Taminiau, A.H.M., Hogendoorn, P.C.W., Bove  , J.V.M.G., 2009. Aberrant heparan sulfate proteoglycan localization, despite normal exostosin, in central chondrosarcoma. *Am. J. Pathol.* 174, 979–988.
- Schr  der, E., Gebel, L., Eremeev, A.A., Morgner, J., Grum, D., Knauer, S.K., Bayer, P., Mueller, J.W., 2012. Human PAPS synthase isoforms are dynamically regulated enzymes with access to nucleus and cytoplasm. *PLoS One* 7, e29559.
- Schukz, M., Fuster, M.M., Brown, J.R., Crawford, B.E., Ditto, D.P., Lawrence, R., Glass, C.A., Wang, L., Tor, Y., Esko, J.D., 2008. Surfen, a small molecule antagonist of heparan sulfate. *Proc. Natl. Acad. Sci. U.S.A.* 105, 13075–13080.
- Schulz, M.W., Chamberlain, C.G., de Jongh, R.U., McAvoy, J.W., 1993. Acidic and basic FGF in ocular media and lens: implications for lens polarity and growth patterns. *Development* 118, 117–126.
- Schulz, M.W., Chamberlain, C.G., McAvoy, J.W., 1997. Binding of FGF-1 and FGF-2 to heparan sulphate proteoglycans of the mammalian lens capsule. *Growth Factors* 14, 1–13.
- Seidel, C., B  rset, M., Hjertner,   ., Cao, D., Abildgaard, N., Hjorth-Hansen, H., Sanderson, R.D., Waage, A., Sundan, A., 2000. High levels of soluble syndecan-1 in myeloma-derived bone marrow: modulation of hepatocyte growth factor activity. *Blood* 96, 3139–3146.
- Shi, W., Kaneiwa, T., Cydzik, M., Gari  py, J., Filmus, J., 2020. Glypican-6 stimulates intestinal elongation by simultaneously regulating Hedgehog and non-canonical Wnt signaling. *Matrix Biol.* 88, 19–32.
- Shi, Y., De Maria, A., Lubura, S.,   iki, H., Bassnett, S., 2014. The penny pusher: a cellular model of lens growth. *Investig. Ophthalmol. Vis. Sci.* 56, 799–809.
- Shiau, C.E., Hu, N., Bronner-Fraser, M., 2010. Altering Glypican-1 levels modulates canonical Wnt signaling during trigeminal placode development. *Dev. Biol.* 348, 107–118.
- Shihan, M.H., Kanwar, M., Wang, Y., Jackson, E.E., Faranda, A.P., Duncan, M.K., 2020. Fibronectin has multifunctional roles in posterior capsular opacification (PCO). *Matrix Biol.* 90, 79–108.
- Shimokawa, K., Kimura-Yoshida, C., Nagai, N., Mukai, K., Matsubara, K., Watanabe, H., Matsuda, Y., Mochida, K., Matsuo, I., 2011. Cell surface heparan sulfate chains regulate local reception of FGF signaling in the mouse embryo. *Dev. Cell* 21, 257–272.
- Shirke, S., Faber, S.C., Hallem, E., Makarenkova, H.P., Robinson, M.L., Overbeek, P.A., Lang, R.A., 2001. Misexpression of IGF-I in the mouse lens expands the transitional zone and perturbs lens polarization. *Mech. Dev.* 101, 167–174.
- Shu, D.Y., Lovicu, F.J., 2017. Myofibroblast transdifferentiation: the dark force in ocular wound healing and fibrosis. *Prog. Retin. Eye Res.* 60, 44–65.
- Simirskii, V.N., Wang, Y., Duncan, M.K., 2007. Conditional deletion of β 1-integrin from the developing lens leads to loss of the lens epithelial phenotype. *Dev. Biol.* 306, 658–668.
- Simirskii, V.N., Duncan, M.K., Paltsev, M.A., Suchkov, S.V., 2013. β 1 integrin as the integrating component in cell-cell cooperation for maintenance of lens transparency. *Dokl. Biochem. Biophys.* 453, 297–299.
- Smeters, T.F.C.M., van de Westerlo, E.M.A., Dam, Ten, G.B., Clarijs, R., Versteeg, E.M. M., van Geloof, W.L., Veerkamp, J.H., van Muijen, G.N.P., van Kuppevelt, T.H., 2003. Localization and characterization of melanoma-associated glycosaminoglycans: differential expression of chondroitin and heparan sulfate epitopes in melanoma. *Cancer Res.* 63, 2965–2970.
- Smith, S.M.-L., West, L.A., Govindraj, P., Zhang, X., Ornitz, D.M., Hassell, J.R., 2007. Heparan and chondroitin sulfate on growth plate perlecan mediate binding and delivery of FGF-2 to FGF receptors. *Matrix Biol.* 26, 175–184.
- Smits, N.C., Kobayashi, T., Srivastava, P.K., Skopelja, S., Ivy, J.A., Elwood, D.J., Stan, R. V., Tsongalis, G.J., Sellke, F.W., Gross, P.L., Cole, M.D., 2017. HS3ST1 genotype regulates antithrombin's inflammomodulatory tone and associates with atherosclerosis. *Matrix Biol.* 63, 69–90.
- Song, H.H., Shi, W., Xiang, Y.-Y., Filmus, J., 2005. The loss of glypican-3 induces alterations in Wnt signaling. *J. Biol. Chem.* 280, 2116–2125.
- Song, X., Sato, Y., Felemban, A., Ito, A., Hossain, M., Ochiai, H., Yamamoto, T., Sekiguchi, K., Tanaka, H., Ohta, K., 2012. Equarin is involved as an FGF signaling modulator in chick lens differentiation. *Dev. Biol.* 368, 109–117.
- Song, X., Sato, Y., Sekiguchi, K., Tanaka, H., Ohta, K., 2013. Equarin is involved in cell adhesion by means of heparan sulfate proteoglycan during lens development. *Dev. Dynam.* 242, 23–29.
- Stepp, M.A., Gibson, H.E., Gala, P.H., Iglesia, D.D.S., Pajoohesh-Ganji, A., Pal-Ghosh, S., Brown, M., Aquino, C., Schwartz, A.M., Goldberger, O., Hinkes, M.T., Bernfield, M., 2002. Defects in keratinocyte activation during wound healing in the syndecan-1-deficient mouse. *J. Cell Sci.* 115, 4517–4531.
- Stepp, M.A., Daley, W.P., Bernstein, A.M., Pal-Ghosh, S., Tadvalkar, G., Shashurin, A., Palsen, S., Jurjus, R.A., Larsen, M., 2010. Syndecan-1 regulates cell migration and fibronectin fibril assembly. *Exp. Cell Res.* 316, 2322–2339.
- Stewart, M.D., Sanderson, R.D., 2014. Heparan sulfate in the nucleus and its control of cellular functions. *Matrix Biol.* 35, 56–59.
- Stigliano, I., Puricelli, L., Filmus, J., Sogayar, M.C., Bal De Kier Joff  , E., Peters, M.G., 2009. Glypican-3 regulates migration, adhesion and actin cytoskeleton organization in mammary tumor cells through Wnt signaling modulation. *Breast Cancer Res. Treat.* 114, 251–262.
- Stum, M., Davoine, C.S., Vicart, S., Guillot-No  l, L., Topaloglu, H., Carod-Artal, F.J., Kayserili, H., Hentati, F., Merlini, L., Urtizberea, J.A., Hammouda, E.H., 2006. Spectrum of HSPG2 (perlecan) mutations in patients with schwartz-Jampel syndrome. *Hum. Mutat.* 27, 1082–1091.
- Sulochana, K., Ramakrishnan, S., Vasanthi, S., Madhavan, H., Arunagiri, K., Punithan, R., 1997. First report of congenital or infantile cataract in deranged proteoglycan metabolism with released xylose. *Br. J. Ophthalmol.* 81, 319–323.
- Sun, C., Liu, M., Sun, P., Yang, M., Yates, E.A., Guo, Z., Fernig, D.G., 2019. Sulfated polysaccharides interact with fibroblast growth factors and protect from denaturation. *FEBS Open Bio* 9, 1477–1487.
- Sz  tm  ri, T., Mundt, F., Kumar-Singh, A., M  bus, L.,   tv  s, R., Hjerpe, A., Dobra, K., 2017. Molecular targets and signaling pathways regulated by nuclear translocation of syndecan-1. *BMC Cell Biol.* 18, 34–20.
- Tanihara, H., Inatani, M., Koga, T., Yano, T., Kimura, A., 2002. Proteoglycans in the eye. *Cornea* 21, S62–S69.
- Tao, C., Makrides, N., Chuang, J.-Z., Wu, Y., Brooks, S.E., Esko, J.D., Sung, C.-H., Zhang, X., 2022. Chondroitin sulfate enhances the barrier function of basement membrane assembled by heparan sulfate. *Development* 149, dev200569.
- Tholozan, F.M., Gribbon, C., Li, Z., Goldberg, M.W., Prescott, A.R., McKie, N., Quinlan, R. A., 2007. FGF-2 release from the lens capsule by MMP-2 maintains lens epithelial cell viability. *Mol. Biol. Cell* 18, 4222–4231.
- Tian, F., 2014. Rapamycin-induced apoptosis in HGF-stimulated lens epithelial cells by AKT/mTOR, ERK and JAK2/STAT3 pathways. *Int. J. Mol. Sci.* 15, 13833–13848.
- Tillo, M., Charoy, C., Schwarz, Q., Maden, C.H., Davidson, K., Fantin, A., Ruhrberg, C., 2016. 2-and 6-O-sulfated proteoglycans have distinct and complementary roles in cranial axon guidance and motor neuron migration. *Development* 143, 1907–1913.
- Tsen, G., Halfter, W., Kroger, S., Cole, G.J., 1995. Agrin is a heparan sulfate proteoglycan. *J. Biol. Chem.* 270, 3392–3399.
- Tumova, S., Woods, A., Couchman, J.R., 2000. Heparan sulfate chains from glypican and syndecans bind the Hep II domain of fibronectin similarly despite minor structural differences. *J. Biol. Chem.* 275, 9410–9417.
- Uechi, G., Sun, Z., Schreiber, E.M., Halfter, W., Balasubramani, M., 2014. Proteomic view of basement membranes from human retinal blood vessels, inner limiting membranes, and lens capsules. *J. Proteome Res.* 13, 3693–3705.
- Umeda, I.O., Nakata, H., Nishigori, H., 2004. Identification of protein phosphatase 2C and confirmation of other protein phosphatases in the ocular lenses. *Exp. Eye Res.* 79, 385–392.
- Utani, A., 2003. The laminin α 3 LG4 domain promotes cell adhesion through syndecans. *Connect. Tissue* 35, 187–192.
- Vallet, S.D., Clerc, O., Ricard-Blum, S., 2021. Glycosaminoglycan-protein interactions: the first draft of the glycosaminoglycan interactome. *J. Histochem. Cytochem.* 69, 93–104.
- VanSlyke, J.K., Boswell, B.A., Musil, L.S., 2018. Fibronectin regulates growth factor signaling and cell differentiation in primary lens cells. *J. Cell Sci.* 131, 22.
- Waghmare, I., Wang, X., Page-McCaw, A., 2020. Dally-like protein sequesters multiple Wnt ligands in the *Drosophila* germlarium. *Dev. Biol.* 464, 88–102.
- Walker, J., Menko, A.S., 2009. Integrins in lens development and disease. *Exp. Eye Res.* 88, 216–225.
- Wang, Q., Stump, R., McAvoy, J.W., Lovicu, F.J., 2009. MAPK/ERK1/2 and PI3-kinase signalling pathways are required for vitreous-induced lens fibre cell differentiation. *Exp. Eye Res.* 88, 293–306.
- Wang, Q., McAvoy, J.W., Lovicu, F.J., 2010. Growth factor signaling in vitreous humor-induced lens fiber differentiation. *Investig. Ophthalmol. Vis. Sci.* 51, 3599–3610.

- Wang, S., Qiu, M., Xia, W., Xu, Y., Mao, Q., Wang, J., Dong, G., Xu, L., Yang, X., Yin, R., 2016. Glypican-5 suppresses Epithelial-Mesenchymal Transition of the lung adenocarcinoma by competitively binding to Wnt3a. *Oncotarget* 7, 79736–79746.
- Wawersik, S., Purcell, P., Rauchman, M., Dudley, A.T., Robertson, E.J., Maas, R., 1999. BMP7 acts in murine lens placode development. *Dev. Biol.* 207, 176–188.
- Weber, G.F., Menko, A.S., 2006. Actin filament organization regulates the induction of lens cell differentiation and survival. *Dev. Biol.* 295, 714–729.
- Webster Jr., E.H., Searls, R.L., Hilfer, S.R., Zwaan, J., 1987. Accumulation and distribution of sulfated materials in the maturing mouse lens capsule. *Anat. Rec.* 218, 329–337.
- West-Mays, J.A., Pino, G., Lovicu, F.J., 2010. Development and use of the lens epithelial explant system to study lens differentiation and cataractogenesis. *Prog. Retin. Eye Res.* 29, 135–143.
- Wilcox-Adelman, S.A., Denhez, F., Goetinck, P.F., 2002. Syndecan-4 modulates focal adhesion kinase phosphorylation. *J. Biol. Chem.* 277, 32970–32977.
- Williams, E.H., Pappano, W.N., Saunders, A.M., Kim, M.-S., Leahy, D.J., Beachy, P.A., 2010. Dally-like core protein and its mammalian homologues mediate stimulatory and inhibitory effects on Hedgehog signal response. *Proc. Natl. Acad. Sci. U.S.A.* 107, 5869–5874.
- Winkler, J., Wirbelauer, C., Frank, V., Laqua, H., 2001. Quantitative distribution of glycosaminoglycans in young and senile (cataractous) anterior lens capsules. *Exp. Eye Res.* 72, 311–318.
- Winzen, U., Cole, G.J., Halfter, W., 2003. Agrin is a chimeric proteoglycan with the attachment sites for heparan sulfate/chondroitin sulfate located in two multiple serine-glycine clusters. *J. Biol. Chem.* 278, 30106–30114.
- Wishart, T.F.L., Lovicu, F.J., 2021. An atlas of heparan sulfate proteoglycans in the postnatal rat lens. *Investig. Ophthalmol. Vis. Sci.* 62, 5.
- Wistow, G., Bernstein, S.L., Wyatt, M.K., Behal, A., Touchman, J.W., Bouffard, G., Smith, D., Peterson, K., 2002. Expressed sequence tag analysis of adult human lens for the NEIBank Project: over 2000 non-redundant transcripts, novel genes and splice variants. *Mol. Vis.* 8, 171–184.
- Won, J.-H., Tsogtbaatar, O., Tsogtbaatar, O., Son, W., Singh, A., Choi, K.-W., Cho, K.-O., 2015. Cell type-specific responses to wingless, hedgehog and decapentaplegic are essential for patterning early eye-antenna disc in *Drosophila*. *PLoS One* 10, e0121999.
- Wormstone, I.M., 2002. Posterior capsule opacification: a cell biological perspective. *Exp. Eye Res.* 74, 337–347.
- Wormstone, I., Wride, M.A., 2011. The ocular lens: a classic model for development, physiology and disease. *Phil. Trans. Biol. Sci.* 366, 1190–1192.
- Wride, M.A., 2011. Lens fibre cell differentiation and organelle loss: many paths lead to clarity. *Phil. Trans.: Biol. Sci.* 366, 1219–1233.
- Wu, W., Tholozan, F.M., Goldberg, M.W., Bowen, L., Wu, J., Quinlan, R.A., 2014. A gradient of matrix-bound FGF-2 and perlecan is available to lens epithelial cells. *Exp. Eye Res.* 120, 10–14.
- Xian, X., 2010. Syndecans as receptors and organizers of the extracellular matrix. *Cell Tissue Res.* 339, 31–46.
- Yan, D., Lin, X., 2009. Shaping morphogen gradients by proteoglycans. *Cold Spring Harbor Perspect. Biol.* 1, a002493–a002493.
- Yates, E.A., Gallagher, J.T., Guerrini, M., 2019. Introduction to the molecules special edition entitled ‘Heparan Sulfate and heparin: challenges and controversies’: some outstanding questions in Heparan Sulfate and heparin research. *Molecules* 24, 1399.
- Ylikärppä, R., Eklund, L., Sormunen, R., Kontiola, A.I., Utriainen, A., Määttä, M., Fukui, N., Olsen, B.R., Pihlajaniemi, T., 2003. Lack of type XVIII collagen results in anterior ocular defects. *Faseb. J.* 17, 2257–2259.
- Yoneda, A., Couchman, J.R., 2003. Regulation of cytoskeletal organization by syndecan transmembrane proteoglycans. *Matrix Biol.* 22, 25–33.
- Young, R.W., Ocumpaugh, D.E., 1966. Autoradiographic studies on the growth and development of the lens capsule in the rat. *Invest. Ophthalmol.* 5, 583–589.
- Yu, P., Pearson, C.S., Geller, H.M., 2018. Flexible roles for proteoglycan sulfation and receptor signaling. *Trends Neurosci.* 41, 47–61.
- Zhang, Y., Ryan, D.S., Bower, K.S., Ilan, N., Vlodavsky, I., Laurie, G.W., 2010. Focus on molecules: heparanase. *Exp. Eye Res.* 91, 476–477.
- Zhang, Y., Fan, J., Ho, J.W.K., Hu, T., Kneeland, S.C., Fan, X., Xi, Q., Sellarole, M.A., de Vries, W.N., Lu, W., Lachke, S.A., Lang, R.A., John, S.W.M., Maas, R.L., 2016a. Crim1 regulates integrin signaling in murine lens development. *Development* 143, 356–366.
- Zhang, X., Wang, F., Sheng, J., 2016b. “Coding” and “Decoding”: hypothesis for the regulatory mechanism involved in heparan sulfate biosynthesis. *Carbohydr. Res.* 428, 1–7.
- Zhang, Y., You, J., Ren, W., Lin, X., 2013. *Drosophila* glypicans Dally and Dally-like are essential regulators for JAK/STAT signaling and Unpaired distribution in eye development. *Dev. Biol.* 375, 23–32.
- Zhou, F., 2018. Glypican-3: a promising biomarker for hepatocellular carcinoma diagnosis and treatment. *Med. Res. Rev.* 38, 741–767.
- Zhou, M., Li, X.M., Lavker, R.M., 2006. Transcriptional profiling of enriched populations of stem cells versus transient amplifying cells: a comparison of limbal and corneal epithelial basal cells. *J. Biol. Chem.* 281, 19600–19609.
- Zhou, Z., Wang, J., Cao, R., Morita, H., Soininen, R., Chan, K.M., Liu, B., Cao, Y., Tryggvason, K., 2004. Impaired angiogenesis, delayed wound healing and retarded tumor growth in perlecan heparan sulfate-deficient mice. *Cancer Res.* 64, 4699–4702.
- Zoeller, J.J., Whitelock, J.M., Iozzo, R.V., 2009. Perlecan regulates developmental angiogenesis by modulating the VEGF-VEGFR2 axis. *Matrix Biol.* 28, 284–291.
- Zong, F., Fthenou, E., Wolmer, N., Hollósi, P., Kovalszky, I., Szilák, L., Mogler, C., Nilsson, G., Tzanakakis, G., Dobra, K., 2009. Syndecan-1 and FGF-2, but not FGF receptor-1, share a common transport route and Co-localize with heparanase in the nuclei of mesenchymal tumor cells. *PLoS One* 4, e7346.