CELL SURFACE PROTEOGLYCANS CONTROL ASTROCYTE MIGRATION AND RETINAL ANGIOGENESIS BY REGULATING BASEMENT MEMBRANE ASSEMBLY

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Elaborate vascularization of the retina is crucial for the development and functioning of the eye. The proper patterning of astrocytes is a key event preceding retinal angiogenesis by providing guidance cues for endothelial cells, yet how this is regulated still remains obscure. The dual function of proteoglycans in both extracellular matrix (ECM) composition and cell signal transduction suggests their potential in the regulation of astrocyte migration. The current study demonstrated that non-cell-autonomous regulation by neuroretina cell surface proteoglycan is crucial for PDGF-A regulated astrocyte migration. Ablation of glycosaminoglycan side chains of proteoglycans in neuroretina led to impaired astrocyte migration, incomplete retinal angiogenesis, and hyaloid vessel persistence. This is followed by severe photoreceptor degeneration as a result of reactive gliosis, which cannot be rescued by constitutively activated Kras signaling. Notably, inner limiting membrane (ILM), the basement membrane of the retina, was breached in proteoglycan-deficient retinae prior to the formation of astrocytic network. Herein we propose that cell surface proteoglycans are essential for the initial assembly of ILM, and this cannot be compensated by secreted ECM proteoglycans. In support of this, after removal of ILM in retinal explant by Collagenase digestion, establishment of a new ILM can be achieved by incubation with exogenous laminin-supplemented

Matrigel. This basement membrane reconstitution failed, however, in proteoglycan-deficient retinae or in wild type samples digested with a combination of Heparinase and ChABC in addition to Collagenase. Taken together, our study reveals a novel function of neuroretinal cell surface proteoglycans in the initial assembly of basement membrane which subsequently serves as a permissive substratum necessary for astrocyte migration.

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APC	Astrocyte progenitor cell
BM	Basement membrane
BMP	Bone morphogenetic protein
BrdU	5′-bromo-2′ deoxyuridine
ChABC	Chondroitinase ABC
Col XVIII	Type VXIII collagen
CNS	Central nerves system
CS	Chondroitin sulfate
Dll4	Delta-like 4
DS	Dermatan sulfate
ECM	Extracellular matrix
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GalNAc	*N*-acetylgalactosamine
GCL	Ganglion cell layer
GFAP	Glial fibrillary acidic protein
GlcNAc	*N*-acetylglucosamine
GFAP	Glial fibrillary acidic protein
GPI	Glycosylphosphatidylinositol
HA	Hyaluronic acid
HS	Heparin sulfate

ILM	Inner limiting membrane
INL	Inner nuclear layer
KS	Keratin sulfate
LIF	Leukemia inhibitory factor
MMP	Matrix metalloproteinase
O-2A	Oligodendrocyte-type-2 astrocyte
ONH	Optic nerve head
PDGF	Platelet-derived growth factor
PDGF-receptor α	Pdgfrα
PG	Proteoglycan
PI3K	Phosphatidylinositol 3′ kinase
PLC γ	Phospholipase C-γ
rER	Rough endoplasmic reticulum
RGC	Retinal ganglion cell
ROP	Retinopathy of prematurity
Shh	Sonic hedgehog
TGF-β	Transforming growth factor-beta
Ugdh	UDP-glucose 6-dehydrogenase
VEGF	Vascular endothelial growth factor
Wnt	Wingless-int

CHAPTER 1. INTRODUCTION

Retina is the multilayered photosensitive tissue lining the inner surface of the eye. Dedicated to light sensing and vision processing, retina is one of the most metabolically active and energy demanding tissue in the body, with an oxygen consumption rate higher than even some parts of the brain (Anderson and Saltzman 1964, Ames 1992, Schmidt, Giessl et al. 2003). Even mild hypoxia, acute or chronic, would significantly affect the vision performance of humans (Wiedman and Tabin 1999, Karakucuk, Oner et al. 2004, Connolly, Barbur et al. 2008). The supply of oxygen and nutrients by a proper vasculature system is thus crucial to the normal development and functioning of the eye. Abnormal ocular vascularization can lead to pathologies from mild vision impairment to complete blindness in all age groups, from retinopathy of prematurity (ROP) (Chen and Smith 2007), diabetic retinopathy (DR) (Durham and Herman 2011), to age-related macular degeneration (AMD) (Bhutto and Lutty 2012).

Mechanism of vascularization in the retina

In most adult mammals including humans, the retina is nourished by two independent circulatory systems: the outer retina via choroid and the inner parts by retinal vasculatures. Early in development, however, the retinal vasculatures are initially absent, and the retina is supported instead by choroid and hyaloid vessels. First observed in mice at E10.5, hyaloid vessels are a transient arterial vascular system embedded in the primary vitreous between the lens and the retina (**Figure 1**, A). At certain stage of development—immediately after birth in mice and 16

weeks of gestation in humans (Sapieha 2012), retinal vasculature invades from the optic nerve and spreads on the inner retina surface from the center to the periphery, and then downwards to inner and outer edges of the inner nuclear layer, forming the intermediate and deep layer plexuses, respectively **(Figure 1**, B and D). In the meanwhile, the hyaloid vessel that supports the embryonic retina undergoes progressive regression (Ito and Yoshioka 1999); (**Figure 1,** B and C). Macrophages induce apoptosis in those endothelial cells through the canonical WNT pathway via a short range paracrine mechanism of Wnt7b (Lobov, Rao et al. 2005). This might also be accompanied by autophagic processes (Kim, Kim et al. 2010). Hyaloid vessel regression usually finishes by 34 weeks of gestation in humans and postnatal day (PD) 16 in mice (Ito and Yoshioka 1999), coinciding with the full vascularization of the retina at 36-40 weeks of gestation in human (Stahl, Connor et al. 2010) and PD14 – PD21 in mice. Failure of hyaloid vessel regression in humans causes persistent fetal vasculature (PFV) syndrome, which is associated with incomplete retinal angiogenesis and retinal dysplasia (Jones 1963). This is exemplified in Norrie Disease where bilateral persistent hyaloid vessels may cause blindness in both eyes (Dhingra, Shears et al. 2006).

The timing and directionality of retinal vasculature morphogenesis is consistent with the onset of the central-to-peripheral differentiation wave of retinal cells, the thickening of the retina, and the "physical hypoxia" as the photoreceptor metabolism begins (Akula, Hansen et al. 2007, Berkowitz, Roberts et al. 2007). The completion of the primitive three-layered retinal vascular system in mouse pups by

P14 coincides with the time of eye opening and vision onset, indicating a correlation between vasculature morphogenesis and functionality of the retina.

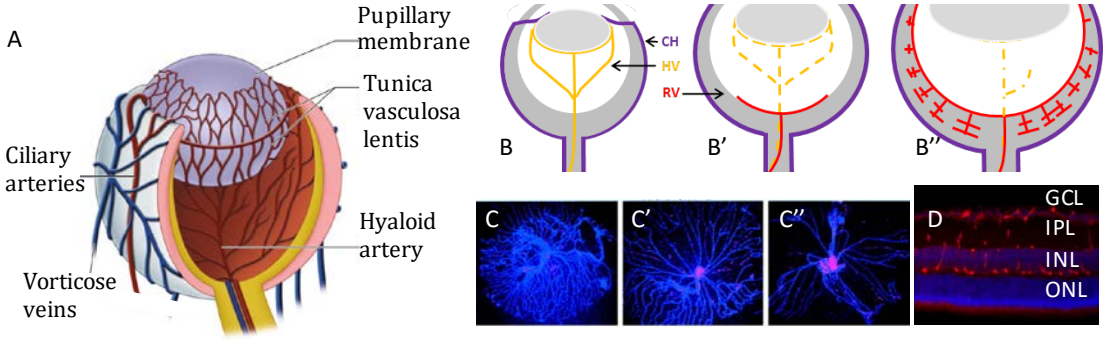


Figure 1. Illustration of the circulatory systems of the retina.
A. An embryonic retina is supported by two independent circulatory systems: choroid vessels (ciliary arteries and vorticose veins) and hyaloid vessels (pupillary membrane, tunica vasculosa lentis and hyaloid artery) (after Sebag et al., 2014, *Vitreous: in Health and Disease*, pp231). **B**. Retinal angiogenesis accompanied with hyaloid vessel regression gives rise to the three-layered intraretinal vasculature network. **C.** Hyaloid vessels undergo progressive regression in postnatal eyes. **D.** The superficial, intermediate and deep plexus shown in a section stained with Isolectin B4 (IB4). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer.

The formation of new vasculatures can be achieved mainly through two distinct mechanisms: vasculogenesis and angiogenesis (Risau, Sariola et al. 1988, Patan 2000). Vasculogenesis refers to the in situ differentiation of endothelial progenitor cells and de novo assembly of blood vessels (Risau and Flamme 1995), which, though not exclusive to embryonic stage, gives rise to the first primitive vasculature network in the embryo. Angiogenesis, on the other hand, takes place by the expansion and re-modeling from pre-existing vasculatures (Risau 1997, Carmeliet 2000). During angiogenesis, two major processes, sprouting and intussusception, works in concert to build the complex vascular systems.

While lack of evidence for endothelial precursors in mouse retina indicates vascularization is exclusively mediated by angiogenesis (Fruttiger 2002), in humans, the mechanism had been controversial. Though the observation of "spindle-shaped cells" migrating from the optic disc indicates the presence of invading "angioblasts" that are characteristic of vasculogenesis (Ashton 1970), in murine they were later shown by Fruttiger *et al.* to be astrocyte precursor cells (APCs) instead. It was not until recent that the use of CD39/ecto-ADPase as a marker for human vascular precursors convincingly showed that the formation of primordial vessels in the central retina is indeed mediated by vasculogenesis (Chan-Ling, McLeod et al. 2004). Nevertheless, angiogenesis is still responsible for the peripheral vascularization of the inner retina, as well as the intermediate and deep plexus morphogenesis (Hughes, Yang et al. 2000).

In my project I will exclusively focus on angiogenesis, which is the predominant mechanism for postnatal retinal vessel morphogenesis, and also the major cause for neovascularization that lead to various ocular pathologies. While mouse retina closely mimic that of humans and offers a valuable model studying angiogenesis in both physiological and pathological conditions, cautions should be taken about the species differences when the conclusions are to be generalized from animal studies to humans.

VEGF signaling in retinal angiogenesis

The first clue for the correlation between hypoxia and retinal angiogenesis came from emerging cases of a blinding disease in neonatal clinics in 1940s, where

preterm babies experienced abnormal retinal vessel growth that caused hemorrhages, retinal detachment and even vision loss(Terry 1942). Later named as retinopathy of prematurity (ROP), this disease was eventually traced to the use of supplemental oxygen to relieve respiratory distress in preterm neonates (Ashton, Ward et al. 1953, Ashton, Ward et al. 1954). Vascular endothelial growth factor (VEGF), the mysterious "factor X" known by that time, was found to be the major regulator of retinal angiogenesis, and also culprits for the pathological neovascularization in ROP and a number of other retinopathies (Alon, Hemo et al. 1995).

VEGF is a homodimeric, disulfide-bound glycoprotein with a high specificity for endothelial cells (Thomas 1996). Induced by hypoxia, VEGF binds VEGF receptor 2 (VEGFR-2, Flk-1, KDR) to promote endothelial cell proliferation, migration and survival, and hence triggers angiogenesis through a tyrosine kinase signaling pathway (Gerhardt, Golding et al. 2003). VEGF/Flk-1 interaction in the leading endothelial tip cells upregulates the expression of Delta-like 4 (Dll4), which interacts with Notch1 receptors on adjacent endothelial cells to turn down their expression of VEGFR-2 in those cells, thereby specifying tip/stalk cell fate (Hellstrom, Phng et al. 2007). By inducing the expression of arterial marker ephrin B2 and suppressing the expression of venous marker EphB4, Notch signaling downstream of VEGF also participated in determining arteriovenous specification (Lawson, Scheer et al. 2001). In cells destined to become veins, COUP-TFII suppresses Nrp1 expression and reduces VEGFR2 signaling (You, Lin et al. 2005). Furthermore, crosstalk with Notch1 signaling also upregulates VEGF receptor 1

(VEGFR-1 or flt-1) (Funahashi, Shawber et al. 2010), which negatively regulates endothelial cell division (Kearney, Ambler et al. 2002) but positively promotes vessel sprouting and branching (Kearney, Kappas et al. 2004, Chappell, Taylor et al. 2009), adding complexity to this hierarchical organization of vasculature. Subsequently, the retinal vessels undergo stabilization via platelet-derived growth factor-B (PDGFB)-dependent pericyte recruitment, giving rise to mature vessels that are insensitive to VEGF withdrawal (Benjamin, Hemo et al. 1998).

During retinal development, VEGF is successively expressed at the ganglion cell layer (GCL) and later at the inner nuclear layer (INL), perfectly matching the onset of the formation of superficial, intermediate and deep vessels in the retina (Stone, Itin et al. 1995).

Astrocytes in neuroretina

Astrocytes are star-shaped glial cells that represent the largest cell population in CNS (Tsai and Miller 2002, Molofsky, Krencik et al. 2012). Initially thought to be merely the non-excitable "brain glue" that passively supports and nurtures neurons (Somjen 1988), astrocytes are now realized to participate in almost every aspect of development and functioning in CNS.

Much of our early understanding about cells of astrocytic lineage in the visual system is attributed to studies with the optic nerve culture (Raff, Abney et al. 1983, Raff, Abney et al. 1984, Miller, Ffrenchconstant et al. 1989), which yielded three types of macroglials with distinctive morphological and immunolabeling hallmarks:

oligodendrocytes, type-1 and type-2 astrocytes. In vitro, these three cell populations arise from two independent lineages and debut at different developmental stages: Type-1 astrocytes derive from astrocyte progenitor cells (APCs), whereas oligodendrocytes and type-2 astrocytes diverge from oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells (Raff, Abney et al. 1983). Unlike the O-2A cells that develop postnatally and remain excluded from neural retina (Small, Riddle et al. 1987, Ffrench-Constant, Miller et al. 1988), astrocytes of type 1 lineage represent the only type of macroglial in the embryonic optic nerve that later invades the neuroretina as well (Hart, Richardson et al. 1989, Ye and Hernandez 1995).

Astrocytes are not native residents of the neuroretina. In rodents, cells of the astrocytic lineage migrate into retina through the optic nerve head (ONH) as a mixture of precursor cells and immature perinatal astrocytes, and then spread across the nerve fiber layer toward peripheral margins of the retina. On this journey, the $Pax2^{+}$/$vimentin^{+}$ APCs undergo at least three stages of differentiation. The first stage is defined as immature perinatal astrocytes that express glial fibrillary acidic protein (GFAP) in addition to Pax2 and vimentin. This is followed by the emergence of mature perinatal astrocytes that lose vimentin expression, but retain Pax2, S100, and GFAP. After the final stage of development, while adult astrocytes exhibit robust expression of GFAP and S100β, they have lost expression of Pax2. Both APCs and immature astrocytes population exhibit proliferative and migratory capacity. However, in vitro studies of rat retina showed a greater proliferative index in perinatal immature astrocytes, while APCs exhibit high mobility (Miller, David et al. 1985, Orentas and Miller 1996, Chu, Hughes et al. 2001,

Chan-Ling, Chu et al. 2009). This creates a spatial pattern that most GFAP$^+$ immature astrocytes are clustered in the central retina, surrounded by a halo of less differentiated APCs at the leading edge of the migratory front. This small but distinct margin persists around birth until all APCs complete maturation.

The migration of astrocytes is closely regulated by neuroretina. Several lines of evidence have suggested that Platelet-derived growth factor A (PDGFA) secreted by retinal ganglion cells (RGCs) is critical to the patterning of the retinal astrocyte network. PDGF receptor α (PDGFRα)-expressing cells at the optic nerve head do not start to migrate toward peripheral retina until PDGFA mRNA is detected in RGCs (Mudhar, Pollock et al. 1993). Inhibition of PDGF signaling by either a blocking antibody or a soluble extracellular fragment of PDGFRα results in significant but incomplete inhibition of astrocyte migration and a reduced branching pattern of the astrocyte network (Fruttiger, Calver et al. 1996). Overexpression of PDGFA in RGCs, on the other hand, leads to a dose-dependent increase in the astrocytic population that migrates more slowly across the retina, forming a denser astrocytic network (Fruttiger, Calver et al. 1996, Reneker and Overbeek 1996). It is not known exactly why astrocytic hyperplasia is accompanied by a delay in migration. It is possible that excessive PDGF signal results in more differentiated astrocytic linage, with a decreased mobility. Meanwhile, PDGFA and PDGFRα expression persist in RGCs and retinal astrocytes respectively throughout life, suggesting RGC-derived PDGF may be required for long-term regulation of astrocytes.

Astrocyte regulation of retinal angiogenesis: a classic model

Astrocytes are found ubiquitously in richly vascularized retinae of mice, rats and humans but not in avascular retinae such as those of echidnas, guinea pigs, and horses (Schnitzer 1987, Stone and Dreher 1987). In early postnatal mouse retina, the advancing superficial retinal vasculatures almost precisely follows the path of astrocytic meshwork in the front. They are also closely associated with the radially oriented ganglion cell axons (**Figure 2**).

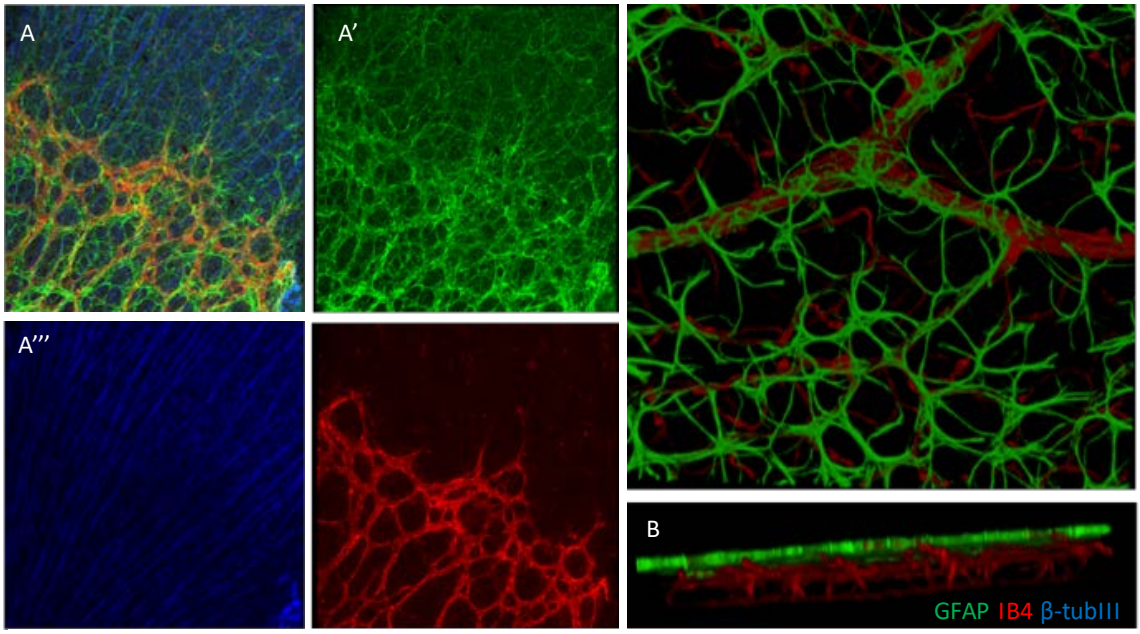


Figure 2. Spatial correlation of neuron, glia and endothelial cells of the mouse retina.
PD 4 (**A**) and PD 21 (**B**) retinae were stained by GFAP, IB4 and β-tubulin III to mark astrocytes, vasculature and RGC axons, respectively. **B'**. A 3D image stack of (**B**) to show close association of astrocytes to the superficial retinal vasculatures.

Based on the spatial and temporal presence of neuron, glial and endothelial cells, and their expression of angiogenic factors, a classic model of retinal angiogenesis has been put forward:

Retinal ganglion cell-derived platelet derived growth factor A (PDGFA), through interaction with PDGF Receptor-alpha (PDGFRα) on astrocytes, regulates the proliferation and migration of the latter across the surface of the inner retina (Fruttiger, Calver et al. 1996, Fruttiger, Calver et al. 2000). The advancing astrocytes beyond the vasculature front experience hypoxia, and by producing a gradient of VEGF, they promote the proliferation, migration and survival of vessel endothelial cells. During this process, a transient expression of cell surface-adhesion molecule R-cadherin from the GCL facilitates the interaction between astrocytes and endothelial cells, making the astrocytic processes a scaffold for the subsequent retinal vessels (Stone, Itin et al. 1995, Dorrell, Aguilar et al. 2002). Invasion of endothelial cells, which are a main source of leukemia inhibitory factor (LIF), in turn promotes astrocyte maturation, as LIF has been demonstrated to stimulate astrocyte differentiation in vitro by augmenting promoter activity of GFAP, a key marker for dedicated astrocytes (Yoshida, Satoh et al. 1993, Nakagaito, Yoshida et al. 1995, Richards, Kilpatrick et al. 1996, Bonni, Sun et al. 1997, Mi and Barres 1999, Nakashima, Yanagisawa et al. 1999, Galli, Pagano et al. 2000). As the oxygen tension is relieved with the expansion of the blood vessels, the production of VEGF by the astrocytes decreases (**Figure 3**). Meanwhile, R-cadherin expression at GCL waned along with the vascularization of the superficial retina. Their new site of expression at the inner and outer edge of INL at P7 and P14 respectively, coincides with the

onset of intermediate and deep plexus formation (Honjo, Tanihara et al. 2000, Usui, Westenskow et al. 2015).

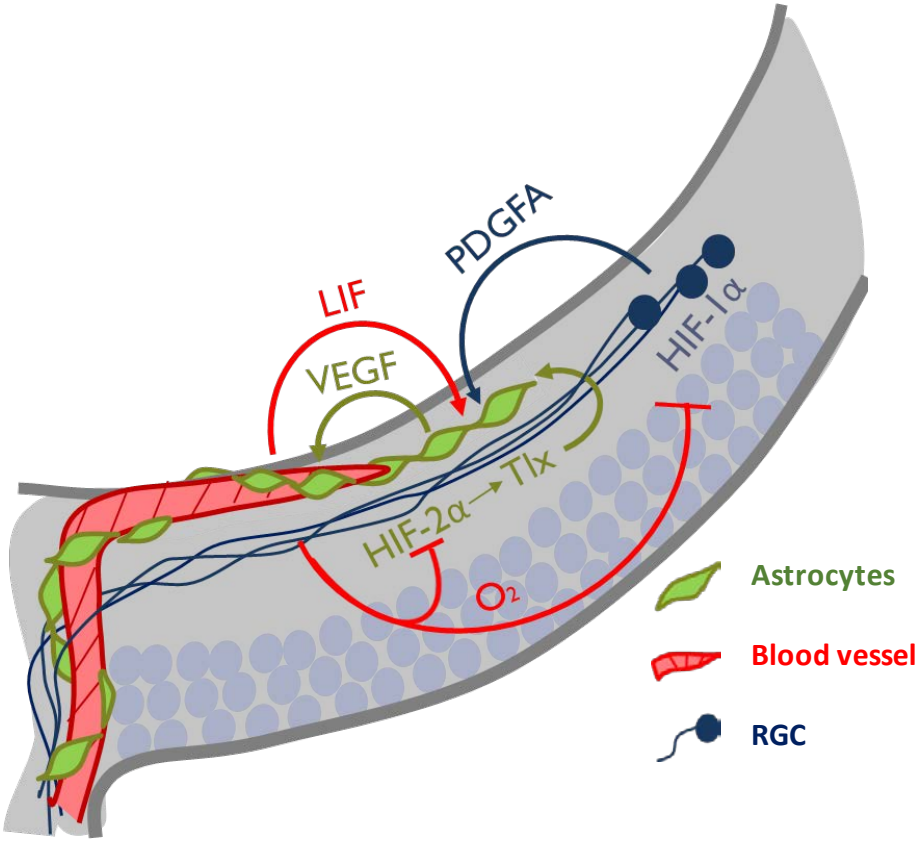


Figure 3. Angiogenesis mediated by interplay among astrocytes, endothelial cells and neuroretina.
Oxygen deprivation sensed by HIF-1α in neuroretina induces retinal ganglion cells to express PDGFA, promoting the proliferation and migration of astrocyte to populate the retina. Astrocytes in turn secrete VEGF in an HIF- and Tlx-dependent manner, attracting endothelial cells during angiogenesis. Invading endothelial cells relieve the oxygen tension and express LIF to promote maturation of astrocytes.

Retinal extracellular matrix regulates astrocyte migration and vasculature patterning

The extracellular matrix (ECM) is a three-dimensional, non-cellular suprastructure. Composed of interstitial matrix and basement membrane, ECM not

only provides structural support for tissue integrity but also actively participate in a variety of cellular events (Kim, Turnbull et al. 2011).

Neural retinal provides the permissive ECM essential for astrocyte migration. In a microchemotaxis assay, type 1 astrocytes from rat brain migrated toward lower chamber filled with laminin (Armstrong, Harvath et al. 1990). Genetic deletions of laminin α1, β2 and γ3 chains in retina disrupted astrocyte migration and spatial distribution (Edwards, Mammadova-Bach et al. 2010, Gnanaguru, Bachay et al. 2013). It is proposed that laminins act as haptotactic factors in vitro in an isoform-specific manner, inducing astrocyte migration and promoting astrocyte differentiation (Gnanaguru, Bachay et al. 2013).

Proteoglycans, a major component of the extracellular matrix

Among the ~300 proteins that makes up the proteome of ECM, 36 belongs to the family of proteoglycans (Hynes and Naba 2012). Proteoglycans are heavily glycosylated proteins with a wide range of molecular weight. Each proteoglycan is composed of a core protein to which one or multiple glycosaminoglycans (GAGs) covalently link as side chains. GAGs are large unbranched heteropolysaccharides comprised of repeating disaccharide units, whose structure classifies GAGs into five main categories—chondroitin sulfate (CS), heparin sulfate (HS)/heparin, dermatan sulfate (DS) keratin sulfate (KS), and hyaluronic acid (HA). Those disaccharides are derivatives of either glucose-glucose or galactose-glucose, and, with the exception of KS, consist of an amino sugar [*N*-acetylglucosamine (GlcNAc) or *N*-

acetylgalactosamine (GalNAc)] and a choice of either uronic acid (glucuronic acid or iduronic acid) or galactose (**Table 1**).

The biosynthesis of proteoglycans is initiated by the formation of the core polypeptide, followed by glycosylation. After formation of the trisaccharide linkage region, the actual polysaccharide is assembled by alternate transfer of D-glucuronosyl and N-acetyl-D-hexosaminyl moieties from the appropriate UDP-sugars to the non-reducing end of the growing chain. Following these glycosylation reactions, the polysaccharide chains undergo enzymatic modification, which includes the incorporation and secondary modifications of sulphate groups and to yield the mature proteoglycan.

While the core protein is synthesized by ribosomes and translocated into the lumen of the rough endoplasmic reticulum (rER), its glycosylation occurs in the Golgi apparatus in multiple enzymatic steps.

Ubiquitously expressed by all cells, proteoglycans exhibit enormous structural diversity and tissue specificity. This is attributable to variations in **1)** the type of core protein **2)** the stoichiometry and heterogeneity of GAG side chains and **3)** the secondary N- or O-linked sugar modifications. Even a defined proteoglycan may adopt different structure and biological properties due to the selective use of one of more attachment sites for GAG chains, as in the case of sydecan-1 (Eriksson and Spillmann 2012).

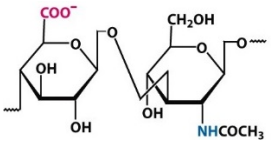
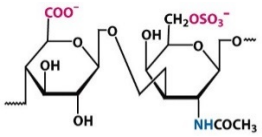
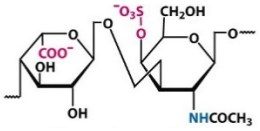
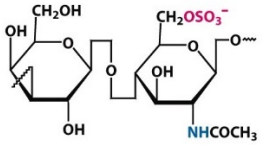
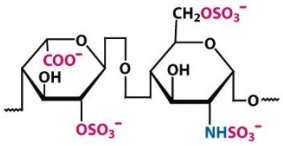
GAG (M.W. Range)	Amino Sugar	Uronic Acid	Type of Sulfate Linkage	
Hyaluronate (4 - 80 x 10^6)	D-Glucosamine	D-Glucuronate	None	
Chondroitin Sulfate (5000 - 50,000)	D-Galactosamine	D-Glucoronate	4-O and/or 6-O-sulfate on galactosamine	
Dermatan Sulfate (15,000 - 40,000)	D-Galactosamine	L-Iduronate, D-Glucuronate	4-O-sulfate on galactosamine; 2-O-sulfate on iduronate	
Keratan Sulfate (4000 – 19,000)	D-Glucosamine	None (but contains D-Galactose)	6-O-sulfate on both carbohydrate residues	
Heparan Sulfate (10^4 to 10^5) or Haparin (10^3 to 10^6)	D-Glucosamine	D-Glucuronate or L-Iduronate	6-O-sulfate and N-sulfate (or N-acetyl) on glucosamine; 2-O-sulfate on iduronate	

Table 1. Summary of major category of GAGs. (After Bhagavan & Ha, *Essentials of Medical Biochemistry: With Clinical Cases,* Table 10.2 and Berg et al., *Biochemistry, 7th Edition,* Figure 11.18)

Biological functions of glycosaminoglycan side chains

The biological functions of proteoglycans are to a large extent determined by the properties of the GAG chains due to the large amount of sugar residues they carry (Esko, Kimata et al. 2009). GAGs hold a large number of water molecules and assume extended structures in aqueous solutions because of their strong hydrophilic nature. This is endowed by their large number of negative charges owing to the presence of acidic sugar residues and/or modification by sulphate

groups. Proteoglycans can thus buffer pressure changes in joints and connective tissues. GAGs are also crucial for organization of ECM. For example, HS is important for the barrier function of ECM against cell transmigration (Benhamron, Nechushtan et al. 2006); KS regulates arrangement of collagen fibril to ensure skin tensility and cornea transparency (Chakravarti, Magnuson et al. 1998). The mucopolysaccharidoses (MPSs), a heterogeneous group of lysosomal storage diseases, are caused by defective catabolism of GAGs that eventually leads to abnormality in bones, joints and connective tissues (Muenzer 2004).

Apart from water molecules, GAGs have binding affinity with a wide range of ECM components and signaling molecules, participating in regulation of various biological activities. This is most studied in heparan sulfate (HS) and heparin, as they serve as

- Matrix receptors to regulate cell adhesion and migration in concert with integrin (Saoncella, Echtermeyer et al. 1999)
- Co-receptors for a variety of receptor tyrosine kinases (RTK)s by facilitating ligand-receptor interaction (Yayon, Klagsbrun et al. 1991)
- Generator of morphagen gradient by sequestering the otherwise freely diffusible HS-binding ligands to cell surface (Jasuja, Allen et al. 2004)
- Repository for HS-binding factors that can be liberated at later stage by shedding of HS chains (Vlodavsky, Goldshmidt et al. 2002)

Specifically, Heparan sulphate proteoglycans (HSPGs) have been shown to interact with a variety of factors involved in the regulation of angiogenesis (**Table 2**).

HS-binding factors	Functions
Wnt	Endothelial cell (EC) fate specification, proliferation, and survival
Shh	Promotes neovascularization
BMP	Stimulates angiogenesis
FGF-2	Promotes proliferation & differentiation of ECs, SMCs and fibroblasts
VEGF-A	Proliferation, migration and survival of ECs
PDGF-BB	Pericyte recruitment
TGF-β	Vascular remodeling
Angiopoietin-3	Inhibits EC proliferation and survival; induces retraction and loss of integrity of the endothelial monolayer
Slit3	Promotes EC proliferation and motility; simulates vessel sprouting
Endostatin	Anti-angiogenic
MMPs	ECM remodeling; release of HS-bound factors

Table 2. Examples of HS-binding factors that are involved in angiogenesis

The importance of GAGs in regulation of angiogenesis is exemplified in VEGF. Exon 6 and 7 of VEGF gene encodes heparin sulfate (HS) binding domains, alternative spicing of which gives rise to three isoforms of VEGF-A: $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ in humans ($VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$ in mice). ECM sequestration of $VEGF_{188}$, which has two HS binding sites and thus high affinity to ECM components, led to formation of a very steep gradient. $VEGF_{164}$ generates an intermediate gradient due to its possession of a single HS binding domain. $VEGF_{120}$, in which both HS binding domains are missing, is most diffusible and produces the shallowest gradient. Knock-in mice that selectively expressing any single isoform of VEGF exhibited retinal vascular phenotype with different severity, among which mice exclusively expressing $VEGF_{120}$ exhibited the most profound vessel outgrowth defects; mice with only expression of $VEGF_{164}$ exhibited normal angiogenesis

comparable to wildtype; and mice solely expressing $VEGF_{188}$ had normal venular development but aborted arterial outgrowth (Stalmans, Ng et al. 2002). Notably, defects in hyaloid vessel regression were observed in mice that exclusively expressed either $VEGF_{120}$ or $VEGF_{188}$.

Key questions and research summary

My project is dedicated to the function of neuroretina-derived proteoglycans in retinal angiogenesis and astrocyte migration. This idea came from the previously known facts that 1) HSPGs bind to a variety of angiogenesis-related proteins, 2) PDGF, a potent mitogen for astrocytes, has isoforms with or without HSPG binding affinity and 3) as major structural components of ECM, proteoglycans may affect basement membrane-mediated haptotaxis of cell migration.

It has been shown that proteoglycans can interact with a number of ECM components such as laminin, fibronectin and collagen and facilitates the stabilization of ECM, but whether they directly affect basement membrane assembly is not known. Furthermore, it has been difficult to distinguish functions of secreted ECM proteoglycans versus the cell surface proteoglycans, due to the close proximity of the two.

By ablating the synthesis of proteoglycans specifically in neuroretina, we demonstrated that proteoglycans are essential for astrocyte migration and retinal angiogenesis. We further confirmed the indispensable role of PDGF-PI3K pathway in astrocyte migration, and developed an ex-vivo explant assay to visualize astrocyte migration towards PDGF-coated beads. We also investigated the novel function of

neuroretinal cell surface proteoglycans in the assembly of the ILM, whose integrity is a prerequisite for PDGF-A-directed astrocyte migration and retinal angiogenesis.

CHAPTER 2. MATERIALS AND METHODS

Animals

Ugdh^{flox} and *Ndst1*^{flox} mice have been previously reported (Grobe, Inatani et al. 2005, Qu, Pan et al. 2012). *Ndst2*^{KO}, *α-Cre* and *Six3-Cre* mice were kindly provided by Drs. Lena Kjellén (University of Uppsala, Uppsala, Sweden), Ruth Ashery-Padan (Tel Aviv University, Tel Aviv, Israel) and Yasuhide Furuta (M.D. Anderson Cancer Center, Houston, TX), respectively (Forsberg, Pejler et al. 1999, Furuta, Lagutin et al. 2000, Marquardt, Ashery-Padan et al. 2001). *LSL-Kras*^{G12D} mice were obtained from the Mouse Models of Human Cancers Consortium (MMHCC) Repository at National Cancer Institute (Tuveson, Shaw et al. 2004). *PDGFRα*^{flox} (Stock No: 006492), *PDGFRα*^{GFP} (Stock No: 007669) and *GFAP-Cre* (Stock No: 004600) mice were from Jackson laboratory (Bar Harbor, ME) (Zhuo, Theis et al. 2001, Hamilton, Klinghoffer et al. 2003, Tallquist and Soriano 2003). All mice were maintained in mixed genetic background and experiments were performed in accordance with guidelines of the Institutional Animal Care and Use Committee of the Columbia University Medical Center.

Histology and Cre reporter assay

Eyeballs from mice of various developmental stages were enuleated and fixed in 4% paraformaldehyde (PFA) for 5 minutes. Cuts were made in cornea with spring scissors, and retinae were carefully separated from the iris, sclera and the pigmented epithelium. Lens along with the hyaloid vessels were removed using two forceps.

To examine the expression of EGFP under pax6-α-*Cre*, four radial incisions were made on the isolated neuroretinae, which were then flat mounted with n-propyl gallate (NPG) and examined for GFP under a dissection microscope (M165 FC, Leica). Otherwise the eye balls were embedded in O.C.T. compound (#4583, Sakura Finetek) at -20°C and subject to sectioning using a cryostat (CM1950, Leica). Sections at 10μm were dehydrated with ddH2O, mounted with NPG and examined under an upright fluorescent microscope (DM5000-B, Leica).

To visualize the Cre expression in a R26R strain, β-galactosidase (β-gal) staining was conducted as described previously (Pitulescu, Schmidt et al. 2010).

Ligand and carbohydrate engagement (LACE) assay

HSPGs was detected by LACE assay as previously described (Allen and Rapraeger 2003, Pan, Woodbury et al. 2006). Briefly, enucleated eyeballs were fixed in 4% paraformaldehyde (PFA) overnight at 4°C prior to paraffin embedding. Sections at 10 μm were deparaffinized and rehydrated, followed by incubation in 0.5mg/ml $NaBH_4$ for 10 minutes, 0.1M glycine for 30 minutes and PBS washing for 3 times. After blocking with 2% bovine serum albumin (BSA) for 1 hour at room temperature, sections were incubated with 20 μM recombinant human FGF-10 (#345-FG, R&D systems), 20 μM human FGF R2α (IIIb)/Fc chimera (#663-FR, R&D systems) in Dulbecco's Modification of Eagle's Medium (DMEM, #10-013-CV, Corning) with 10% fetal bovine serum (FBS) at 4°C overnight. Cy3-labelled anti-human Fc 2nd antibody was applied the next day after PBS washing. The slides may be further processed for regular immunohistochemistry and mounted with NPG

reagent for examination. For LACE assay on whole mount retina, retinae were fixed in 4% PFA for 1 hour at 4°C, washed with PBS and blocked by 2% BSA, followed by incubation with FGF-FGFR mixture overnight and visualized by cy3-labelled anti-human Fc 2nd antibody as described above.

Immunohistochemistry

Whole retina fixed in 4% PFA or 10 μm rehydrated cryosections were blocked with 10% normal goat serum (NGS) for 1 hour at room temperature and incubated with 1st antibody overnight at 4°C. After washing with PBS, samples were incubated with 2nd fluorescent-conjugated antibody in 2% BSA for 1 hour at room temperature in dark. Isolectin GS-IB_4 (IB4) conjugated with Alexa Fluor 488 (#I21411, Thermo Fisher Scientific) was applied to visualize the vasculature. Samples were washed and mounted with n-propyl gallate (NPG) anti-fading reagent and examined under a Leica DM5000-B fluorescent microscope. Antibodies used were: anti-BrdU (G3G4, Developmental Studies Hybridoma Bank); anti-Brn3a (#MAB1585, Chemicon); anti-Chx10 (X1179P, Exalpha); anti-Col IV (#AB756P, Millipore); anti-GFAP (#Z0334, Dako); anti-Ki-67(#550609, BD Pharmingen); anti-laminin (#L9393, Sigma-Aldrich); anti-NF165 (2H3, DSHB); anti-pax2 (#RB-276P, Covance); anti-pax6 (#PRB-278P, Covance); Alexa Fluor 488 Phalloidin (#A12379, Life Technologies); anti-rhodopsin (O4886, Sigma-Aldrich); , and anti-Sox2 (sc-17320, Santa Cruz). Anti-perlecan antibody was a kind gift from Dr. Peter Yurchenco (Rutgers University, Piscataway, NJ).

BrdU staining on retinal whole mount

Mouse pups were injected intraperitoneally with 5′-bromo-2′ deoxyuridine (BrdU, #550891, BD Pharmingen) dissolved in PBS at 0.1 mg BrdU per 1 g body weight. Pups were sacrificed after BrdU pulse of 2 hours and retinae were dissected as previously described. After 2 hour fixation with 4% PFA, retinae were washed and incubated with 50% formamide in saline sodium citrate solution for 1 hour at 65 °C, followed by incubation with 2N HCl solution for 30 minutes at 37 °C. DNA denaturation was stopped by neutralizing the retina with 0.1 M Tris-HCl (pH 8.0) followed by PBS wash. Regular immunohistochemistry staining with anti-BrdU antibody was carried out as previously described. Cell proliferation were calculated as $BrdU^+$ cells versus $DAPI^+$ cells and analyzed by the student's t test.

TUNEL staining

TUNEL staining was performed with an *in situ* cell-death detection kit (#11684795910, Roche). Rehydrated cryo-/paraffin sections were incubated with 20μg/ml proteinase K for 15 min at room temperature. Sections were washed and blocked with PBS containing 3% BSA and 20% NGS for 1 hour. TUNEL reaction mixture was applied to the section. After 2 hour incubation, the sections were washed with PBS and mounted with Vectashield antifade mounting medium with DAPI (H-1200, Vector). Apoptosis rate was calculated as $TUNEL^+$ cells versus $DAPI^+$ cells.

RNA in-situ hybridization on retinal whole mounts

RNA *in situ* hybridization was performed as previously described (Pan, Woodbury et al. 2006, Cai, Feng et al. 2010).

Whole-mount RNA *in situ* hybridization was performed as previously described (West, Richardson et al. 2005). Briefly, isolated retinae were fixed in 4% PFA and dehydrated with graded methanol series. After rehydration and wash with PBS containing 0.1% Tween-20 (PBT), retinae were digested with proteinase K (10μg/ml) for 5 minutes, followed by extensive wash with PBT and radioimmunoprecipitation assay (RIPA) buffer. Postfixation was performed in 4% PFA/0.2% glutaraldehyde in PBT for 20 minutes. After 1 hour wash in hybridization buffer at 65°C, retinae were hybridized at 65°C overnight with DIG-labeled probes diluted in hybridization buffer. Unbound probes were removed by extensive wash with SSC/FA/Tween20 at 65°C, then with TBST and MABT at room temperature. After blocking, retinae were incubated with alkaline phosphatase (AP)-conjugated anti-DIG antibodies and visualized with BM purple (11442074001, Roche) according to the manufacturer's instructions.

VEGFA probe was a kind gift from Dr. Marcus Fruttiger (University College London, London, UK). *Perlecan* probe was generated from a full length cDNA clone (IMAGE:3497930). After wash with PBS, the samples were further incubated with IB4 to visualize blood vessels.

Affi-gel bead assay

Retinae from postnatal day 1 pups were dissected in DMEM, quartered to petals and flat mounted onto 0.45 µm membrane filters (#HAWP01300, Millipore) with the vitreal side facing up. Affi-Gel Blue Gel agarose beads (#1537302, Bio-Rad) pre-incubated with 100 µg/ml $PDGFA_L$ (#221-AA, R&D systems) or $PDGFA_S$ (#1055-AA, R&D systems) were carefully placed on the periphery of the retinal using forceps (**Figure 4**). Beads pre-incubated with 2% BSA were included as controls. Liquid-air interface was maintained by floating the membrane filters on DMDM supplemented with 10% FBS and cultured at 37°C with 5% CO_2. After 2 days, the retinal whole mounts were fixed with 4% PFA for 1 hour and processed for immunohistochemistry as described above.

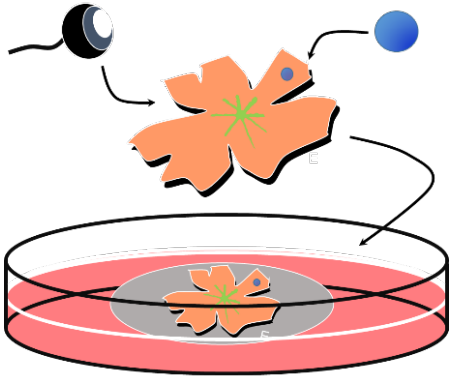


Figure 4. A diagram of Affi-gel bead assay.

Time-lapse imaging

Flat-mounted retina was placed on a 24 mm transwell insert (#3450, Corning) with vitreal side facing up. Pre-coated beads were placed at peripheral retina as previously described. The insert was placed on a 35 mm glass-bottomed imaging dish (#81156, ibidi) filled with DMEM-10% FBS and cultured in a stagetop

incubator chamber with environmental control unit (IV-ECU-HC, In Vivo Scientific) to maintain the temperature at 37°C and CO_2 level at 5%. The culture was subject to imaging with an inverted fluorescent microscope (ECLIPSE Ti, Nikon) with a Perfect Focus System (PFS) for 2 days at an interval of 10 minutes.

qRT-PCR

Total RNAs were extracted from postnatal day 3 retinal or kidney samples using TRIZOL (#15596, Life Technologies) as instructed by the product manual, and converted to cDNA using the SuperScript III Reverse Transcriptase kit (#18080, Invitrogen).

Two sets of primers were designed to detect the isoform-specific expression of *PDGFA*. The first set targets exon 6 which is absent from *PDGFA* short isoform due to alternative splicing (sense: ACCAGGACGGTCATTTACG; antisense: TTTACCTGATTCCCTACGCC); the second set targets exon 7 which is present in both long and short isoforms. (sense: GTCCAGGTGAGGTTAGAGGA; antisense: TCACGGAGGAGAACAAAGAC).

qRT-PCR was performed with the SYBR Green PCR Master Mix (4367659, Applied Biosystems) and analyzed on a StepOnePlus Real-Time PCR instrument (Applied Biosystems). Relative standard curves were generated by serial dilutions, and all samples were run in triplicates.

Matrigel reconstitution assay

Retinae from newborn pups were dissected out and digested with 20 U/ml Collagenase from *Clostridium histolyticum* (#C0773, Sigma Aldrich) alone or in combination with 1 U/ml Heparinase I and III Blend from *Flavobacterium heparinum* (#H3917, Sigma Aldrich) and/or 1 U/ml Chondroitinase ABC from *Proteus vulgaris* (ChABC, #C2905, Sigma Aldrich) in DMEM for 20 hours at 37°C with 5% CO_2. Digestion was stopped by transferring retinae to DMEM with 10% FBS. Retinae were quartered and flat mounted onto membrane filter (#HAWP01300, Millipore) with the vitreal side up. 10 µl Matrigel matrix (#356234, Corning) supplemented with 100 µg/ml laminin (#CB-40232, Fisher Scientific) was added on top of each retinal whole mount. After the Matrigel solidified, membrane filters were floated on DMEM-10% FBS and incubated at 37°C with 5% CO_2 for 2 days.

After the incubation the culture was washed in ice cold PBS for 30 minutes to remove the Matrigel, and fixed with 4% PFA at 4°C for 1 hour. Immunohistochemistry on whole mount retinae was conducted as described above. ILM reconstitution rate was quantified by NIS-Elements AR Software (Nikon) as the ratio of laminin$^+$ area versus the total area of retinal whole mount imaged. At least 3 images were taken from different regions of one retina. Data from different digestion groups were compared using one-way ANOVA.

CHAPTER 3. TISSUE-SPECIFIC REQUIREMENT FOR PROTEOGLYCANS IN ASTROCYTE MIGRATION AND RETINAL ANGIOGENESIS

Introduction

Proteoglycans are not only structural components of ECM but also active regulators of a variety of signaling pathways crucial for development (See **Chapter 1**), and most biological functions of proteoglycans are attributable to their negatively charged GAG side chains.

UDP-glucose 6-dehydrogenase (Ugdh) is a four-electron transferring oxidoreductase that converts the 6-hydroxyl group of UDP-α-d-glucose (UDP-Glc) to UDP-α-d-glucuronic acid (UDP-GlcA) in a two-step NAD^+-dependent reaction:

$$\text{UDP-Glc} + 2NAD^+ + H_2O \rightarrow \text{UDP-GlcA} + 2NADH + 2H^+$$

In animals, this pathway provides the only source of UDP-GlcA (Hempel et al., 1994), which is the donor substrates for a variety of transferases that incorporates the D-glucuronosyl moiety into the growing GAG side chains. As a matter of fact, Ugdh has been suggested to be the rate-limiting enzyme for GAG biosynthesis (De Luca et al. 1976). UDP-GlcA produced in cytosol has to be transported to the inner face of the endoplasmic reticulum/Golgi cisternal membrane to meet the downstream transferases before being added to the core protein for production of sulfated GAGs (heparin/HS, DS, KS).

Ugdh polymorphisms in human are associated with cardiac valve malformation (Hyde, Farmer et al. 2012). In mice, ablation of Ugdh led to lethality at early gastrulation phase (Garcia-Garcia and Anderson 2003).

In this chapter, by ablating *Ugdh* to block the biosynthesis of GAGs in different parts of the eye, we aim to investigate the requirement of proteoglycans for astrocyte migration and retinal angiogenesis, and how this affects the long term development and homeostasis of the retina.

Results

Neuroretina-derived GAGs are indispensable for astrocyte migration and retinal angiogenesis

Ablation of proteoglycan biosynthesis was achieved by deleting Ugdh, a key enzyme for the production of GAG side chain. Glia-specific knock out of *Ugdh* was constructed by crossing a GFAP-*Cre* line with *Ugdh*-floxed line. Surprisingly, no obvious mis-patterning of astrocyte network or vascularization defects were observed at postnatal post 3 (PD3) (**Figure 5**, B), suggesting that GAGs were not required within astrocytes for their migratory behavior. When Ugdh was ablated in the central retina in addition to astrocytes using *Six3-Cre*, however, rudimentary sprouts of blood vessels were confined to the central retina and astrocytes congregated around the optic disc, apparently unable to migrate into the peripheral retina (**Figure 5**, C). This indicates a non-cell-autonomous regulation by GAGs is essential for astrocyte migration. To further confirm this finding, we took advantage of another Cre deletor, *α-Cre*, which was restricted to the peripheral neural retina (**Figure 5**, D). Although *α-Cre* was not active in astrocytes, we again observed that astrocytes failed to invade the peripheral retina, which were instead attached with residual hyaloid vessels (**Figure 5**, D, asterisk). Taken together, these results

showed that neuroretina-derived proteoglycans were non-cell-autonomously required for migration of astrocytes and endothelial cells.

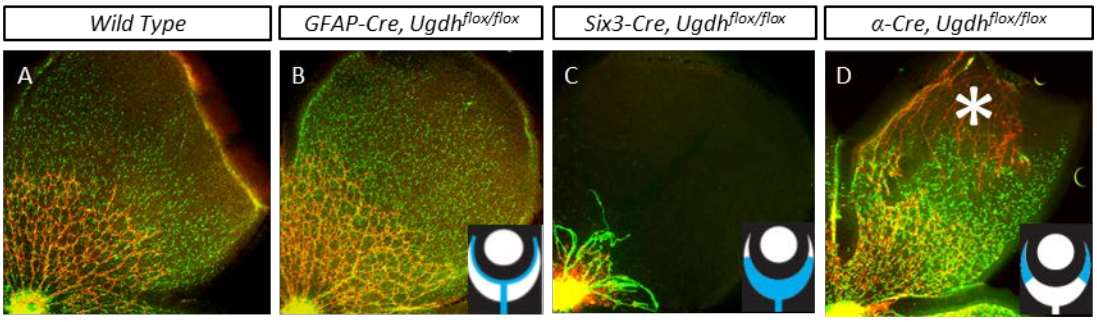


Figure 5. Cell type specific requirement for GAGs in astrocyte migration. Compared to the wild type (**A**), glia-specific knockout of *Ugdh* did not disrupt astrocyte patterning and retinal angiogenesis (**B**), while PG ablation in the central retina and the optic stalk by *Six3-Cre* (**C**) severely impaired astrocyte and vasculature development. (Inset**:** Expression pattern of different tissue-specific *Cre* in the eye. *GFAP-Cre* is expressed in astrocytes, *Six3-Cre* is expressed in the central retina as well as astrocytes, and α-Cre is expressed in the peripheral neuroretina but not astrocytes.)

Loss of GAGs in peripheral retinae of α-Cre, Ugdh$^{flox/flox}$

To understand the cause of astrocyte migration and angiogenesis defects, we focused on *α-Cre; Ugdh$^{flox/flox}$* mutants. Driven by the α enhancer of Pax6, *α-Cre* transgene starts expression from E10.5, showing a mosaic expression pattern with strong expression in peripheral retinae throughout three nuclear layers (**Figure 6,** A-B). The central retina does not express Cre and can thus serve as an internal control. A ligand and carbohydrate engagement (LACE) assay was conducted to examine the presence of functional HSPG as an indication for overall proteoglycans. Consistent with the Cre-reporter assay, LACE signal was lost in the peripheral retina

of *α-Cre, Ugdh*$^{flox/flox}$ but not in wild type (**Figure 6**, C-D'), indicating successful ablation of HSPGs in affected area.

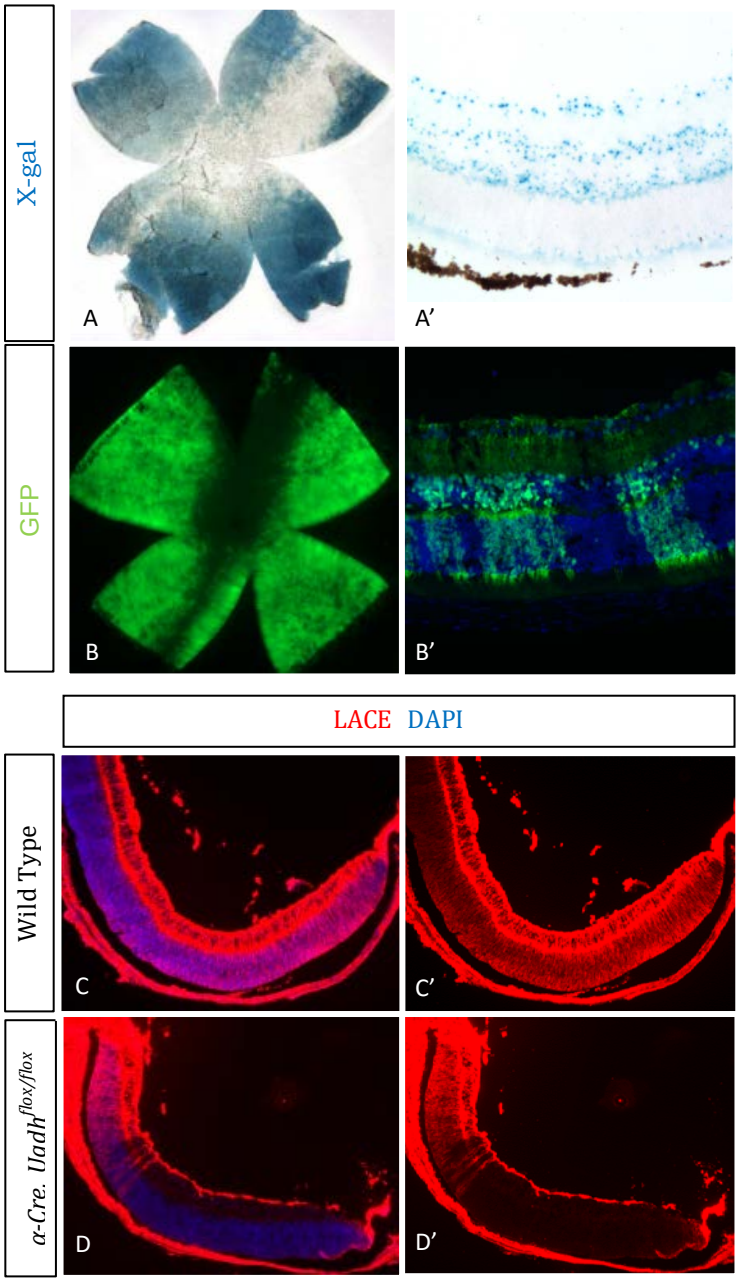


Figure 6. Expression pattern of α-Cre recombinase and loss of PGs in the peripheral retina of α-Cre, Ugdh$^{flox/flox}$
A-B. Reporter assay on whole mount retina. **C-D**. LACE assay on retinal sections.

α-Cre, Ugdh$^{flox/flox}$ exhibited impaired astrocyte migration and incomplete retinal angiogenesis accompanied by hyaloid vessels persistence

As described previously, at postnatal day 3, astrocytes in wild type retina has extended well ahead of the vasculature front and reached the edge of the retina. Hyaloid vessels in the vitreous were readily detachable during retinal whole mount preparation. In *α-Cre, Ugdh$^{flox/flox}$* retinae, however, migration of astrocytes was abruptly halted. The peripheral retina was instead attached with hyaloid vessels (**Figure 7**, A-B'). Note that hyaloid vessels can be distinguished from retinal vessels by its characteristic arterial, enlarged and less branched morphology. As astrocytes were sweeping toward the peripheral retina, BrdU labeling experiment failed to detect any obvious changes in their proliferation rate (**Figure 7**, C-E). The advancing endothelial cells, however, displayed significant reduction in BrdU incorporation as compared to wild type controls (data not shown). The proliferation and migration of retinal endothelial cells are driven at least in part by VEGFA secreted by astrocytes in front of the vascular plexus, and these astrocytes sharply downregulate *VEGFA* expression after the passing of the endothelial wave front (**Figure 7**, F-G). *Ugdh* mutant retinae instead were devoid of *VEGFA* ahead of the endothelial cells (**Figure 7**, B-B'), which correlated with lack of astrocytes in the peripheral retina. Considering the critical role of astrocytes in retinal angiogenesis, we concluded that failure of astrocyte migration can account for the vasculature defects.

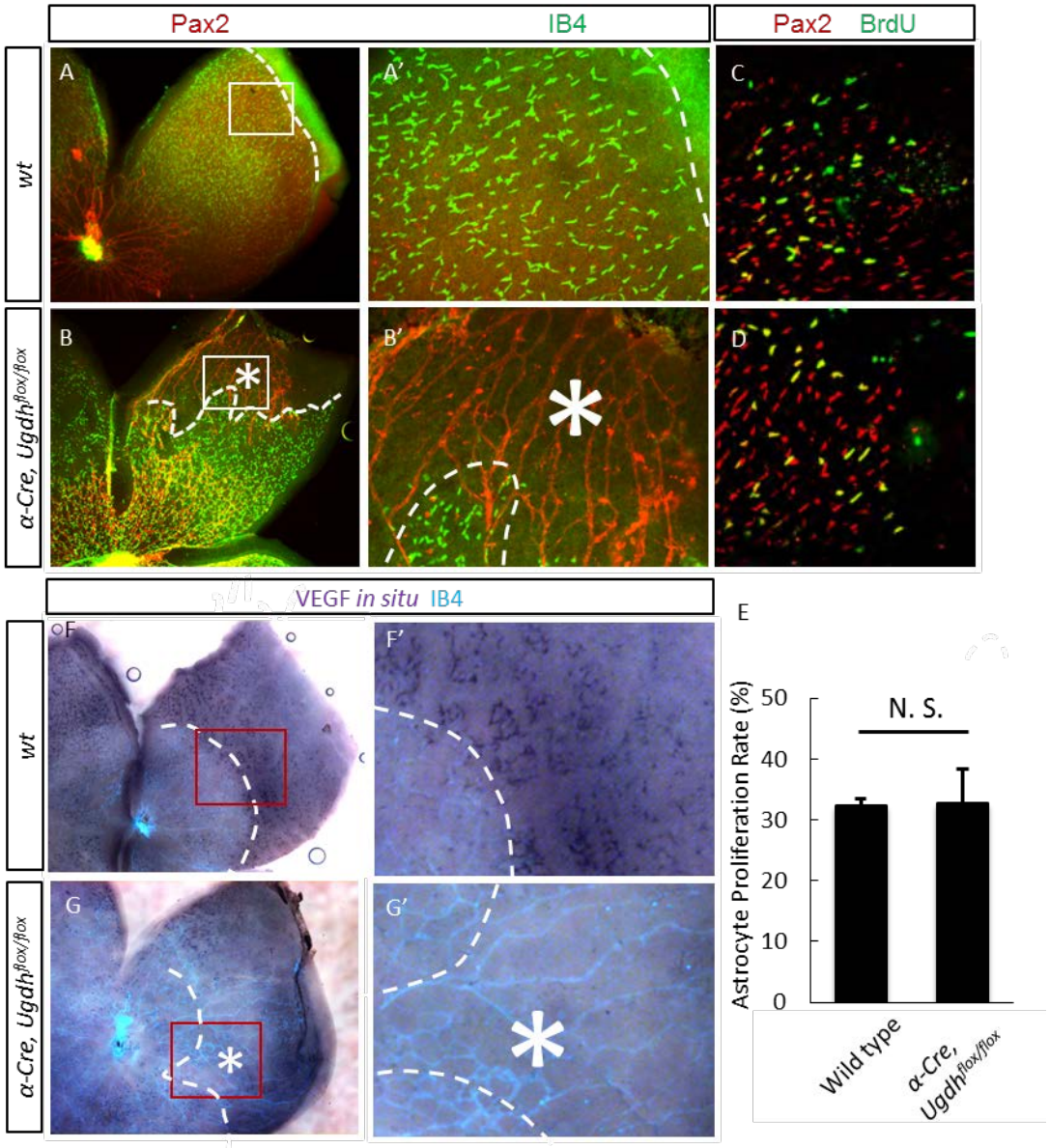


Figure 7. Characterization of α-Cre; Ugdh$^{flox/flox}$.
A-B. Astrocyte migration in a P3 α-Cre; *Ugdh$^{flox/flox}$* retina was blocked in areas occupied by hyaloid vessels (asterisk). Curvy dash demarcates the astrocyte front). **C-E**. Astrocyte proliferation was intact in *Ugdh* mutants. **F-G**. The upregulation of VEGF expression beyond the retinal vasculature front was lost in hyaloid vessel-occupied area in α-Cre; *Ugdh$^{flox/flox}$* retina. (Dashed curve: retinal vasculature front. Asterisk: persistent hyaloid vessels).

Of note, in *Ugdh* mutant, peripheral retina was never occupied by retinal vessels (**Figure 8**. A-D), and hyaloid vessels eventually fused with the incompletely developed retinal vessel, a phenotype also observed in human patients of persistent fetal vasculature (PFV) syndrome (Schwab and Schriever 1977).

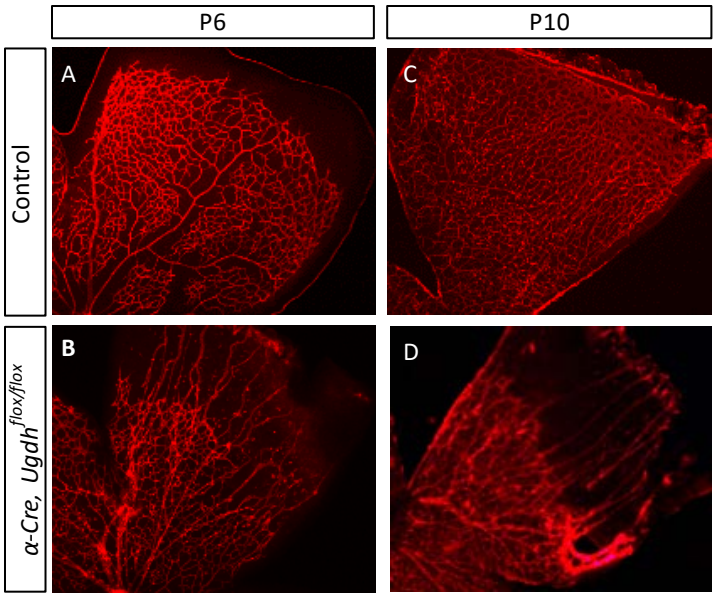


Figure 8 Vasculatures of *α-Cre; Ugdh*$^{flox/flox}$
Unlike its wild type control (**A** and **C**), the full coverage of retinal vessels at peripheral retina of *Ugdh* mutant was never achieved (**B** and **D**).

Proteoglycan deficiency causes retinal degeneration without affecting cell differentiation

Retinal neurogenesis in mammals begins with retinal ganglion, amacrine and horizontal cells born embryonically, followed by later appearance of rod photoreceptors, bipolar and Müller cells. To exclude the possibility that impaired astrocyte migration at early postnatal stage is secondary to altered retina differentiation, we examined retinae for markers of major retina cell types. At postnatal day 3 when astrocyte migration defect first became visible, α-*Cre*;

Ugdh$^{flox/flox}$ retinae appeared morphological identical to wild type controls. Using Brn3a as a marker for retinal ganglion cells, Pax6 for amacrine cells, Chx10 for bipolar cells, NF165 for horizontal cells, Rhodopsin for photoreceptors and Sox2 for Müller glia, we did not detect any cell differentiation defects (**Figure 9,** A). By P10 when the birth of retinal neurons was complete, the number of each retinal cell types remained similar to those of wild type controls, except that GFAP positive astrocytes were missing from the superficial layer in the distal retina (**Figure 9,** B, arrows). It was notable, however, there were rosettes formed in the photoreceptor cell layer throughout the peripheral retina, whereas the central retina were unaffected.

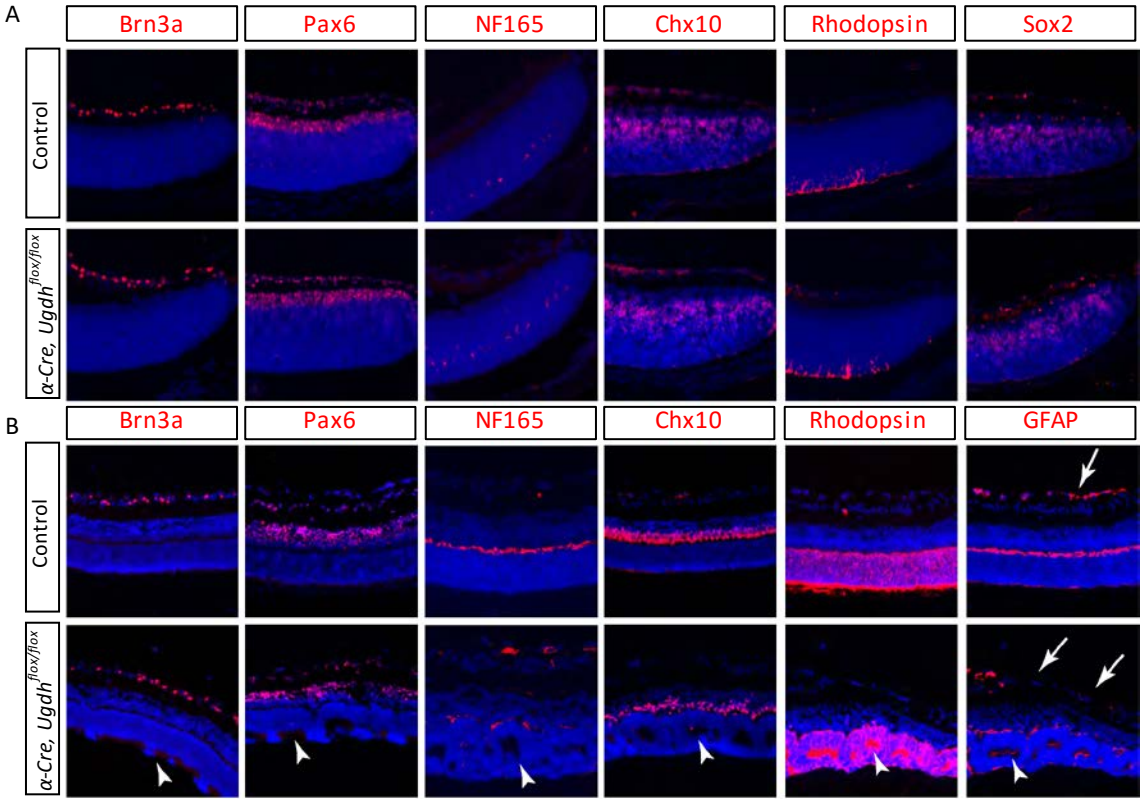


Figure 9. Gross cell fate of α-Cre; Ugdh$^{flox/flox}$ retina is unchanged.
Expression pattern of the major cell types in the retina at P3 (**A**) and P10 (**B**), during and at the end of the differentiation wave of retinal cells, respectively.

Since rosette formation is a hallmark of retinal degeneration, we next examined cell death by TUNEL staining. Physiological cell death is known to occur in retina during the first week after birth (**Figure 10**, A-B), but it should have ceased by P14. In contrast, extensive cell death was still detectable in P14 mutant retinae (**Figure 10**, C-D). As a result, the peripheral retinae in 3 months old *Ugdh* mutants were severely hypoplastic with massive cell loss (**Figure 10**, E-F). Therefore, proteoglycan deficiency resulted in extensive retinal degeneration.

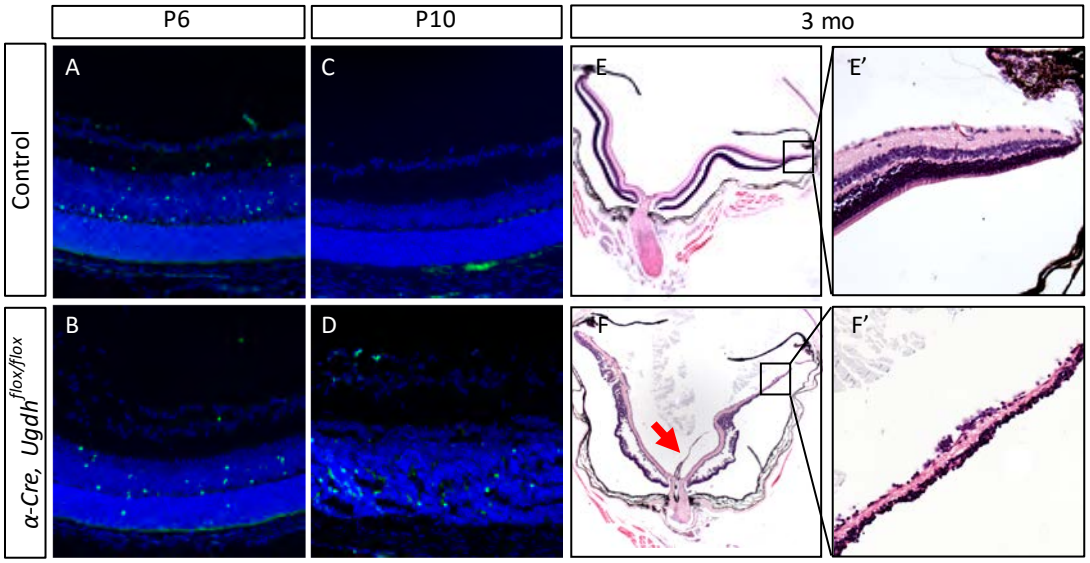


Figure 10. Abnormal apoptosis in *α-Cre; Ugdh*$^{flox/flox}$ retina at late postnatal stage led to retinal degeneration.
A-D. TUNEL staining of retinae at P6 and P10. **E-F**, H&E staining of adult retinae. Note that at 3 months old, hyaloid vessel still persisted in α-Cre; *Ugdh*$^{flox/flox}$ retina (arrow).

Reactivation of MAPK/Kras signaling cannot rescue Ugdh phenotype

We have previously shown that ablation of heparan sulfate (HS) N-sulfotransferase genes (Ndst1 and Ndst2) disrupts FGF signaling, leading to optic

stalk dystrophy and retinal degeneration. These phenotypes could be partially rescued by constitutive activation of downstream Ras-MAPK signaling. In *Ugdh* mutants, however, we did not detect any apparent loss of FGF signaling by phospho-ERK staining (data not shown). Furthermore, using an inducible allele of oncogenic Kras ($Kras^{G12D}$), we showed that constitutively active Ras signaling was unable to ameliorate astrocyte migration defects in α-Cre; $Ugdh^{flox/flox}$; $LSL\text{-}Kras^{G12D}$ retinae (**Figure 11**, A-C, asterisks). The stalled astrocytes in both mutants displayed significant increase in GFAP expression at P8 (**Figure 11**, D-F, arrows), which was also visible in the cell body and the end feet of Müller glia by P24 (**Figure 11**, G-I, arrow and arrow heads, respectively). The elevation of GFAP expression in astrocytes and Müller cells indicated massive reactive gliosis, which likely contributed to retinal degeneration in adult animals.

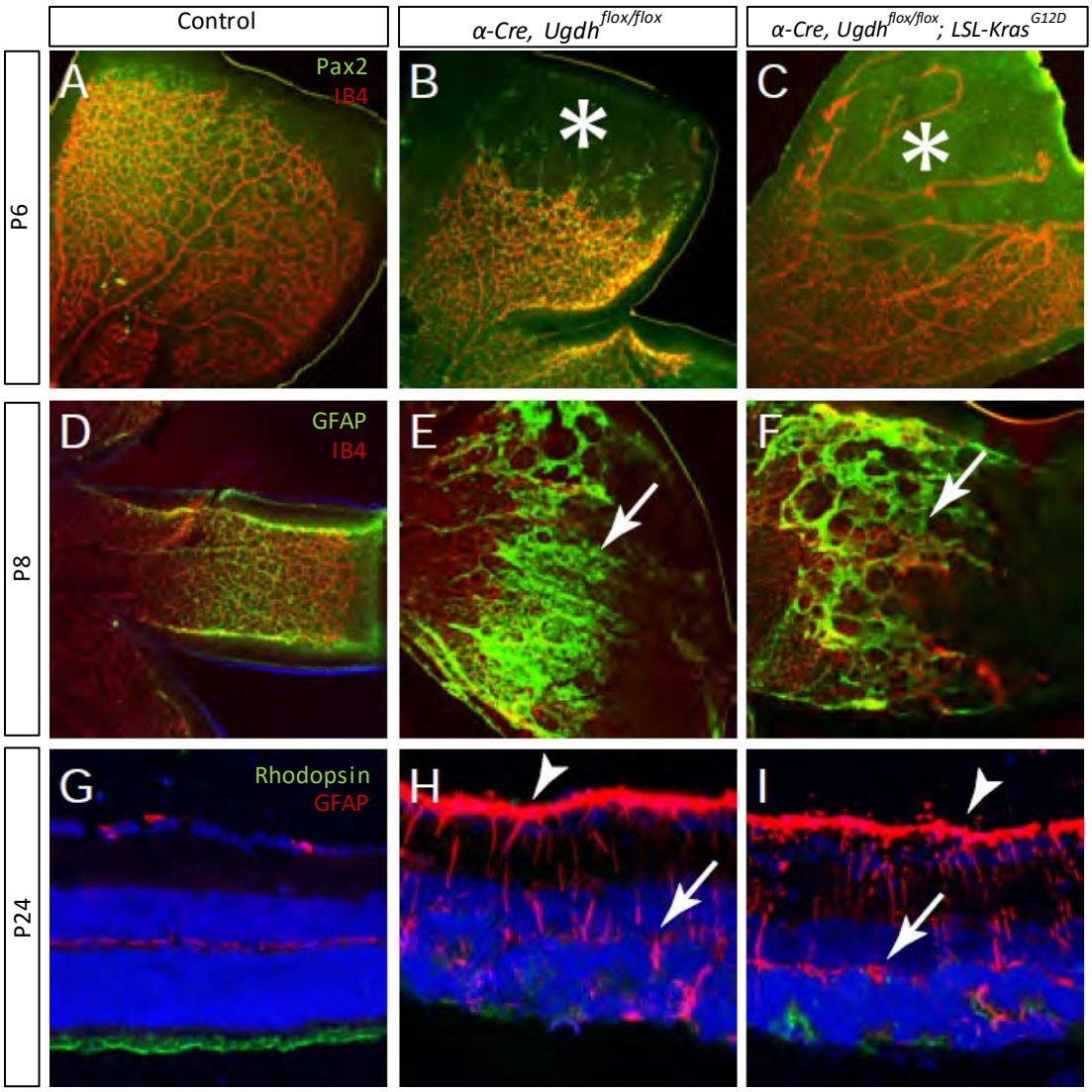


Figure 11. Constitutive activation of Ras signaling was unable to rescue astrocyte migration defects in *α-Cre; Ugdh$^{flox/flox}$*.

A-C. At early postnatal days, *α-Cre; Ugdh$^{flox/flox}$*; *LSL-Kras^{G12D}* retinae exhibited same defects in astrocyte migration, retinal angiogenesis and hyaloid vessel regression as α-Cre*; Ugdh$^{flox/flox}$*. **D-F.** Upregulation of GFAP in *Ugdh* mutant peripheral retina is indicative of reactive gliosis. **G-I**, Sections of retinae from young adult mice showing upregulated GFAP signal in *Ugdh* mutant from both astrocytes and Müller cells (arrows and arrowheads), and resettle formation accompanied with loss of Rhodopsin expression in the photoreceptor layer.

CHAPTER 4. PDGF SIGNALING IN ASTROCYTE MIGRATION

Introduction

In the previous chapter we showed that retinal angiogenesis and degeneration defects in *Ugdh* mutants were preceded by failure of astrocyte migration. This could be due to defective PDGF signaling, which has been implicated in astrocyte development (see **Chapter 1**). However, this notion was confounded by studies in which injection of PDGFRα neutralizing antibody or knockout of *PDGFA* perturbed astrocyte patterning in retina, but extensive astrocytic network still remained. Rather, retinal astrocytes from transgenic mice that overexpressed PDGFA in neurons exhibited hyperplasia but reduced migration (Fruttiger, Calver et al. 1996), indicating a mitogenic rather than chemotactic function of PDGFA.

In this chapter, we revisited the role of PDGFA in astrocyte migration. By disrupting the potential downstream PI3K pathway, we aim to dissect the mechanism underlying PDGF-directed astrocyte migration. Furthermore, we developed an ex-vivo whole mount culture system to visualize the astrocyte response to PDGF guidance cue by live imaging.

Results

Retinal astrocyte migration directed by PDGFA-PI3K pathway

To confirm the role of PDGF signaling in astrocyte migration, we used the astrocyte-specific *GFAP-Cre* to ablate PDGFRα. Whole mount immunostaining showed that while wild type retina was fully covered by an astrocytic network at postnatal day 3, only a few scattered astrocytes appeared in the center of *GFAP-Cre;*

PDGFRα$^{flox/flox}$ retina (**Figure 12**, A-B). Close examination of *PDGFRα* mutants revealed a few rudimentary vascular sprouts, which aligned precisely with isolated groups of astrocytes (**Figure 12**, B'-B''). This is consistent with the idea that retinal astrocytes provide the critical guidance cue for endothelial cells, further supporting that the angiogenesis defect in *Ugdh* retina is secondary to the mispatterning of astrocyte network.

PDGF-directed cell migration can be mediated via either phosphatidylinositol 3′ kinase (PI3K)(Onishi, Higuchi et al. 2007) or phospholipase C-γ (PLC γ) signaling (Tallquist and Kazlauskas 2004). To further elucidate the downstream mechanism of PDGF signaling, we ablated the two catalytic subunits of PI3K, P110 αβ, in astrocytes. *GFAP-Cre*; *P110α*$^{flox/flox}$*β*$^{-/-}$ retinae phenocopied *GFAP-Cre; PDGFRα*$^{flox/flox}$ in abolished astrocyte migration and vascularization (**Figure 7**. C-C'), demonstrating that PDGF-PI3K pathway is required for retinal astrocyte migration.

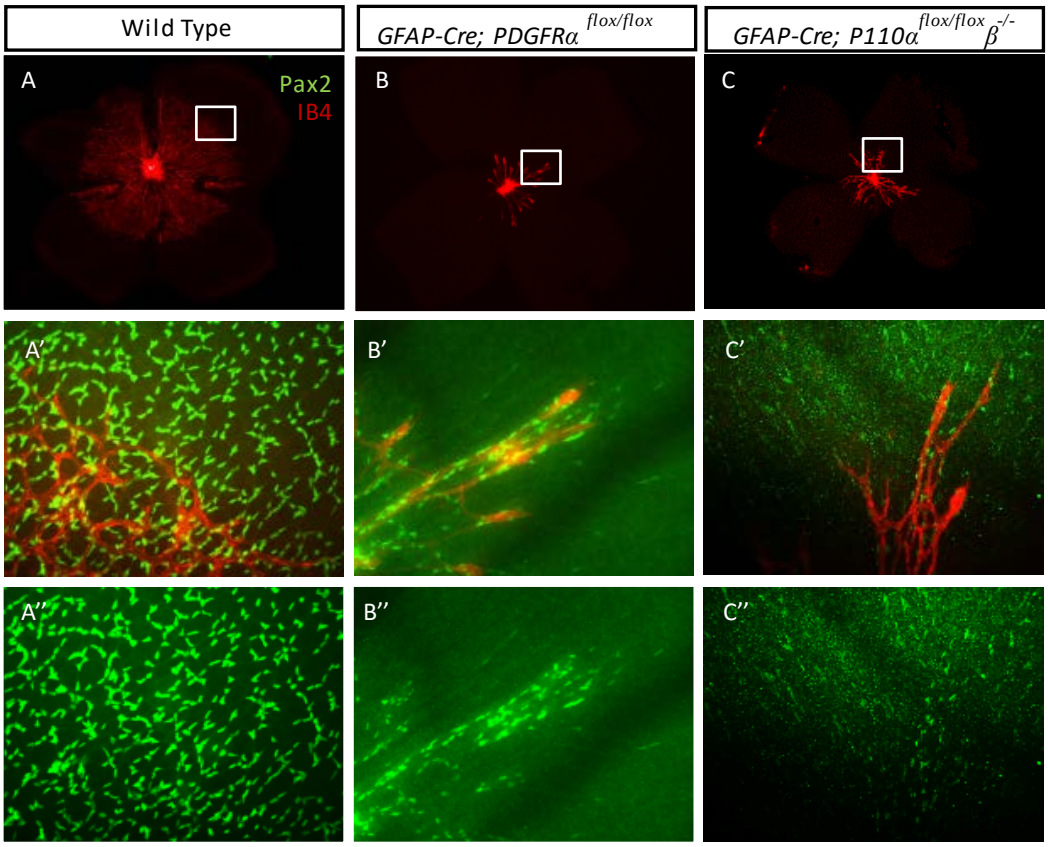


Figure 12. PDGFA-PI3K signaling is essential for retinal astrocyte migration. Ablation of PDGFRα (**B-B''**) or P110 αβ subunits (**C-C''**) in astrocytes severely impaired astrocyte patterning and subsequent retinal angiogenesis. The astrocyte network in mutants (B, C) failed to expand from the optic nerve head, while retina vasculatures could not migrate beyond the existent astrocyte network.

PDGF signaling acts independent of PGs to direct astrocyte migration

Proteoglycans may serve as co-receptors for PDGF in signal-recipient cells, but we consider this mechanism to be unlikely in the context of astrocyte migration, because astrocyte-specific ablation of GAGs in *GFAP-Cre; Ugdh*$^{flox/flox}$ retina failed to produce any phenotype (**Figure 5**, B). Another potential mechanism is that proteoglycans may bind directly to PDGF, converting it from a freely diffusible chemoattractant to a substrate-bound haptotactic signal. Indeed, murine PDGFA are expressed in two isoforms as a result of alternative splicing of exon 6, which

encodes a cell retention motif that confers binding affinity to extracellular matrix molecules including HSPGs. However, Fruttiger *et al.*, previously reported that only the "short" isoform of PDGFA ($PDGFA_S$) was present in P3 retina (Fruttiger, Calver et al. 1996). We confirmed this finding by RT-PCR using primers targeting exons 4 and 7 (**Figure 13**, A), showing that $PDGFA_S$ was expressed in brain, kidney and retina. In contrast, the "long" isoform of PDGFA ($PDGFA_L$) detectable by primers against exons 4 and 6 was not expressed in retina. This result was further quantified by qPCR, which also showed that the amount of $PDGFA_S$ was similar between wild type and *Ugdh* mutant retinae (**Figure 13**, B-C),

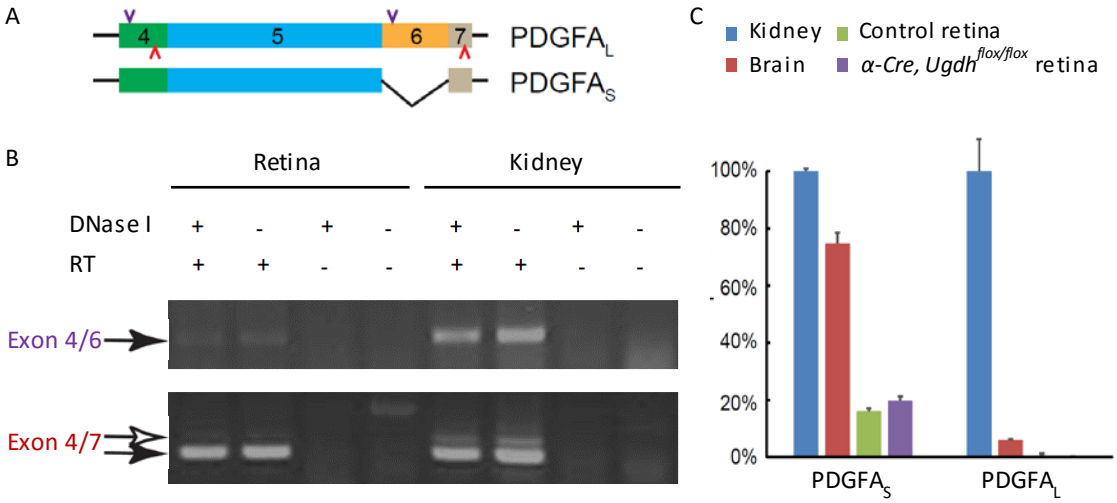


Figure 13. $PDGFA_S$ is the predominant PDGFA isoform expressed in retina. **A**. An illustration of primer design strategy for detection of long and short isoforms of PDGFA. **B-C**. RT-PCR and qPCR quantification demonstrating the dominance of $PDGF_S$ expression in the retina.

Finally, we established an ex vivo astrocyte migration assay to examine the functional requirement of PDGF isoforms, taking advantage of a $PDGFR\alpha^{GFP}$ knock-in allele that expresses GFP specifically in astrocytes. Neonatal $PDGFR\alpha^{GFP/+}$ retina

removed of the lens and the retinal pigmented epithelium was placed on a transparent filter. After insertion of PDGF soaked beads at the peripheral retina, the explant was incubated over culture medium and the GFP-expressing astrocytes were monitored by an inverted microscope (**Figure 14**, A). Time lapse imaging showed that astrocyte gradually migrated toward the PDGF beads over 48 hours, eventually forming a dense halo around the beads (**Figure 14**, C and E). As a control, FGF, EGF or BSA soaked beads failed to affect astrocyte migration (**Figure 14**, B, D and data not shown). Although $PDGFA_S$ did not have the proteoglycan binding motif, it was equally efficient as $PDGFA_L$ in attracting astrocytes (**Figure 15**, quantification shown in **Figure 18**, F). Consistent with time-lapse recording, Ki67 staining of the whole mount culture revealed that PDGFA, regardless of long or short isoforms, induced migration prior to proliferation (**Figure 9**, F-H"). Taking together, these experiments demonstrated that PDGF is necessary and sufficient to promote astrocyte migration in retina, but it does not require interaction with proteoglycans for its chemoattractive function.

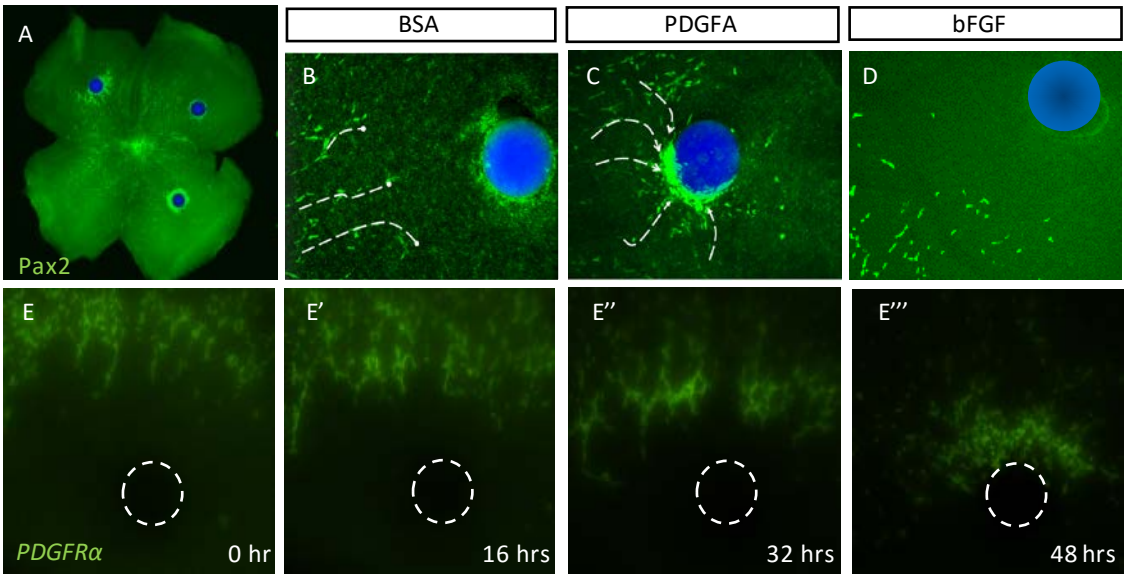


Figure 14. Illustration of astrocyte migration assay and time-lapse imaging

A. A diagram of the retinal whole mount culture with Affi-gel beads. Astrocyte response to BSA (**B**), PDGFA (**C**) and bFGF (**D**) after 2 day incubation was examined. **E-E'''**. Representative images showing astrocyte migration path in response to PDGFA on a 2-day time scale.

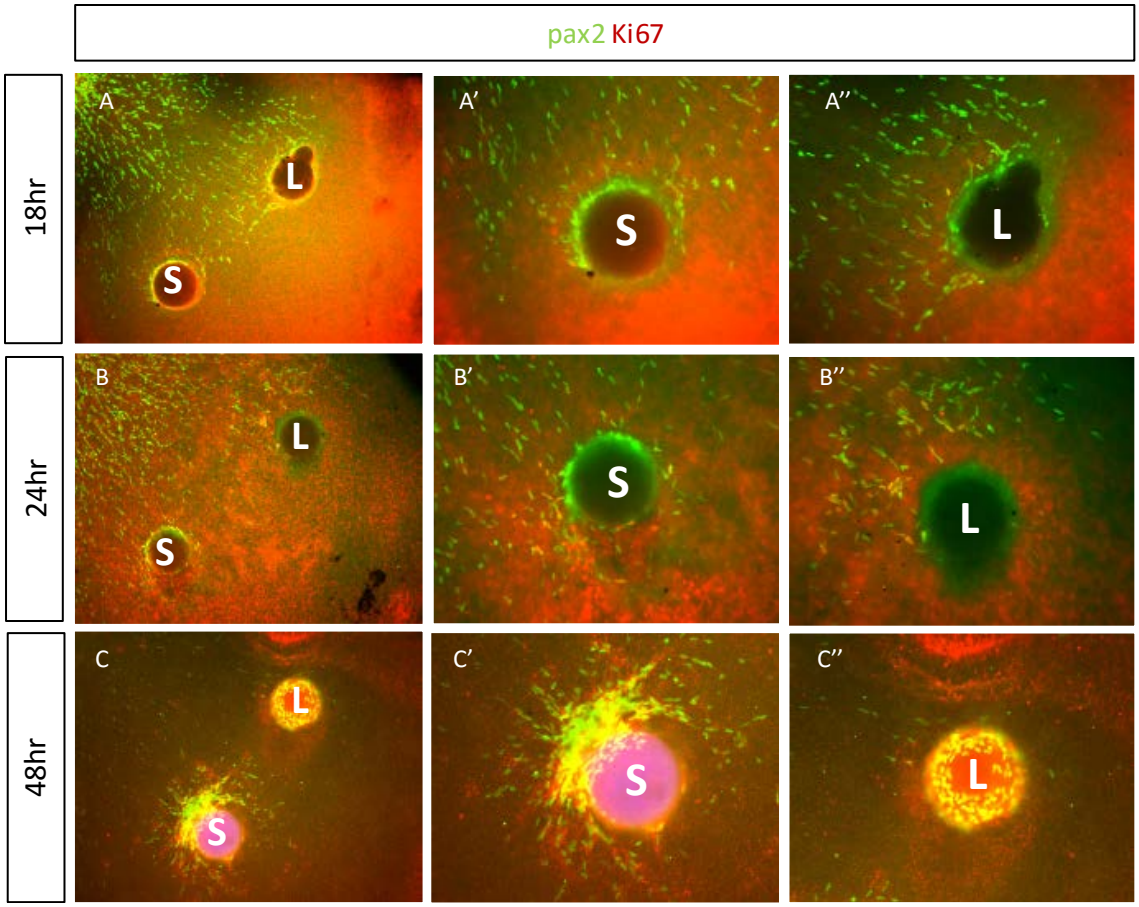


Figure 15. Both $PDGFA_L$ and $PDGFA_S$ were able to attract astrocytes.
Beads coated with either long (L) or short (L) isoforms of PFGFA were placed on the same retinal petal, and astrocytes were examined on a 2-day time scale. Migration is the dominant event during the 1st day of culture (**A**, **B)**, while on the 2nd day extensive proliferation takes place in astrocytes close to PDGF-coated beads (**C**).

CHAPTER 5. INNER LIMITING MEMBRANE ASSEMBLED BY NEURORETINA-DERIVED PROTEOGLYCANS ARE CRUCIAL FOR RETINAL ASTROCYTE MIGRATION

Introduction

Directed cell migration requires not only guidance cues but also appropriate migratory substrates. Basement membrane (BM) is a cell-associated, tightly crosslinked network of ECM, whose integrity has been shown crucial for astrocyte migration (see **Chapter 1**). Composition of BM is tissue-specific. Inner limiting membrane (ILM) is the BM of the retina that separates neuroretina from the vitreous. It includes members of the laminin family, nidogen1 and 2, collagen IV and three heparan sulphate proteoglycans: agrin, perlecan and collagen XVIII (Halfter et al., 2000).

Among all the BM components, laminin is the most fundamental one, as ablation of laminin expression led to embryonic lethality at E5.5 (Miner et al., 2004), much earlier than mutations of other BM proteins (Hohenester and Yurchenco, 2013). Deletion of β2 and γ3 chains of laminin heterotrimers led to the loss of laminin network as haptotactic factors, which subsequently impaired astrocyte migration (Gnanaguru et al., 2013).

During BM assembly, laminin polymerizes to form the first layer of the matrix, to which Collagen IV (Col IV) network is added through linkage of perlecan and nidogens. Anchorage of laminin to the cell surface through interactions with cellular receptors such as integrin and dystroglycans is essential for polymerization,

possibly by increasing the surface laminin concentration above the "nucleation threshold" (Hohenester and Yurchenco, 2013).

As we have ruled out the role of proteoglycans in PDGF-induced chemotaxis in the previous chapter, we next turned to its potential involvement in cell adhesion. Since our genetic analysis suggested that proteoglycan functions non-cell-autonomously in astrocyte migration, we asked in this chapter whether neuronal-derived proteoglycans indirectly regulate the extracellular matrix that interacts with astrocytes.

Results

The ILM is disrupted in GAG-deficient retina

To evaluate the integrity of ILM, we examined the distribution of laminin and collagen IV, two major components of the basement membrane. Whole mount immunostaining using a pan-laminin antibody (L9393) revealed a smooth sheet of laminin network in wild type retina, but the distal retina in *α-Cre; Ugdh*$^{flox/flox}$ mutants displayed many large holes, which were usually associated with persistent hyaloid vessels (**Figure 16**, arrows). The disruption of ILM was further confirmed by immunostaining of retinal sections, which showed significant gaps in both laminin and collagen IV networks (**Figure 16**, arrow heads), through which retinal cells travel ectopically to invade the vitreous humor. Taken together, these results

showed that the loss of neuroretina-derived proteoglycans disrupted the integrity of the inner limiting membrane.

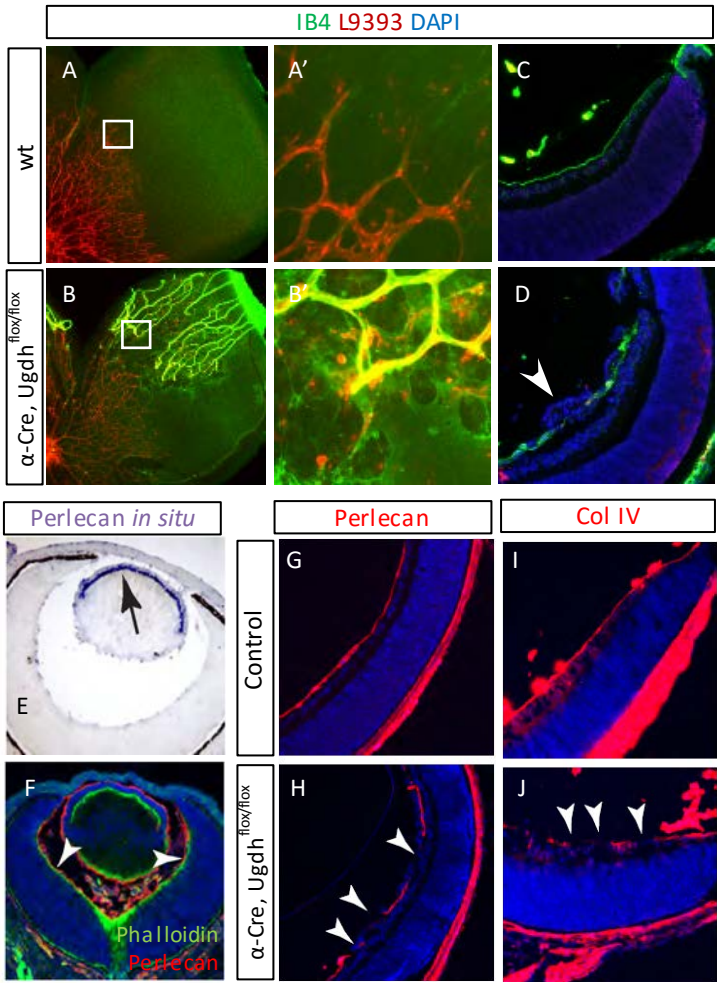


Figure 16. ILM was disrupted in PG-deficient retina.
A-D. While the laminin network in ILM was smooth and continuous in wild type retina, the ILM in *Ugdh* mutants was ruptured in hyaloid vessel-occupied area. Retinal cells escape into the vitreous body through breaks in the ILM of α-*Cre; Ugdh*$^{flox/flox}$ (arrowheads). Incorporation of two other major components of ILM, perlecan (**G,H**) and Col IV (**I, J**), were also impaired in *Ugdh* mutant. Perlecan mRNA is produced by the lens but not by the retina (**E**, arrow), despite its abundance in the wild type ILM (**F**, arrowhead).

Notably, secreted proteoglycans such as perlecan can serve as cross linkers within the basement membrane, which is especially important in mechanically stressed tissues. However, perlecan was not produced by the neuroretina itself.

Instead, it was exclusively produced by the lens, stored in vitreous and deposited onto neuroretina during ILM assembly (**Figure 16**, E, F). Hence, retinal specific knockout of *Ugdh* was not expected to affect the biosynthesis and glycosylation of perlecan.

PGs are essential in the initial assembly but not the maintenance of ILM

Proteoglycans may be regulating ILM through two mechanisms: they might be required for the initial assembly of ILM, or they might be essential for the maintenance of ILM. To test the latter hypothesis, retinae explants were incubated with different digestive enzymes. Collagenase treatment massively disrupted ILM (**Figure 17**, A). Removal of HSPG or CSPG, by Heparinase I & III or ChABC, respectively, did not impair the integrity of ILM, indicating that removal of PGs did not affect pre-existent ILM (**Figure 17**, B and data not shown).

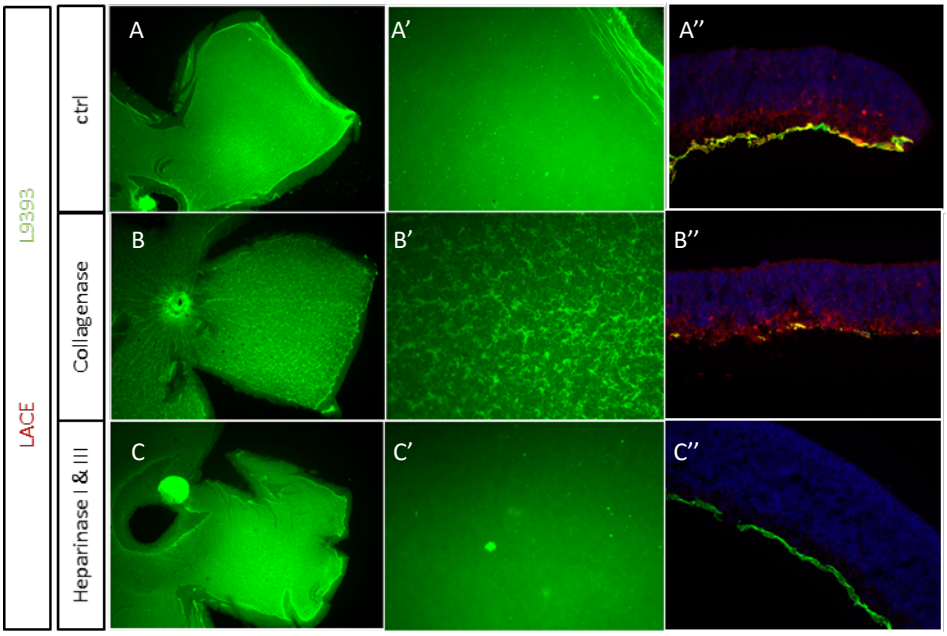


Figure 17. PGs are not required for maintenance of ILM integrity after ex-vivo enzymatic digestion.
A-B. Treatment by Collagenase massively degraded the ILM in retinal organ culture. **C.** HeparinaseI & III however, did not disrupt the existent laminin meshwork in the ILM.

To evaluate the role of retinal proteoglycans in basement membrane assembly, neonatal retinae were first removed of the ILM by Collagenase treatment, followed by incubation in Matrigel, a protein mixture rich in ECM components including laminin, Col IV, secreted HSPGs and nidogen. After 2 day incubation, immunostaining revealed a smooth sheet of laminin on top of wild type retinae (**Figure 18**, B). In contrast, *Ugdh* mutants only showed fragmented laminin staining, reminiscent of the pattern in vivo (**Figure 18**, C). This suggested that *Ugdh* deficient retina was intrinsically defective in assembling laminin network. Since Ugdh enzyme participates in the biosynthesis of both HS and CS, we next sought to identify which GAG chain was required for laminin assembly by retina. Interestingly, after removal of endogenous ILM by Collagenase, single digestion of

wild type retina with either Heparinase or ChABC only partially disrupted the reconstitution of laminin network in our assay (**Figure 18**, D, E). Combined treatment of both Heparinase and ChABC, however, resulted in numerous large holes in laminin staining, similar to what was observed in *Ugdh* mutant retina (**Figure 18**, F, G). Altogether, these data supported that retinal GAGs were important for the assembly of laminin matrix.

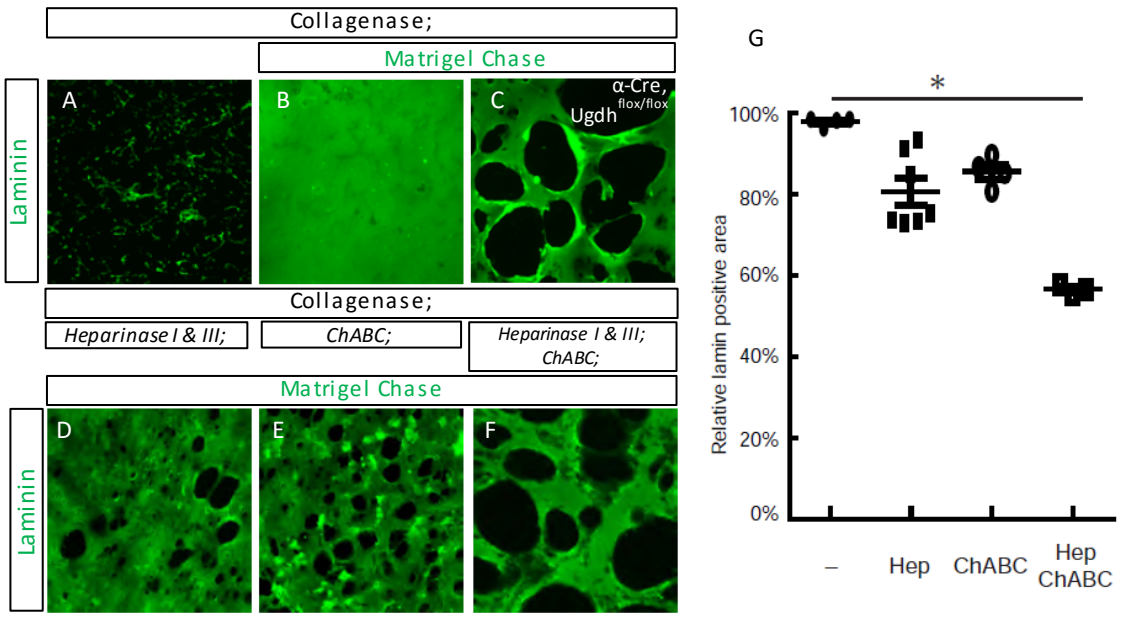


Figure 18. ILM reconstitution after enzymatic digestion of the retina.
A. Endogenous ILM was removed after digestion with Collagenase. **B**. In wild type retina, incubation with Matrigel led to a complete reconstitution of laminin sheet. **C**. Reconstitution was poor in α-*Cre; Ugdh*$^{flox/flox}$ mutant. After digestion of wild type retina with Heparinase I & III (**D**) or ChABC (**E**) in addition to Collagenase, Matrigel chase led to a partial reconstitution of ILM. Triple digestion with Heparinase I & III and ChABC and Collagenase (**F**) worsened the reconstitution, to an extent comparable to (**C**). **G.** A quantitative analysis of the reconstitution outcome.

According to our model, retinal astrocytes can sense the chemoattractive PDGF signal produced by retinal ganglion cells, but they are unable to migrate into the proteoglycan-deficient region due to lack of intact ILM as substrate (**Figure 19**,

A). It further predicts that astrocytes will traverse wild type retina unimpededly in response to exogenous PDGF, but these cells will stall immediately upon encountering proteoglycan-deficient retina. To test this idea, we performed the *ex vivo* astrocyte migration assay on *α-Cre; Ugdh*$^{flox/flox}$ retinae, followed by LACE staining to mark the boundary of proteoglycan ablation. In agreement with our model, When PDGFA beads were placed at the edge of mutant area, astrocytes migrated toward the beads as efficiently as those observed in wild type control (**Figure 19**, B and C). As PDGFA beads were moved further away into mutant region, however, astrocytes remained accumulated at the boundary of wild type retina, apparently unable to move further (**Figure 19,** D-F). The astrocyte exclusion zone coincided with the breach of ILM in mutant area, supporting our model that proteoglycan-mediated assembly of ILM is essential for migration of astrocytes.

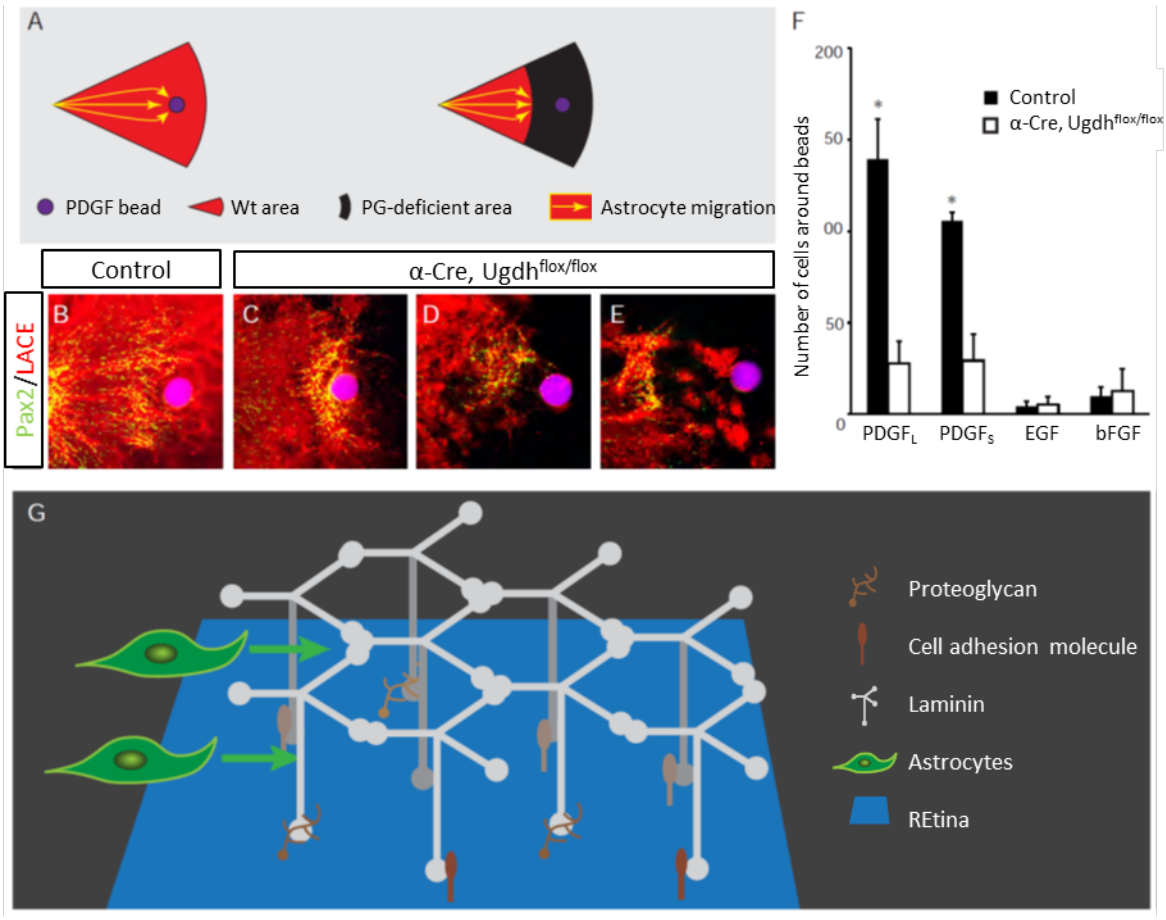


Figure 19. An illustration of our model for PG-regulated astrocyte migration

A. A diagram of astrocyte migration pattern towards PDGF-coated beads placed in different area of the retina, exemplified by wild type area (**B**), border of wild-type/mutant area (**C**), mutant area close to (**D**) and far away from (**E**) wild type/mutant boundary. **F**. Statistics of cells attracted to different growth factors are counted **G**. A model of cell surface proteoglycan regulation of ILM assembly and astrocyte migration.

Conclusions

In the classic model of ILM assembly, the function of proteoglycans lies in **1)** anchoring laminin to cell surface and **2)** bridging the two layers of laminin and Collagen IV networks. Both functions are carried out by the secreted ECM proteoglycans such as perlecan and agrin.

Herein we proposed novel functions of cell surface proteoglycans in the assembly of ILM and regulation of astrocyte migration, which cannot be compensated by secreted ECM proteoglycan or other cell surface receptors.

CHAPTER 6. DISCUSSIONS AND FUTURE DIRECTIONS

As a part of ECM, basement membrane is the frontline barrier and supportive substratum critical for adhesion, migration and survival of cells. Loss of integrity in basement membrane underlies a variety of pathologies such as hemorrhage, inflammation, neovascularization, tumor invasion and metastasis. Without attempting to be exhaustive, I would like to discuss below some aspects that might shed light on future studies in this project.

Potential proteoglycans regulating ILM assembly

In the previous chapter we proposed that cell surface proteoglycans are important for the initial assembly of ILM, and their loss cannot be compensated by the presence of secreted ECM proteoglycans.

Syndecans and glypicans are the two major families of cell surface proteoglycans, yet knowledge about them is still limited. Four syndecans and six glypicans have been identified in mammals, and they are anchored to the cell surface via transmembrane domains or glycosylphosphatidylinositol (GPI)-linkages, respectively.

While to date no ILM phenotypes has been reported in mammalian mutants of glypican (Jen, Musacchio et al. 2009), syndecan involvement in basement membrane assembly has been implied in several studies. Syndecan 2 is essential for the de-novo assembly of ECM on Chinese Hamster Ovary cells (Klass, Couchman et al. 2000). The satellite cells in syndecan-4 null mice exhibited thinning and disorganization in basal laminae (Cornelison, Wilcox-Adelman et al. 2004). General

basement membrane defects in other tissues have not been reported in any single syndecan mutation (Alexopoulou, Multhaupt et al. 2007), probably because of compensation from other members of syndecan family. In the eye, for example, syndecan-1, 2, and 4 are all expressed in the neuroretina (Inatani, Honjo et al. 2002, Koso, Iida et al. 2008, Regatieri, Dreyfuss et al. 2010). Compound knockout of multiple members of syndecan/glypican family might be necessary to reveal the functional redundancy of those cell surface proteoglycans.

In contrast to perlecan and collagen XVIII, agrin is the only ECM proteoglycan that is produced by neuroretina (Halfter, Dong et al. 2008). With binding affinity to laminin and α-dystroglycan, ECM agrin functions to anchor the laminin to the cell surface in the early phase of basement assembly. Agrin has been shown to be critical in cartilage ECM formation (Eldridge, Nalesso et al. 2016). In addition to this well studied secreted form, agrin is also produced as a transmembrane proteoglycan due to an alternative NH2-terminus (Neumann, Bittcher et al. 2001). Transmembrane agrin (TM-agrin) is abundantly expressed in CNS and has been indicated in regulating neuronal filopodia (Burgess, Skarnes et al. 2000). Considering the enrichment of TM-agrin in RGC axons and the laminin-anchorage function of its secreted counterpart, it would be interesting to investigate its involvement in ILM assembly. Again we should bear in mind that agrin might work in synergy with other cell surface proteoglycans during this process.

ILM integrity under stress

Although Heparinase/ChABC digestion of wild type retinae explant did not lead to disruption of the ILM, we cannot exclude the possibility that proteoglycans are required for the maintenance of ILM under mechanical stress. Perlecan, for example, has been shown crucial for the integrity of basement membrane in heart and cartilage where mechanical stress is frequently encountered (Costell, Gustafsson et al. 1999). The use of atomic force microcopy (AFM) would be a promising method to study the mechanical stability of proteoglycan-deficient basement membrane. Over time, the impaired integrity of basement membrane may exasperate the pathological condition induced by diseases and aging. This will be an important topic warranting further studies in neurodegenerative diseases.

It is also worth noting that, in a variety of maculopathies, ILM peeling is beneficial to prevent future retinal detachment caused by vitreoretinal tangential traction (Almony, Nudleman et al. 2012). What is curious is that the loss of ILM does not appear to impair long term retinal function at this stage, although there were clinical reports that the nerve fiber layer thickness and visual acuity were temporarily reduced (Baba, Yamamoto et al. 2012). Considering that production of the ILM components is supposed to be turned off in adulthood (Halfter, Dong et al. 2008), how the adult retina copes with the loss of basement membrane is another interesting topic worth pursuing.

Hyaloid vessel persistence

Hyaloid vessel persistence, always accompanied by incomplete retinal angiogenesis, can be caused by several different reasons. As introduced in **Chapter 1**, three isoforms of VEGF exist as a result of the alternative splicing of exon 6 and 7, giving rise to isoforms from the most diffusible $VEGF_{120}$ to the most strongly cell-associated $VEGF_{188}$. Hyaloid vessel persistence has been observed in transgenic mice that exclusively expressed $VEGF_{120}$ ($VEGF^{120/120}$) or $VEGF_{188}$ ($VEGF^{188/188}$). It was proposed that hyaloid vessel persistence in $VEGF^{120/120}$ mice was caused by the leak of soluble VEGF into the vitreous, while in $VEGF^{188/188}$ mice, hyaloid vessels were attracted to retinal surface by the matrix-associated VEGF (Carmeliet 2003).

In our *Ugdh* mutants, the simplest explanation for hyaloid vessel persistence might be that the failure of occupation by VEGF-producing astrocytes in the peripheral retina prevented the establishment of retinal vasculature in those areas, leading to increased oxygen tension. As a result, hyaloid vessels are attracted to the retinal surface in response to hypoxia. This is also consistent with our preliminary observation from an oxygen-induced retinopathy (OIR) experiment that, after exposure to hyperoxia from postnatal day 7 to 12, no persistent hyaloid vessels are observed in *α-Cre; Ugdh*$^{flox/flox}$ retinae (data not shown). However, the fact that hyaloid vessels are found to be in contact with *Ugdh* mutant neuroretina as early as postnatal day 2 casts doubt on oxygen tension as the main reason, because at this early stage even wild type retina is also devoid of astrocyte coverage in the periphery, but no hyaloid is ever observed to be tethered to the retina. In this view, the lack of hyaloid vessels in *Ugdh* mutants after hyperoxia might be due to the

obliteration of hyaloid vessels that are already attached to the retinal surface. Nevertheless, the fact that hyperoxia prevents hyaloid vessel persistence indicates that hyaloid vessel penetration is associated with hypoxia in the peripheral retina.

Col XVIII is a HSPG abundantly expressed in the ILM. Proteolytic cleavage of Col XVIII releases endostatin, a fragment with anti-angiogenesis activity (Dhanabal, Ramchandran et al. 1999). Mutations in Col XVIII/endostatin have been shown to cause defects in hyaloid vessel regression and retinal angiogenesis (Fukai, Eklund et al. 2002). Although in *Ugdh* mutant the production of Col XVIII is expected to be unchanged because they are mainly produced in ciliary body, their deposition/incorporation into the ILM is likely to be affected in the wake of the overall ILM disruption. Notably, cell surface glypicans are low-affinity endostatin receptors (Karumanchi, Jha et al. 2001). The gross abnormality of ILM, along with the loss of glypican, may impair the anti-angiogenic properties of the ILM and make the PG-deficient retina attractive to hyaloid vessel attachment.

Unlike retinal vasculatures, hyaloid vessels normally lack astrocyte coverage. In Ugdh mutant retinae, astrocyte migration stopped at the boundary of wild-type/proteoglycan-deficient region. Days later, however, astrocytes cling to the hyaloid vessel remnants in the periphery, which by that time have made connections to the native retinal vasculatures (**Figure 11**). Consistent with our hypothesis, astrocytes initially failed to migrate into the periphery of Ugdh mutant retina because of the disruption in ILM, which serves as a permissive substratum for cell migration. Astrocytes are later attracted to hyaloid vessels, maybe because of

the high expression of laminin on the latter (**Figure 16**), which serves as an alternative source of ECM and accessible path for migration. However, abnormal astrocyte migration onto the persistent hyaloid vessels was also observed in a laminin null mutant (Gnanaguru, Bachay et al. 2013). It would be thus curious to test whether any ECM components other than laminin are also enriched in hyaloid vessels as well.

Vitreous hemorrhage

Although lacking astrocyte investment, hyaloid vessels in normal conditions are not leaky (Hamming et al., 1977), probably owing to the presence of pericyte-endothelial tight junctions (Jo et al., 2013). In the Ugdh mutant, however, vessel leakage in hyaloid vessel is frequently observed (data not shown). Vitreous hemorrhage from the persistent hyaloid artery is not uncommon in human patients (Chen and Yarng 1993, Onder, Cossar et al. 2000), yet no consensus has been reached on the exact mechanism of this pathology. The simplest explanation is the rupture of the vasculatures caused by vitreous traction. Loss of tight junction during hyaloid vessel regression has also been suggested as a reason for vitreous hemorrhage (Chen and Yarng 1993). In normal conditions, blood flow ceases during hyaloid vessel regression (Jones 1963). A fluorescein angiography examination would be helpful to determine the precise site of leakage in our Ugdh mutants and the status of blood circulation in hyaloid vessels. It would also be interesting to examine the status of pericytes and tight junctions on hyaloid vessels.

Intermediate and deep plexuses

Intermediate and deep plexuses extend from the primary plexus in response to a transient expression of VEGF from amacrine and horizontal cells of the INL (Usui, Westenskow et al. 2015). Unlike their superficial counterpart, those two layers of blood vessels are not associated with astrocyte networks (**Figure 2**, B'). In *GFAP-Cre, PDGFRα$^{flox/flox}$* , intermediate and deep plexuses never arose because there lacked the superficial plexus as the sprouting source (data now shown). In α-Cre, *Ugdh$^{flox/flox}$* mutants, however, the peripheral retina sometimes does possess vasculatures sprouting into the intermediate and deep plexus layer, even in the presence of rosette formation in photoreceptor layer (data not shown). Whether those vessels originate from penetrating hyaloid vessels or are they mere extensions from normal retinal vessels from the neighboring wild type area awaits further investigation.

Migration pattern of retinal astrocytes

Astrocyte migration is a complicated process requiring both PDGF-induced chemotaxis and basement membrane, and detailed mechanism underlying this process is just beginning to be unraveled. Time-lapse microscopy with PDGF bead assay on retinal whole mount organ culture offers a great opportunity for us to visualize for the first time the dynamic movement of astrocytes at single-cell resolution.

In the future we will continue to dissect the mechanism of astrocyte migration in retina. The ex-vivo retinal whole mount culture offers a magnitude of potentials to manipulate the ILM and cell surface receptors, and we are excited to take advantage of the cell live imaging to elucidate the astrocyte behavior in response to PDGF-coated beads on those engineered substratum.

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Peer-Reviewed Publications

Qu, X., Carbe, **Tao C.,**, C., Powers, A., Lawrence, R., van Kuppevelt, T. H., Cardoso, W. V., Grobe, K., Esko, J. D. and Zhang, X. (2011) Lacrimal gland development and Fgf10–Fgfr2b signaling are controlled by 2-O- and 6-O-sulfated heparan sulfate. J. Biol. Chem. 286, 14435-14444.

Cai Z., **Tao, C.**, Li H., Ladher R., Gotoh N., Feng GS., Wang F., and Zhang X. (2013) Deficient FGF signaling causes optic nerve dysgenesis and ocular coloboma. Development. 140, 2711-23.

Li, H., **Tao, C.**, Cai, Z., Hertzler-Schaefer, K., Collins, T. N., Wang, F., Feng, G.-S., Gotoh, N. and Zhang, X. (2014). Frs2α and Shp2 signal independently of Gab to mediate FGF signaling in lens development. J. Cell Sci. 127, 571-582.

Tao, C. and Zhang, X. (2016). Retinal Proteoglycans Act as Cellular Receptors for Basement Membrane Assembly to Control Astrocyte Migration and Angiogenesis (Under review)

Review Article

Tao, C., and Zhang, X. (2014). Development of astrocytes in the vertebrate eye. Dev. Dyn. 243, 1501–1510.

Oral Presentations

"Cell Surface Proteoglycans Control Astrocyte Migration and Retinal Angiogenesis by Regulating Basement Membrane Assembly", Gordon Research Conference, Proteoglycans: Diverse Regulators of Health and Disease, Andover, NH. Jul. 2014

"Chain migration of astrocytes in retinal development", CBRG meeting, Columbia University, Dec. 2015

Poster Presentations

Tao, C. and Zhang X. "Proteoglycan Regulation of Retinal Astrocyte Migration and Angiogenesis", Gordon Research Conference "Directed Cell Migration" Galveston, TX. 2013

Tao, C. and Zhang X. "Cell Surface Proteoglycans Control Astrocyte Migration and Retinal Angiogenesis by Regulating Basement Membrane Assembly", Gordon Research Conference "Glial Biology: Functional Interactions Among Glia & Neurons" Ventura, CA 2015