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Development of Astrocytes in the Vertebrate Eye

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Abstract

Astrocytes represent the earliest glial population in the embryonic optic nerve, contributing critically to retinal angiogenesis and formation of brain-retinal-barrier (BRB). Despite of many developmental and clinical implications of astrocytes, answers to some of the most fundamental questions of this unique type of glial cells remain elusive. This review provides an overview of the current knowledge about the origination, proliferation and differentiation of astrocytes, their journey from the optic nerve towards the neuroretina, and their involvement in physiological and pathological development of the visual system.

Keywords

Astrocyte; Optic Disc; Optic Stalk; Shh; RA; Bmp; Fgf; Pdgf; Vegf; Lif; Hif; Pax2; GFAP

INTRODUCTION

Astrocytes are star-shaped glial cells that make up the largest cell population in the central nervous system (CNS) (Tsai and Miller, 2002; Molofsky et al., 2012). Initially thought to be merely the non-excitable “brain glue” that passively supports and nurtures neurons (Somjen, 1988), astrocytes are now known to participate in almost every aspect of development and function in CNS. Its functions range from the establishment of the blood–brain barrier and the maintenance of ionic homeostasis to active roles in regulating brain vascular tone, synaptic plasticity and neuronal communication (Kettenmann and Ransom, 2005; Volterra and Meldolesi, 2005; Allaman et al., 2011; Lavialle et al., 2011; Bernardinelli et al., 2014).

The abnormal differentiation and migration of astrocytes are culprits in a variety of human neuropathies (Markiewicz and Lukomska, 2006; De Keyser et al., 2008). In many parts of the CNS, astrocyte dysfunction can be fatal due to brain infiltration and aggressive growth potential of glial cells (Kleihues and Sobin, 2000). In the visual system, outcome is less life-threatening because of the confinement of astrocytes to the optic tract and the inner surface of the retina (Ramón y Cajal, 1893). However, patients may still suffer from devastating pathologies such as coloboma, optic nerve dysplasia and various retinopathies, with

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outcomes ranging from mild vision impairment to complete blindness (Friedlander, 2007). Whilst a wealth of studies have been dedicated to oligodendrocytes because of their implication in myelination of the optic nerve (Ffrenchconstant and Raff, 1986; Pringle et al., 1992; Redwine and Armstrong, 1998; Fancy et al., 2004; Ligon et al., 2006), the specific origination, differentiation and migration of astrocytes, the earliest glial cells in the optic nerve that are crucial for retinal angiogenesis (Gariano, 2003), remain enigmatic. Advances in genetic engineering and fate mapping in recent years have enabled more elaborate examination of this lineage of glial cells *in vivo*, and have provided substantial new insights. In this review, we focus on astrocytes in the optic nerve and retina during embryonic and perinatal development, a time window without interference from other neuroglia.

ORIGIN OF OPTIC NERVE ASTROCYTES

Since Cajal first described astrocytes in retina back in nineteenth century, the origin of those cells remained controversial for a long time (Ramón y Cajal, 1893). Some proposed astrocytes are transformed from the retinal Müller cells (Reichenbach and Wohlrab, 1986), just like radial glia giving rise to astrocytes in the cerebral cortex (Schmechel and Rakic, 1979; Culican et al., 1990). Alternatively, astrocytes might arise in-situ from the retinal neuroepithelium, as they do in avian spinal cord grafts (Pringle et al., 1998). Glial restricted precursors (GRPs), a specific group of $A2B5^+$ cells in the embryonic rat spinal cord, are able to generate both oligodendrocytes and $CD44^+$ astrocyte precursors *in vitro* and *in vivo* (Rao et al., 1998; Herrera et al., 2001; Liu et al., 2004). Such kind of precursors, however, has not been found in the optic system.

Much of our early understanding about cells of astrocytic lineage in the visual system can be attributed to studies with the rat optic nerve culture (Raff et al., 1983; Raff et al., 1984; Miller et al., 1989), which yielded three types of macroglials with distinctive morphological and immunolabeling hallmarks: oligodendrocytes, $Ran2^+$ $A2B5^-$ (type-1) and $Ran2^-$ $A2B5^+$ (type-2) astrocytes. *In vitro*, these three cell populations arise from two independent lineages and debut at different developmental stages (Fig. 1). $Pax2^+$ $A2B5^+$ astrocyte progenitor cells (APCs), derived from the optic stalk neuroepithelium, give rise to type 1 astrocytes upon stimulation of ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) (Mi and Barres, 1999; Mi et al., 2001), and migrate into the retina before retinal angiogenesis takes place (Ling and Stone, 1988; Watanabe and Raff, 1988; Ling et al., 1989; Huxlin et al., 1992). On the other hand, NG2+ glia precursor isolated from early postnatal optic nerve gave rise to oligodendrocytes by default and to type 2 astrocytes upon induction of CNTF in the presence of extracellular matrix, both lineages remaining excluded from neuroretina (Small et al., 1987; Ffrench-Constant et al., 1988). The existence of these bipotential glia precursors, the so-called oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells, however, has been under questioning, as attempts to isolate such precursors *in vivo* failed in the following years (Butt and Ransom, 1989; Skoff, 1990; Fulton et al., 1991).

In the wake of controversies, advanced fate mapping techniques have emerged to provide new insights into these old questions. Studies using Cre-lox based lineage tracing demonstrated that $NG2^+$ glia precursors gave rise to either oligodendrocytes or protoplasmic astrocytes, but not both, in embryonic brain and spinal cord (Zhu et al., 2008a; Zhu et al.,

2008b; Zhu et al., 2011), while in postnatal CNS they are exclusively oligogenic under normal development conditions (Dimou et al., 2008; Rivers et al., 2008; Kang et al., 2010; Zhu et al., 2011). Though a valuable tool, Cre-lox fate mapping has its own limitations: the specificity of the Cre recombinase expression; the efficacy of the Cre reporter in different cell types, and the inability to trace the origin of the differentiated cells back to a single multipotent progenitor cell population or a heterogeneous progenitor pool that shared the transcriptional control of a same promoter. In an alternative approach, a recent study using retrovirus-based clonal lineage tracing suggested that a shared multipotent progenitor cell in chick gave rise to both retinal astrocytes and oligodendrocytes (Rompani and Cepko, 2010). It is still not clear whether this conclusion can be generalized into rodents, which, unlike that of chick, lacks oligodendrocytes in retina (Meyer, 1977). The idea that there might be multiple lineages of astrocytes, however, remains possible and awaits further exploration.

PATTERNING AND MORPHOGENESIS OF THE OPTIC STALK

In vertebrates, formation of the neural component of the eye is initiated with the bilateral evagination from the anterior portion of the neural tube, giving rise to the distal optic vesicle and the proximal optic stalk (For reviews,Jean et al., 1998; Chow and Lang, 2001; Martinez-Morales and Wittbrodt, 2009; Fuhrmann, 2010). Optic vesicle further invaginates upon approaching the surface ectoderm to form the double-layered optic cup (Fig. 2). The asymmetric invagination of the neural tube leaves a temporary groove in the ventral optic cup (retinal fissure) and optic stalk (optic groove), collectively known as optic fissure (Morcillo et al., 2006). It is through this groove that the vascular mesenchyme invades the optic cup to form hyaloid vessels that nourish the developing eye during the embryonic stage. Later, lips of the lateral expanding optic cup and optic stalk margin fuse, and the interface of the two constitutes the optic disc (OD, also known as optic nerve head or ONH). Projection of retinal ganglion cell (GRC) axons through the OD into the optic stalk characterizes the formation of the optic nerve, a pathway that transmits visual information from the eye to the brain.

The optic nerve serves as the source of neuroepithelial cells from which APCs derive, thus the presence of the optic stalk is the prerequisite of astrocyte development. Optic nerve hypoplasia is almost always accompanied by impaired development of APCs and their descendent astrocytes (Dutton, 2004). A variety of signaling molecule and transcription factors are involved in optic stalk morphogenesis. In zebra fish, TGFβ family member Nodal plays crucial roles in early proximal-distal patterning of the optic vesicle (Take-uchi et al., 2003), at least partially through modulating Shh signaling pathway (Muller et al., 2000). Optic vesicles are lost or defective, state- and dose-dependently, in chick embryos treated with either Cyclopamine or a partial inhibitor of Nodal signaling (Mercier et al., 2013). In amniotes, however, the dependence on Nodal signaling in optic vesicle patterning is less clear. Optic vesicle formation is lost in Nodal deficient mice (Varlet et al., 1997; Lowe et al., 2001). Cyclopia is observed in murine postgastrulation embryos mutated in either Smad2, a downstream effector of Nodal (Heyer et al., 1999), or in Smad2/Nodal trans-heterozygous mutants with incomplete penetrance (Nomura and Li, 1998), demonstrating potential requirements of TGFβ signaling for early eye patterning.

The ventral midline derived sonic hedgehog (Shh) is crucial for the initial axial patterning of the optic cup. Germline disruption of Shh in mice leads to cyclopia, defective dorsoventral patterning and absence of the optic stalk (Chiang et al., 1996). Shh overexpression in chicks, on the other hand, results in an enlarged optic nerve (Nasrallah and Golden, 2001), similar to the mutant overexpressing BMP inhibitory protein Noggin (Adler and Belecky-Adams, 2002), indicating a convergence of Shh and BMP signaling pathway. These results suggest that the magnitude of Shh signaling must be controlled tightly to balance the proximal-distal patterning of optic vesicle. Deletion of BF-1 (also known as Foxg1), a winged-helix transcription factor required for Shh expression in the ventral neural tube, causes a complete conversion of the optic stalk into the optic cup (Huh et al., 1999). Similarly, the retinal pigmented epithelium (RPE) invades the optic stalk domain after genetic ablation of Cdo, a membrane protein that, when works in concert with Patched-1 (Ptc1), plays positive roles in Shh signaling (Zhang et al., 2009). When expressed in a Ptc1-complementary pattern in optic vesicle, however, Cdo functions as a decoy receptor, negatively regulating Shh signaling to demarcate optic stalk/retina boundary (Cardozo et al., 2014). Rb-associated E2F4 transcription factor is another negative regulator of Shh signaling. It prevents the lateral displacement of Shh expression from the ventral midline, which otherwise promotes the expansion of optic stalk markers into the optic cup domain during early eye development (Ruzhynsky et al., 2009). It is also of note that, after the waning of Shh expression from the prechordal plate, the hypothalamus serves as a secondary Shh signaling center under Sox2 and Sox3 regulation to induce optic disc formation (Zhao et al., 2012). Therefore, Shh signaling is important for defining the boundary between the optic stalk and the optic vesicle during early eye development.

Shh signaling regulates the proximal-distal pattern of eye by repressing Pax6 and promoting Pax2 and Vax in cells destined to form the optic stalk (Macdonald et al., 1995; Yang, 2004). Initially co-expressed with Pax6 in the early optic vesicle, Pax2 later retreats from the ventral retina and becomes confined to a ring of cells around OD and the parenchyma of the optic nerve (Chu et al., 2001). The critical role of Pax2 in optic stalk specification is demonstrated by transgenic mice lacking Pax2, which exhibits incomplete optic fissure closure, defective axonal pathways, heavy pigmentation of the optic stalk and optic nerve glia hypoplasia (Ulshafer and Clavert, 1979; Torres et al., 1996). Pax6, on the other hand, resides exclusively in neuroretina and RPE, playing essential roles in the optic cup specification. Ectopic expression of Pax6 arises in the optic stalk domain in Pax2 knockouts, while Pax2 expression aggresses the optic cup in Pax6 null mutants, suggesting that Pax2 and Pax6 define the boundary of optic stalk and optic cup by antagonistic interactions (Schwarz et al., 2000). This mutually exclusive pattern of Pax2 and Pax6 expression appears to be established by direct interactions between these two paired-box transcription factors and their respective ocular enhancers. Despite of the high similarity in their consensus DNA binding sequences, gene replacement experiments show that Pax2 and Pax6 have different specificities in *vivo* (Carbe et al., 2013). Pax2 is shown to bind and suppress the retinal α-enhancer of Pax6 and *vice versa*. This retinal α-enhancer of Pax6 is apparently a hot spot for transcriptional regulation, containing additional binding sites for Vax and Coup-TF family transcription factors that are important for maintaining the optic stalk/optic vesicle interface (Schwarz et al., 2000; Mui et al., 2005; Tang et al., 2010). Deletion of either Vax1/2 or

Coup-TFI/II expands Pax6 expression at the expense of Pax2, causing a complete conversion of the optic stalk to the neuroretina all the way to the diencephalon. Conversely, morpholino knockdown of Zic2a, an Shh-inducible factor in Zebra fish, causes significant expansion of Pax2a into the optic cup, suggesting that Zia2a acts in a negative feedback loop to limit Pax2a expression within the optic stalk (Sanek et al., 2009). The complementary expression of Pax2 and Pax6 are thus the common targets for a cohort of transcription factors to regulate optic nerve morphogenesis.

Independent of the Shh-Pax2 axis, retinoic acid (RA) signaling is another major pathway involved in optic nerve development. Gestational vitamin A (retinol) deficiency is known to cause microphthalmia and coloboma (Dickman et al., 1997). However, despite of the elaborate expression pattern of RA synthetic genes in the retina, only the neural crest specific knockout of retinoic acid receptors (RARs) abrogates optic nerve development, suggesting that the key target of RA signaling is the periocular mesenchyme (POM) (Matt et al., 2008). Although Vax2 and Pax2 are unaffected, Pitx2 expression in the POM is lost. Since deletion of Pitx2 in the neural crest reproduces the optic nerve dystrophy associated with RAR mutants (Evans and Gage, 2005), Pitx2 must be a downstream effector of RA signaling in non-cell-autonomous regulation of optic nerve development. It has been shown that Pitx2 induces Dkk2 to suppress canonical Wnt signaling in the anterior segment of the eye (Gage et al., 2008; Kumar and Duester, 2010), but whether the RA-Pitx2 axis controls a similar paracrine signaling in optic nerve development remains unknown. Interestingly, the germline knockout of AP-2α also displays a failure of optic stalk extension, accompanied by aberrant Pitx2 expression in the POM (Bassett et al., 2010). This is compounded by the expansion of Pax2 expression into the optic cup driven by increasing Shh signaling activity, resulting in the conversion of the RPE to the neuroretina and optic stalk-like tissue. However, since none of these phenotypes were reported in the neural crest-specific deletion of AP-2α, how AP-2α modulates periocular Pitx2 and midline Shh activities remains a conundrum.

ASTROCYTES IN THE OPTIC DISC

Pax2-expressing precursor cells enclose retinal ganglion cell (RGC) axons as a cuff at the interface of the fusing optic fissure and optic cup, giving rise to a unique structure called the optic disc (OD). Previously thought to be an extension of optic stalk cells into the eye cup (Otteson et al., 1998), cells in the OD are now believed to exhibit unique identities of their own, characterized by an overlapping expression of the axon guidance molecule (Netrin 1), the optic stalk markers (Pax2, Vax1) and the ventral retina factors (Vax2, Raldh3) (Morcillo et al., 2006) (Fig. 2). In addition, OD precursors are lost in *Bmp7*-null eyes but expanded in mouse mutants for Bmp antagonist *Smad7*, indicating a direct dependence of these cells on Bmp signaling (Morcillo et al., 2006; Zhang et al., 2013).

Since the observation of higher optic nerve astrocyte proliferation in *Bcl2* transgenic mice which have more RGC axons than their wild-type littermates (Burne et al., 1996), considerable evidence has implicated axon-derived factors in promoting glial cell development (Fields and Stevens-Graham, 2002). One of the major RGC-derived factors was later identified as Shh. The period from E12 to E14 marks the peak of the differentiation

of optic stalk neuroepithelial cells into astrocyte progenitor cells in rats (Kuwabara, 1975). This time window is coincident with the onset of *Shh* expression from rapid differentiating RGCs as well as uniform *Patched* expression in the optic nerve (Dakubo et al., 2003). Although the exact mechanism is not fully understood, it is suggested that Shh from postmitotic RGCs is anterogradely transported into the optic nerve (Wallace and Raff, 1999; Wang et al., 2005a; Dakubo et al., 2008). Conditional ablation of Shh in RGCs results in optic nerve hypoplasia and pigmentation, and a complete loss of OD astrocyte precursor cells, demonstrating a role of RGC-derived Shh in optic nerve astrocyte development (Dakubo et al., 2003; Dakubo and Wallace, 2004; Wang et al., 2005b). It was later shown that BMP and Shh signaling may converge upon Tlx, a member of the *tailless* class of orphan nuclear receptors, reducing its binding on Pax2 promoter to relieve Tlx repression of Pax2 (Sehgal et al., 2009). In the perinatal optic stalk, RGC-derived Shh also promotes astrocyte proliferation via transcriptional control of cell cycle gene *Ccnd1* and *Cdc25b* (Dakubo et al., 2008). In addition, Shh may also induce *Pdgfra* expression in optic disc astrocyte precursor cells, as the peak of *Gli* expression by the $Pax2^+$ optic disc cells coincides with their initial expression of *Pdgfra*, and Gli has been shown to directly activate the expression and phosphorylation of *Pdgfra* in cell lines (Xie et al., 2001; Dakubo et al., 2003).

More recently, we and others have identified fibroblast growth factor (FGF) signaling as another critical pathway for the patterning of the optic fissure, optic disc and optic nerve (Cai et al., 2013; Chen et al., 2013). Our studies demonstrated that double knockout of fibroblast growth factor receptor-1, −2 (Fgfr1/2) does not abrogate BMP or the midline derived Shh signaling, nor does it disrupt the axial patterning of the optic vesicle (Cai et al., 2013). Instead, FGF signaling at the proximal eye cup regulates Pax2 and Mitf expression, thus promoting the APC fate versus the RPE fate. This is reminiscent of the role of FGF signaling in promoting the expression of Chx10 at the expense of Mitf expression in the optic vesicle, biasing the retinal progenitors toward the neuroretina instead of the RPE fate (Nguyen and Arnheiter, 2000; Horsford et al., 2005; Cai et al., 2010). A combined deletion of fibroblast growth factor receptor substrate 2 (*Frs2a*) and protein tyrosine phosphatase *Shp2* phenocopies ocular coloboma, optic nerve hypoplasia/pigmentation and loss of the OD in *Fgfr* knockout, confirming their essential roles in mediating FGF downstream signaling (Cai et al., 2013). On the other hand, genetic depletion of heparan sulfate proteoglycans, the co-receptors for FGF, disrupt the formation of the OD, but not the closure of the optic fissure, showing that OD dysgenesis is separable from optic coloboma (Cai et al., 2014). In both cases, the ocular defects can be ameliorated by constitutional activation of ERK by Ras signaling. Thus, Ras-ERK signaling is the main target of FGF signaling in OD development.

EXPANSION OF ASTROCYTES INTO NEURORETINA

In rodents, cells of the astrocytic lineage migrate into retina through the ONH as a mixture of precursor cells and immature perinatal astrocytes, and then spread across the nerve fiber layer towards peripheral margins of the retina. On this journey the 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 et al., 1985; Orentas and Miller, 1996; Chu et al., 2001; Chan-Ling 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 (Fig 3A).

The migration of astrocytes is closely regulated by neuroretina. Several lines of evidence have suggested that PDGF-A secreted by RGCs is critical to the patterning of the retinal astrocyte network. PDGF receptor α (PDGFRα) expressing cells at the ONH do not start to migrate towards peripheral retina until *PDGF*-A mRNA is detected in RGCs (Mudhar 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 et al., 1996). Overexpression of PDGF-A 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 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, PDGF-A 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.

Neuroretina also provides the permissive extracellular matrix essential for astrocyte migration. In a microchemotaxis assay, type 1 astrocytes from rat brain migrated toward lower chamber filled with laminin (Armstrong et al., 1990). Genetic deletions of the laminin α1, β2 and γ3 chains in retina disrupt astrocyte migration and spatial distribution (Edwards et al., 2010; Gnanaguru 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 et al., 2013).

The migration of astrocyte into neuroretina is followed by the invasion of endothelial cells to form the retinal vasculature, which in turn promotes astrocyte differentiation (Fig. 3B, C). Endothelial cells are the main sources of LIF, which has been demonstrated to stimulate astrocyte differentiation *in vitro* (Yoshida et al., 1993; Nakagaito et al., 1995; Richards et al., 1996; Bonni et al., 1997; Mi and Barres, 1999; Galli et al., 2000). It is consistent with observation that mice lacking LIF receptors have impaired astrocyte differentiation (Koblar et al., 1998). Together with BMP2, LIF induces astrocyte differentiation *in vitro* by augmenting promoter activation of GFAP, a key marker for dedicated astrocytes (Nakashima et al., 1999). Despite the promising role of LIF in *ex-vivo* studies, LIF-deficient mice show a more subtle astrocytic defect than LIF receptors mutants (Kubota et al., 2008), indicating that additional cytokines may be involved in inducing astrocyte differentiation. In culture, APC differentiation can also be induced by CNTF, while type 1 astrocytes

proliferate in response to epidermal growth factor (EGF) (Raff et al., 1983; Purrello et al., 2002) and bFGF (Nakatsuji and Miller, 2001). However, the *in-vivo* roles of these factors have not been established.

The arrival of endothelial cells also relieves the oxygen tension in retina, indirectly promoting astrocyte maturation. Indeed, hypoxia-inducible factor 1 alpha subunit (HIF-1α) in neuroretina, especially retinal progenitor cells, has been implicated in inducing both PDGF-A and VEGF to regulate astrocyte and vasculature network (Nakamura-Ishizu et al., 2012). Although HIF-1α is not required in astrocytes, astrocyte-specific knockout of HIF-2α resulted in partial development of astrocytic network, dramatic loss of primary retinal vasculature and failure of hyaloid vessel regression (Duan et al., 2014). It is thought that hypoxia-sensing in astrocytes maintains a reservoir of astrocyte progenitors by preventing APCs from precocious differentiation. It is notable that oxygen deprivation positively regulates Tlx, which is transiently expressed in migrating retinal astrocytes (Miyawaki et al., 2004). Tlx knockout mice exhibit delayed astrocyte migration, defective fibronectin assembly, sharply increased GFAP expression and an absence of retinal vasculature, resembling HIF-2α mutant phenotypes (Uemura et al., 2006). It would be interesting to investigate whether Tlx is a downstream target of HIF-2α in astrocyte migration and maturation.

CLINICAL IMPLICATIONS

Astrocytes in the retina, like in other parts of CNS, are actively involved in a variety of developmental and pathological conditions. Mammalian eye provides an excellent model for the study of astrocytes in CNS, as the retina shares a common origin with the brain. Visualization of astrocytes, which are confined to the optic nerve and nerve fiber layers of the inner retina and yet in close proximity to retinal vasculatures (Ramón y Cajal, 1893), offers unique advantages for studying interactions between neurons, glial cells and vessels (Chanling, 1994). Furthermore, their appearance before the postnatal emergence of oligodendrocytes and Müller cells makes it possible to study astrocytes without interference of other glial lineages. Cerebrovascular diseases can also be reflected in retinal vasculature changes (Mitchell et al., 2005; Patton et al., 2005), suggesting that studies of retinal astrocyte may shed light on our understanding of development and pathologies of glial cells in other regions of the CNS.

One of the most well studied aspects of astrocyte function is the requirement of the pre-existing astrocyte network for retinal angiogenesis (Gariano, 2003). Astrocytes are found ubiquitously in richly vascularized retinas from 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 support of this idea, overexpressing PDGF resulted in an increase in retinal vasculature, proportional to the extent of astrocyte hypertrophy (Fruttiger et al., 1996). In anencephalic human fetus, elevated apoptosis in ganglion cell layer was accompanied by reduced astrocyte density and attenuated retinal vasculature (Kim et al., 2010). It is thought that the development of retinal vasculature is driven by VEGF produced by astrocytes beyond the vasculature front (Stone et al., 1995; Dorrell et al., 2002). Despite of the elegance of this prevailing model, some discrepancies have arisen recently. In mice

ablated of *brn3b* (Sapieha et al., 2008) or *Math5* (Edwards et al., 2012) where RGCs were mostly depleted, the retinae were completely devoid of vascular plexus, accompanied by persistent hyaloid vessels. The astrocytic networks in those retinae, however, are retained. Loss of VEGF production in astrocytes did not impair, or exhibited only minor impact on, normal development of retinal vasculature (Scott and Fruttiger, 2010; Weidemann et al., 2010), indicating that astrocyte-derived VEGF is largely dispensable for retina neovasculation. This might be explained by the compensatory expression of VEGFA from Müller cells, or by Norrin/Fz4 signaling pathway (Wang et al., 2012; Zuercher et al., 2012), which also participated in regulation of angiogenesis in the superficial retina.

Another major role of astrocyte in retinal angiogenesis is to assemble the extracellular matrix to guide the movement of endothelial cells. It has long been recognized that migrating endothelial cells closely align with the existing astrocytic network, suggesting that the direct interaction between astrocytes and endothelial cells is important for retinal angiogenesis. Although deletion of either fibronectin from astrocytes or its receptor α5β1 integrin from endothelial cells impairs endothelial tip-cell adhesion to the astrocytic network, retinal angiogenesis appears normal (Stenzel et al., 2011). Depletion of both fibronectin and heparan sulfates in astrocytes, however, delays the migration of endothelial cells to the similar extent as the removal of the cell surface retention motif in VEGF, suggesting that astrocytes play a major role in maintaining the chemotactic gradient of VEGF (Ruhrberg et al., 2002; Stenzel et al., 2011). It is further shown that αvβ8 integrin signaling in astrocytes is also necessary for the release of latent TGFβ from extracellular matrix, which regulates endothelial cell sprouting in retinal angiogenesis (Hirota et al., 2011). Nevertheless, the angiogenic defects in all these mutants are considerably weaker than those observed in Tlx and HIF-2α mutants described in the previous section, suggesting that there may exist additional mechanisms by which astrocytes regulate retinal angiogenesis.

Retinopathy of prematurity (ROP) is one of the most common vision-impairing diseases in childhood (Smith, 2004). A ridge of clustering cells forms in response to elevated oxygen delivered to premature infants, demarcating the vascularized retina and the avascular periphery at stage 2. Cells in the ridge were later identified as mainly hyperproliferating astrocyte precursor cells (Gariano, 2010). Neovascularization occurs ahead of the ridge after the baby is sent back into normoxia environment, resulting in fragile and leaky retinal vasculatures that, upon further progression, lead to the detachment of retina and even vision loss (Tasman et al., 2006). Targeted laser ablation of the accumulating astrocytes in the ridge can thus be a potential therapy to alleviate neovascularization (Steinmetz and Brooks, 2002; Ells et al., 2013).

In addition to the induction of retinal vasculature, astrocytes, along with Müller cells, are also crucial for maintaining the integrity of the blood-retinal-barrier (BRB) (Hollander et al., 1991; Tout et al., 1993; Gardner et al., 1997). In cyclic hyperoxia, death of astrocytes precedes neovascularization and vessel leakage into the vitreous humor (Zhang and Stone, 1997). Changed astrocyte reactivity is linked to BRB breakdown in ROP (Chan-Ling and Stone, 1992; Stone et al., 1996) and diabetic retinopathy (Rungger-Brandle et al., 2000; Ly et al., 2011). Increasing expression of water channel protein aquaporin-4 (AQ4) in

astrocytes, for example, contributes to astrocyte swelling and elevated retinal vasculature permeability under hypoxia (Kaur et al, 2007). Protecting retinal astrocytes from degeneration is thus an important venue of research in oxygen-induced retinopathy (Dorrell et al., 2010).

In summary, retinal astrocytes are important for eye development and homeostasis. They attract endothelial cells to guide the retinal vasculature and play important roles in neural protection and degeneration. Further study of these versatile components of the eye will yield significant insights into the ocular development and diseases.

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List of abbreviations

APC	astrocyte progenitor cell
BMP	bone morphogenetic protein
BRB	brain-retinal-barrier
CNS	central nervous system
CNTF	ciliary neurotrophic factor
EGF	epidermal growth factor
FGF	fibroblast growth factor
GFAP	glial fibrillary acidic protein
GRP	glial restricted precursor
HIF	hypoxia-inducible factor
LIF	leukemia inhibitory factor
OD	optic disc
ONH	optic nerve head
O-2A	progenitor cells: oligodendrocyte-type-2 astrocyte progenitor cells
PDGF	platelet-derived growth factor
POM	periocular mesenchyme
RA	retinoic acid
RGC	retinal ganglion cell
ROP	retinopathy of prematurity
RPE	retinal pigmented epithelium
Shh	sonic hedgehog

VEGF vascular endothelial growth factor

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Key findings

- Astrocytes are major component of the optic stalk and contribute to retinal angiogenesis.
- Development of the optic stalk and disc is regulated by Shh, RA, Bmp and Fgf signaling.
- Astrocytes migrate into neuroretina to form a template for later vascular network.
- Reciprocal interaction between astrocytes and endothelial cells controls angiogenesis and astrocyte maturation.

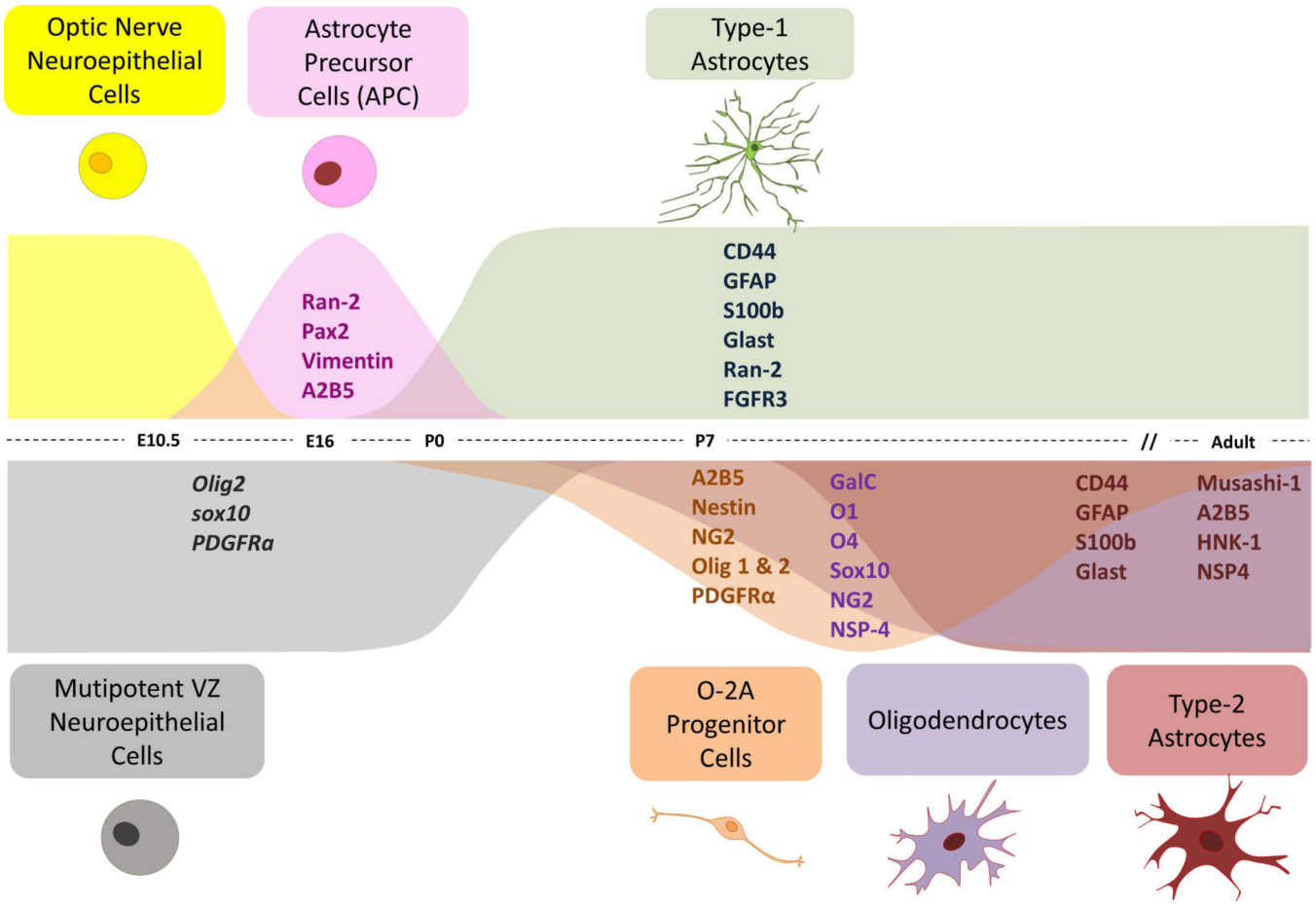

Figure 1. Macroglia lineages in the optic stalk

Type-1 astrocytes are derived from astrocyte progenitor cells (APCs) arisen from the embryonic optic nerve neuroepithelial cells. A common cell lineage of O-2A progenitors derived from ventricular zone (VZ) neuroepithelial cells give rise to both oligodendrocytes and type-2 astrocytes in postnatal optic stalk. Markers for each cell type are indicated.

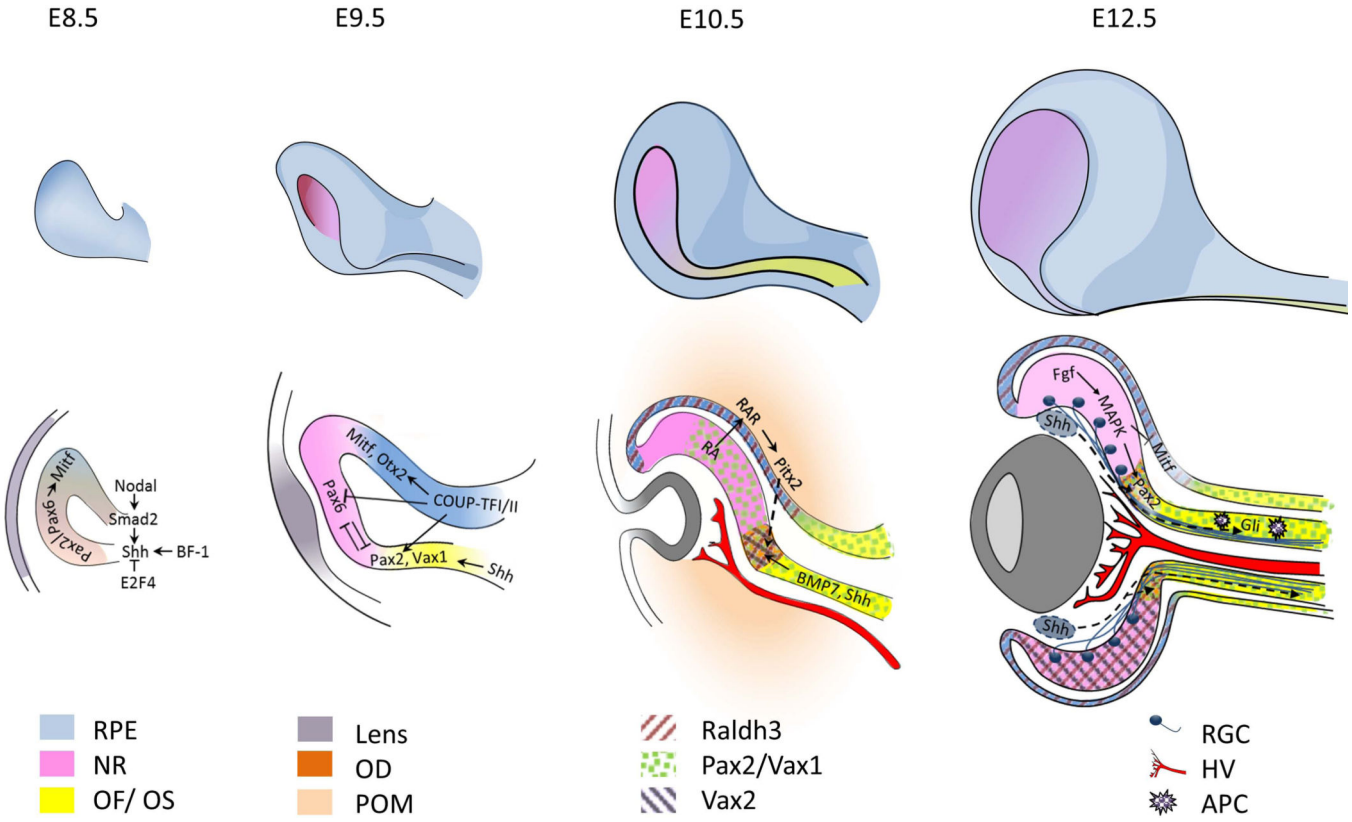

Figure 2. Patterning of the optic stalk and disc

The upper panel depicts the morphogenesis of the optic vesicle. The asymmetrical invagination of the optic vesicle results in the optic fissure at the ventral side, which eventually fuses from the posterior optic stalk to the anterior optic cup. The lower panel shows the cross section of the eye marked with gene regulatory network. RPE, retinal pigmented epithelium; NR, neuroretina; OF/OS, optic fissure/stalk; OD, optic disc; POM, periocular mesenchyme ; RGC, retinal ganglion cell; HV, hyaloid vessel; APC, astrocyte progenitor cell.

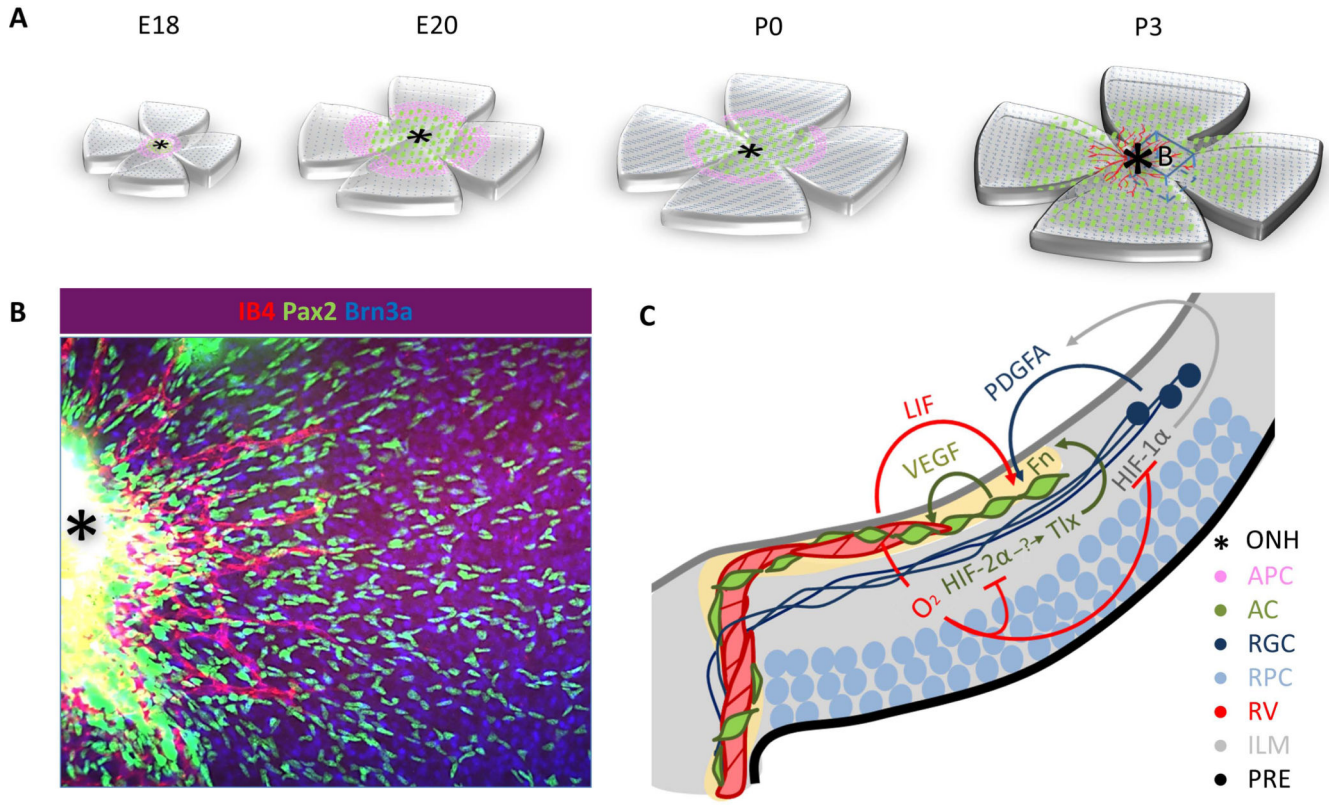

Figure 3. Astrocyte migration and interactions with neuroretina and vasculature

(A) The migration of astrocytes into retina is pioneered by astrocyte progenitor cells (APCs, pink) from the optic nerve head (ONH, marked by asterisk), which differentiated into mature astrocytes (green). This is followed by invasion of endothelial cells (red) that form the retinal vasculature. **(B)** Postnatal day 3 (P3) mouse retina shows astrocytes marked by Pax2 (green), endothelial cells by IB4 staining (red) and retina ganglion cells by Brn3a (blue). **(C)** Interplay among astrocytes, endothelial cells and neuroretina. Oxygen deprivation sensed by HIF-1α in neuroretina induces retinal ganglion cells to express PDGFA, promoting proliferation and migration of astrocyte to populate the retina. Astrocytes secrete VEGF and fibronectin (Fn) in an HIF-2α and Tlx dependent manner, attracting endothelial cells during angiogenesis. Invading endothelial cells relieve the oxygen tension and express LIF to promote maturation of astrocytes. ONH, optic nerve head; APC, astrocyte progenitor cell; RGC, retinal ganglion cell; RPC, retinal progenitor cell; RV, retinal vasculature; ILM, inner limiting membrane; RPE, retinal pigmented epithelium.