Tlx acts as a proangiogenic switch by regulating extracellular assembly of fibronectin matrices in retinal astrocytes

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In response to hypoxia, hypoxia-inducible factors act as the primary proangiogenic triggers by regulating transcription levels of target genes, including *VEGF*. However, little is known about the specific factors that control other components of the angiogenic process, particularly formation of matrix scaffolds that promote adhesion and migration of endothelial cells. We show that in the postnatal mouse retina, the orphan nuclear receptor *tailless* (*Tlx*) is strongly expressed in the proangiogenic astrocytes, which secrete VEGF and fibronectin. *Tlx* expression by retinal astrocytes is controlled by oxygen concentration and rapidly downregulated upon contact with blood vessels. In mice null for *Tlx*, retinal astrocytes maintain *VEGF* expression; however, the extracellular assembly of fibronectin matrices by astrocytes is severely impaired, leading to defective scaffold formation and a complete failure of normal retinal vascular development. This work identifies Tlx as an essential component of the molecular network involved in the hypoxia-inducible proangiogenic switch in retinal astrocytes.

Introduction

During tissue neogenesis in development, increased metabolic activities result in an imbalance between blood supply and oxygen demands in the microenvironment, leading to physiological hypoxia and subsequent activation of specific cell populations in each tissue or organ to induce formation of functional blood vessels. Once the blood supply sufficiently meets the oxygen demands, proangiogenic activities are turned off and blood vessels remain quiescent unless angiogenesis resumes under pathological conditions such as cancer or diabetic retinopathy (1–3). Thus, the extent of angiogenesis is regulated by the switching on or off of proangiogenic activities in response to the microenvironmental oxygen concentration. While the molecular mechanisms controlling the angiogenic processes are vastly heterogeneous, depending on the surrounding cell types (4), hypoxia-inducible factors (HIFs) act as the primary molecular switches to the proangiogenic state in many instances (5, 6). Under hypoxic conditions, activated HIFs transcriptionally upregulate expression levels of a series of proangiogenic genes, including *VEGF* (7–9). Subsequently, vascular sprouts invade the target tissues, utilizing scaffolds composed of extracellular molecules that promote endothelial adhesion, migration, and survival (10, 11). Fibronectin, which interacts with endothelial integrin α5β1, is an essential proangiogenic matrix component as genetic or pharmacological inhibition of fibronectin-integrin α5β1 interactions disrupts angiogenesis in both developmental and pathological conditions (12–15). To date, however, the molecular mechanisms that control formation of proangiogenic scaffolds under hypoxic conditions remain poorly understood.

In the mouse retina, it is well established that astrocytes act as the primary proangiogenic cell type required to establish the retinal vascular system. Prior to the postnatal onset of retinal vascular development, retinal neurons induce an influx of astrocytes via signaling molecules such as PDGF-A. Subsequently, retinal astrocytes secrete VEGF and fibronectin, thereby guiding retinal angiogenesis over the astrocyte network (16–22). After completion of blood vessel formation, astrocytes lose their proangiogenic properties but continue to play a role in vascular homeostasis by promoting the formation and maintenance of the blood-retina barrier (23–25). Thus, retinal astrocytes serve as a unique and simple model to investigate the molecular mechanisms controlling various proangiogenic processes, including the formation of matrix scaffolds. Interestingly, besides being an important stimulus that triggers angiogenesis, hypoxia modulates several aspects of astrocyte behavior, such as mitotic activity (22). However, with the exception of possible HIF involvement, little is known concerning the cell-intrinsic molecular mechanisms regulating astrocyte behavior in response to changes in tissue oxygen concentration.

The orphan nuclear receptor mouse homolog of *Drosophila tailless* (*Tlx*, also known as Nr2e1) is a transcription factor that regulates several genes expressed in astrocytes, such as *glial fibrillary acidic protein* (*GFAP*) (26, 27). In the adult brain, Tlx is expressed in neural stem cells and directly represses the transcription of *GFAP*, resulting in maintenance of pluripotency by preventing commitment into astrocyte lineages (27). In the developing retina, several types of cells, including retinal progenitor cells, astrocytes, and Müller glia, express Tlx in a spatially and temporally restricted manner, and retinas of *Tlx* mutant mice display disorganized astrocyte networks and defective blood vessel formation (26, 28, 29). Taken collectively, the available data indicate that Tlx may play important roles in controlling astrocyte development, although little is known regarding the mechanisms that regulate Tlx expression or the specific role of Tlx in regulating astrocyte function.
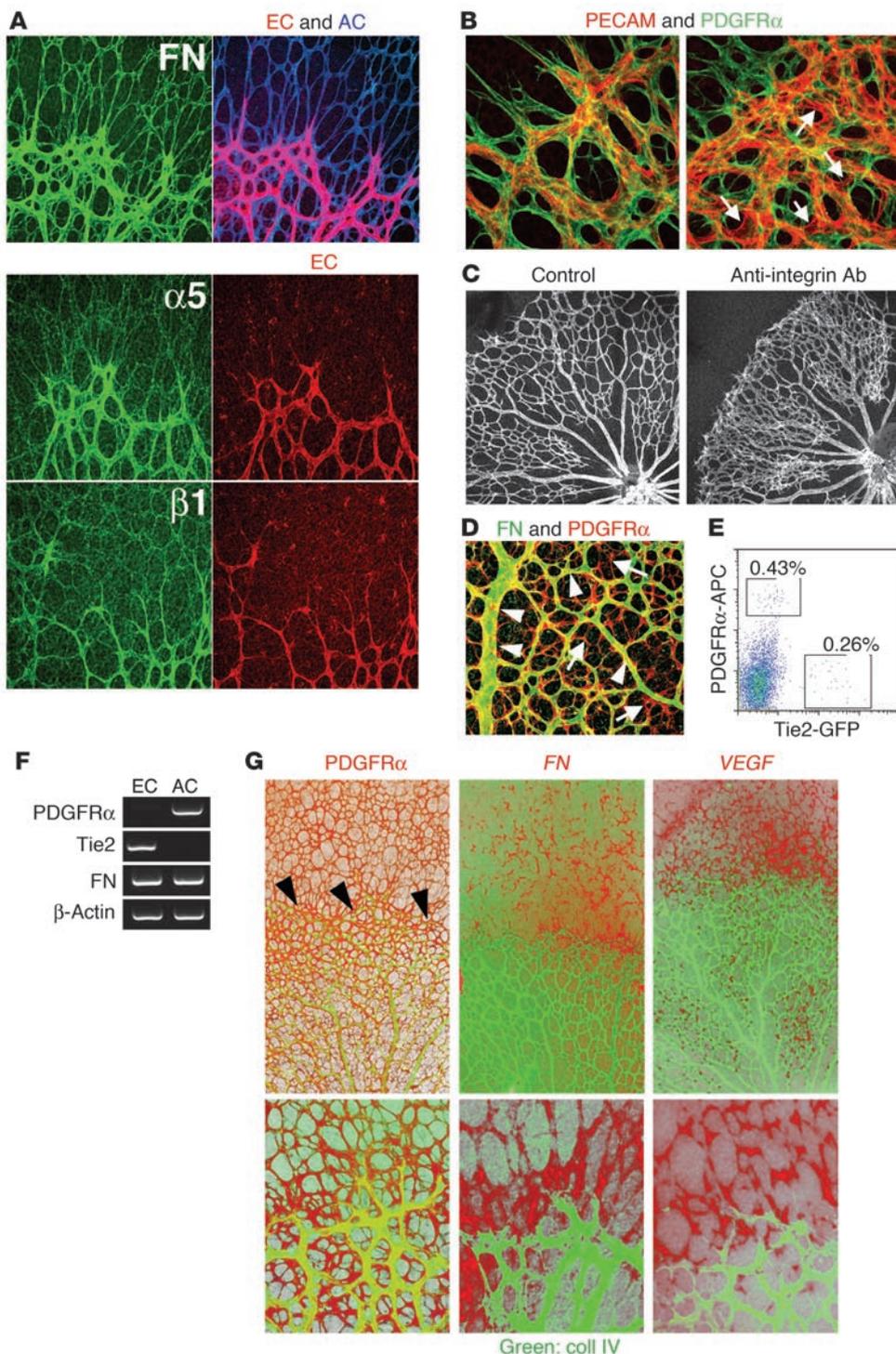
Figure 1
Formation of fibronectin-matrix scaffolds in proangiogenic astrocytes. (**A**) Triple immunostaining (top) for fibronectin (FN, green), PECAM-1 (red), and PDGFRα (blue) and double immunostaining (middle and bottom) for lectin (red) and integrin α5 or β1 (green) at the sprouting vascular edges of P5 retina. (**B** and **C**) Retinal vessels 24 (**B**) or 48 (**C**) hours after intraocular injections of control IgG (left) or anti-integrin β1 antibody (right) at P2. (**B**) Immunostaining for PECAM-1 (red) and PDGFRα (green) at the sprouting vascular edges. Arrows indicate endothelial cells dissociating from astrocyte (AC) scaffolds. (**C**) PECAM-1 staining. (**D**) Immunostaining for PDGFRα (red) and fibronectin (green) in P7 retina. Fibronectin is expressed around blood vessels (arrowheads) but almost absent in astrocyte networks, which are not associated with blood vessels (arrows). (**E**) Flow cytometric analysis of retinal cells from Tie2-GFP transgenic mice at P7. Endothelial cells and astrocytes were sorted as GFP+ (0.26%) and PDGFRα+ (0.43%) fractions, respectively. (**F**) RT-PCR from total RNA of GFP+ and PDGFRα+ cells sorted by FACS. *Fibronectin* mRNA is expressed in both endothelial cells and astrocytes. (**G**) Red immunostaining for PDGFRα (left), ISH for *fibronectin* (middle), or *VEGF* (right) in P4 retinas. Arrowheads indicate the growing edge of the retinal vasculature depicted by immunostaining for type IV collagen (coll IV; green). Lower panels are the magnified views of growing vascular edges. Magnification, ×200 (**A** and **D**), ×400 (**B**), ×70 (**C**), ×63 (**G**, upper), ×252 (**G**, lower).

In this study, we show that *Tlx* is specifically expressed by proangiogenic retinal astrocytes and is rapidly downregulated upon contact with blood vessels. The dramatic regulation of *Tlx* expression is controlled by oxygen concentration. Our analyses of *Tlx* mutant mice reveal that Tlx plays an indispensable role in the formation of the proangiogenic extracellular scaffold, which forms the template for retinal vascular growth through the regulation of the expression and extracellular deposition of fibronectin. Thus, Tlx represents a novel member of the hypoxia-inducible molecular network required for the expression of proangiogenic properties by retinal astrocytes.

Results

Formation of fibronectin matrix scaffolds in proangiogenic astrocytes. It has been suggested that extracellular matrices deposited by astrocytes forms a proangiogenic scaffold, which plays an important role in retinal vascular development (21). However, the mechanisms that regulate the formation of this scaffold and its func-

tional significance have not yet been fully defined. In the present study, we first examined the deposition of fibronectin and the expression of its receptor, α5β1 integrin, at the growing edge of the retinal vasculature. While fibronectin matrices were deposited around the PDGFRα-expressing astrocyte networks ahead of sprouting endothelial cells, both α5 and β1 integrin subunits were strongly expressed in the endothelial cells themselves (Figure 1A). Such complementary expression patterns suggested that the integrin played a critical role in promoting the adhesion of migrating endothelial cells to the fibronectin scaffolds. We therefore injected P2 neonates intraocularly with a monoclonal antibody against integrin β1 subunit to inhibit its binding to extracellular matrices, including fibronectin (30). Twenty-four hours after the intraocular injections, advancement of vascular networks was arrested, and the extension of the endothelial sprouts at the leading edge of the retinal vasculature was decreased (Figure 1B). Furthermore, endothelial cells were no longer strictly associated with the underlying astrocyte network (Figure 1B), leading to enlargement and apparent coalescence of retinal capillaries 48 hours after the treatment (Figure 1C). Thus, integrin-mediated adhesion to the matrix scaffold is an essential part of the development and morphogenesis of the retinal vascular system.

In the course of normal retinal development, fibronectin continued to be present around blood vessels though it was no longer expressed in astrocytes (Figure 1D). This indicates that production of fibronectin associated with blood vessels may shift from astrocytes to endothelial and/or mural cells in the processes of retinal vascular development. Indeed, endothelial cells isolated from P7 retinas also expressed the *fibronectin* mRNA (Figure 1, E and F). To precisely define the expression pattern of *fibronectin* in retinal astrocytes, we performed in situ hybridization (ISH) on whole-mount retinas in conjunction with immunolabeling for PDGFRα or type IV collagen to depict astrocyte or vascular networks, respectively. While astrocytes that form a simple plexus in the unvascularized areas of the retina strongly expressed *fibronectin*, its expression became dramatically downregulated upon contact with blood vessels (Figure 1G). Interestingly, this pattern of *fibronectin* expression was very similar to that of *VEGF* (19, 22, 31, 32), revealing that in the proangiogenic state, retinal astrocytes synchronously express these 2 genes.

Tlx is specifically expressed in proangiogenic astrocytes. While retinal astrocytes strongly express *VEGF* and *fibronectin* in the unvascularized areas, it is reported that *GFAP* is expressed only at very low levels in these astrocytes but strongly expressed in astrocytes associated with blood vessels (22, 31). This indicates that the expression of GFAP is inversely correlated with that of proangiogenic mol-

ecules, suggesting that the molecular mechanisms responsible for regulating GFAP expression might also be involved in regulating the expression of proangiogenic molecules in retinal astrocytes. Therefore, we further examined the regulation of GFAP expression in retinal astrocytes using transgenic mice in which GFP expression was driven by a 2.2-kb 5′ promoter region of the human *GFAP* gene (33). The 2.2-kb *GFAP* promoter was active in all astrocyte populations in the early postnatal period, as shown by staining for GFP (Figure 2A). In contrast, expression of GFAP protein was detectable only in astrocytes near the optic disc (Figure 2A), consistent with the expression pattern of *GFAP* mRNA in retinal whole mounts (data not shown). This discrepancy suggests that GFAP expression is negatively regulated in retinal astrocytes during the early postnatal stages. A good candidate molecule in this regard is the orphan nuclear receptor Tlx, which has been shown to repress *GFAP* expression in neural stem cells (27). In the developing retina, we have reported that Tlx is transiently expressed in astrocytes during late embryonic and early postnatal stages (29). To determine the precise expression pattern of the *Tlx* gene and its relation
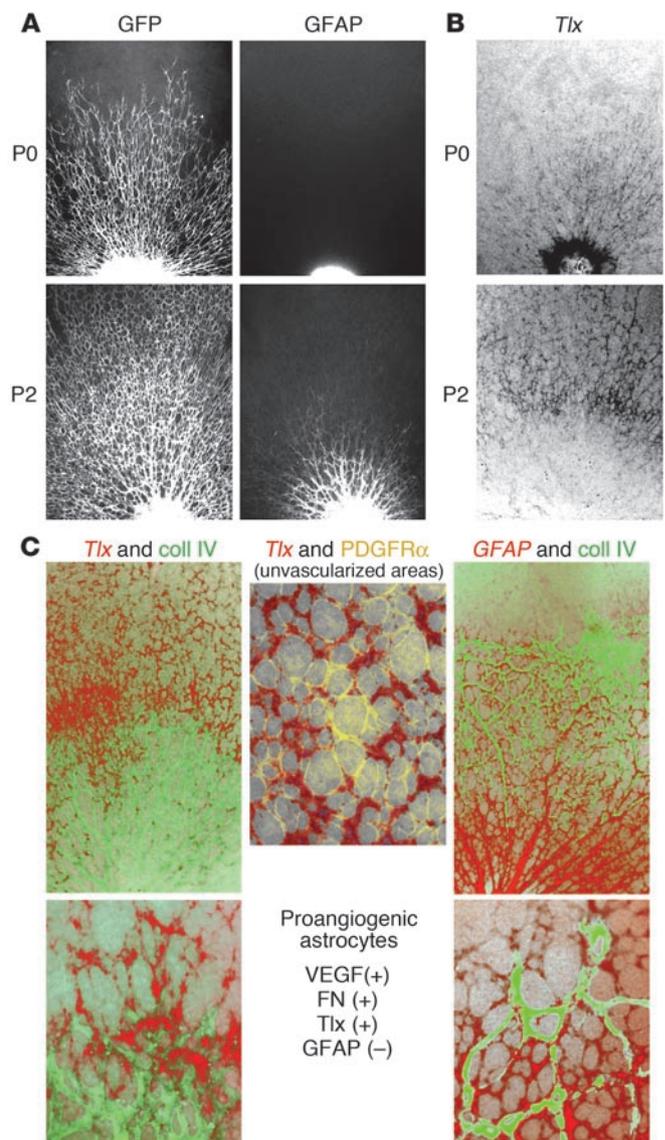

Figure 2
Tlx expression in proangiogenic astrocytes. (**A**) Dual immunostaining for GFP and GFAP in retinas of *GFAP*-GFP transgenic mice at P0 and P2. While the 2.2-kb *GFAP* promoter is uniformly activated in all astrocytes, GFAP expression is seen only in astrocytes around the optic discs. (**B**) Whole-mount ISH for *Tlx* in P0 and P2 retinas. Uniform *Tlx* expression in retinal astrocytes at P0 sequentially becomes downregulated in a central-to-peripheral manner. (**C**) ISH for *Tlx* or *GFAP* (red) in conjunction with immunostaining for type IV collagen (green) or PDGFRα (yellow) on P4 retina. Lower panels are the magnified views of growing vascular edges. *Tlx* mRNA is specifically expressed in proangiogenic astrocytes and displays a pattern complementary to that of *GFAP*. Magnification, ×63 (**A**, **B**, and upper left and upper right panels of **C**), ×252 (lower and middle panels of **C**).
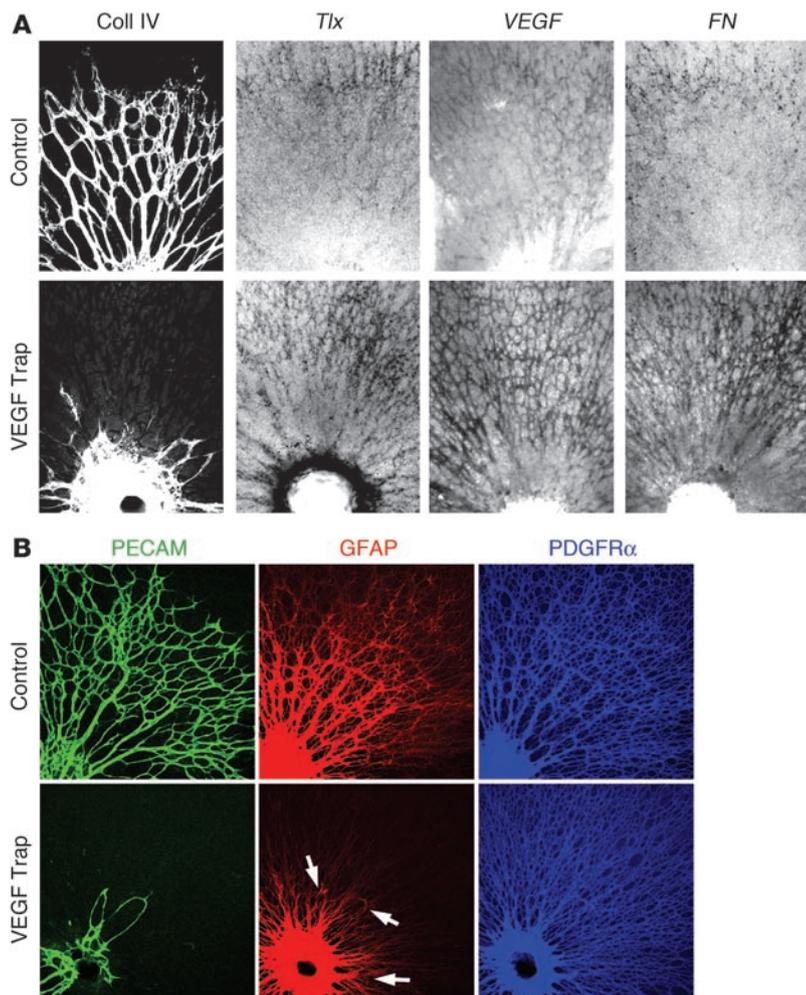
Figure 3
Blood vessels affect expression levels of proangiogenic genes and GFAP in retinal astrocytes. P3 retinas 48 hours after the intraocular injections of Fc control protein (upper panels) or VEGF Trap (lower panels). (**A**) Immunostaining for type IV collagen and ISH for *Tlx*, *VEGF*, or *fibronectin*. In the absence of blood vessels, genes specific to the proangiogenic state are continually expressed in astrocytes. (**B**) Immunostaining for PECAM-1 (green), GFAP (red), and PDGFRα (blue). In the retinas treated with VEGF Trap, GFAP is expressed only around the poorly developed blood vessels (arrows). In the absence of blood vessels, astrocyte networks depicted by PDGFRα staining maintain a simple plexus formation characteristic of the proangiogenic state. Magnification, ×126 (**A**), ×100 (**B**).

to GFAP in retinal astrocytes, we performed whole-mount ISH in mouse retinas from different developmental stages (Figure 2B). At P0, when the endogenous GFAP expression was barely detectable, *Tlx* was almost uniformly expressed in the retinal astrocytes. However, during the course of postnatal development, *Tlx* expression in astrocytes became downregulated in the central retina. To determine whether the transition of *Tlx* expression in retinal astrocytes was closely correlated with the process of blood vessel formation, we evaluated the pattern of *Tlx* expression in conjunction with staining for type IV collagen or PDGFRα (Figure 2C). We found that while *Tlx* was strongly expressed in astrocytes in the unvascularized areas of the retina, its expression was rapidly downregulated upon contact with blood vessels. This pattern of *Tlx* expression in astrocytes was in clear contrast to that of *GFAP* (Figure 2C) and almost identical to those of *VEGF* and *fibronectin* (Figure 1G). Thus, we identified *Tlx* as one of the genes specifically expressed in proangiogenic astrocytes.

Tlx expression is controlled by oxygen concentration in retinal astrocytes. In the developing retina, proangiogenic astrocytes are characterized by the expression of high levels of *VEGF*, *fibronectin*, and *Tlx* and very low levels of *GFAP*. The rapid downregulation of *VEGF*, *fibronectin*, and *Tlx* and simultaneous upregulation of *GFAP* expression seem to be inducible rather than programmed events triggered by the association with blood vessels. Indeed, when the initiation

of retinal vascular growth was delayed by intraocular injections of a VEGF receptor chimeric protein, VEGF Trap (34), astrocytes continued to express *Tlx*, *VEGF*, and *fibronectin*, indicating that these astrocytes remained in the proangiogenic state (Figure 3A). In contrast, GFAP expression was barely detectable except in a few astrocytes associated with blood vessels restricted to the immediate vicinity of the optic nerve head (Figure 3B). Thus, proangiogenic activities and GFAP expression are likely to be coordinately regulated by microenvironmental changes accompanied by blood vessel formation.

Although the mechanisms regulating *Tlx* and *fibronectin* expression in retinal astrocytes remain to be determined, the similarity in their developmental expression patterns to that of *VEGF* suggested that oxygen concentration might play an important role. To investigate this possibility, we placed P2 pups in 80% O_2 for 5 hours, then returned them to normal room air (20% O_2). After a 5-hour exposure to hyperoxic conditions, *Tlx* mRNA expression in retinal astrocytes was markedly suppressed (Figure 4). On the other hand, 18 hours after the return to room air, *Tlx* mRNA levels were fully restored. Throughout the course of this experiment, the *VEGF* expression pattern resembled that of *Tlx*. Thus, in retinal astrocytes, both *Tlx* and *VEGF* appear to be transcriptionally controlled by oxygen concentrations, which would explain their synchronous expression in proangiogenic astrocytes and downregulation upon contact with blood vessels. In contrast, *fibronectin* mRNA was continuously expressed even under hyperoxic conditions, indicating that *fibronectin* transcription is not directly regulated by oxygen concentration. Despite the rapid downregulation of *Tlx* under hyperoxic conditions, *GFAP* mRNA expression was not remarkably upregulated beyond the limits of the retinal vasculature (Figure 4), even when the period of hyperoxia was extended to 24 hours (data not shown), suggesting that relief of Tlx repression alone is not sufficient for upregulation of *GFAP* expression.

Complete absence of retinal angiogenesis in Tlx⁻/⁻ mice. To investigate the functional roles of Tlx in retinal astrocytes, especially those in the proangiogenic state, we analyzed the histological phenotypes of the retinal astrocytes and blood vessels of *Tlx* KO (*Tlx⁻/⁻*) mice.
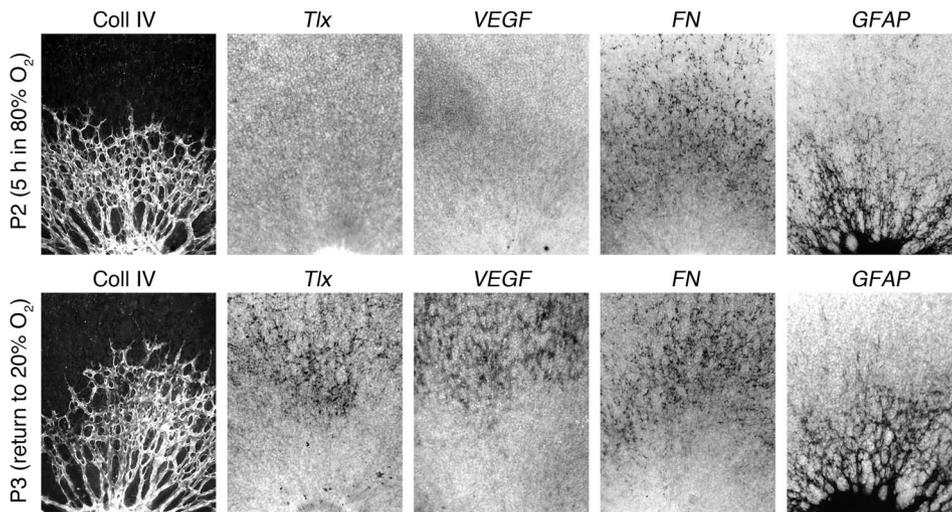
Figure 4
Oxygen concentration synchronously controls expression of *Tlx* and *VEGF* but not *fibronectin* in retinal astrocytes. Immunostaining for type IV collagen and ISH for *Tlx*, *VEGF*, *fibronectin*, and *GFAP*. Expression levels of *Tlx* and *VEGF* mRNA are dramatically downregulated under hyperoxic conditions (upper panels) whereas they are synchronously restored after the return to room air (lower panels). In contrast, *fibronectin* expression is continuously maintained even under hyperoxia. *GFAP* expression is not upregulated beyond the vascularized areas. Magnification, ×126.

The initial formation of the simple astrocyte plexus and subsequent remodeling of the astrocyte network in retinas of *Tlx* heterozygous (*Tlx*+/-) mice was indistinguishable from that of wild-type mice. In contrast, in *Tlx*-/- mice, the radial extension of retinal astrocytes was asymmetric and markedly delayed. Moreover, radial astrocyte networks lacked lateral branches and interconnection in *Tlx*-/- mice, displaying a spoke-like appearance (Figure 5A). As Tlx has been implicated in cell cycle control (27, 29, 35, 36), we performed BrdU incorporation assays to determine whether the impaired mitotic activity in *Tlx*-/- astrocytes might account for the impaired development of the retinal astrocyte networks (Figure 5B). Mitotic activity in retinal astrocytes was most pronounced at the leading edge of the developing network. In P5 retinas, the numbers of BrdU-positive astrocytes in this region were decreased to 73.4% ± 19.4% (mean ± SD, $P = 0.014$) in *Tlx*-/- mice compared with *Tlx*+/- mice. Because astrocyte proliferation was only modestly decreased, impairments in motility and/or adhesion of *Tlx*-/- astrocytes are likely the predominant factors underlying the observed malformation of the astrocyte network in *Tlx*-/- retina.

Although an astrocyte network did develop in the retinas of *Tlx*-/- mice, albeit abnormally, blood vessels were completely absent (Figure 5C). This was attributable to a failure in normal initiation of retinal angiogenesis, as no vessels grew beyond the optic disc (Figure 5D). Thus, *Tlx*-/- astrocytes also lacked 1 or more essential proangiogenic properties. Interestingly, retinal astrocytes uniformly expressed GFAP in *Tlx*-/- mice, despite the absence of blood vessels

Figure 5
Morphogenetic defects in network formation and abortive proangiogenic activities in retinal astrocytes of *Tlx* KO mice. (**A**) Immunostaining for PDGFRα in *Tlx* mutant mice. In *Tlx*-/- mice, astrocyte networks are poorly extended and morphogenetically disorganized, with fewer branchings. (**B**) Double labeling for BrdU (red) and PDGFRα (green) of migrating astrocytes in P5 retina. (**C** and **D**) Double immunostaining for GFAP (red) and PECAM-1 (green) in P5 retina. GFAP is strongly expressed in all retinal astrocytes of *Tlx*-/- mice despite the complete absence of blood vessels (**C**). In *Tlx*-/- mice, blood vessels never sprout from the stem of hyaloid vessels at optic nerve head (arrowheads in **D**). Nuclear staining by TO-PRO-3 iodide appears in blue. Magnification, ×70 (**A**), ×400 (**B**), ×100 (**C**), ×280 (**D**).
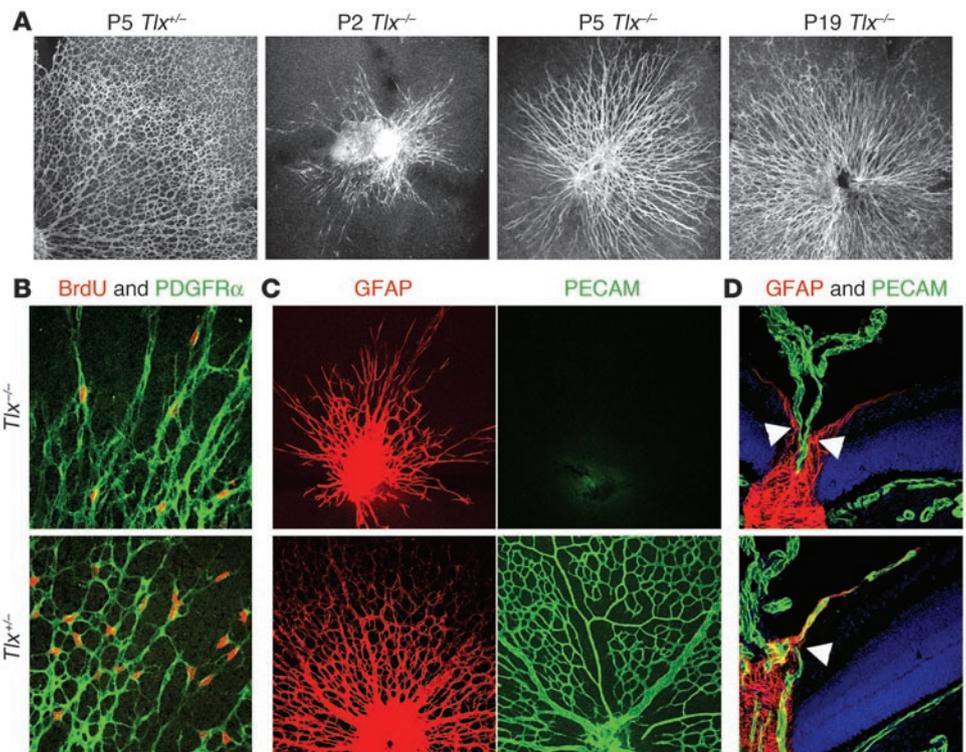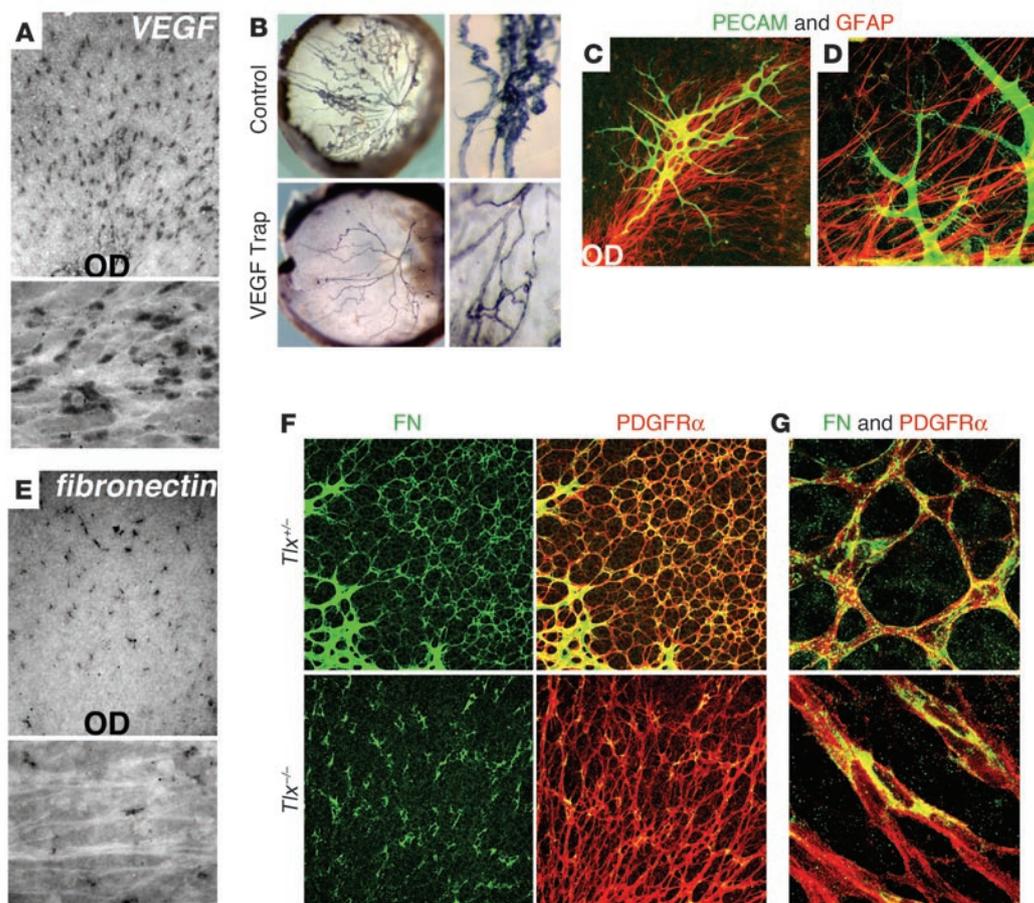
Figure 6

Tlx regulates formation of proangiogenic scaffolds by controlling extracellular deposition of fibronectin matrices in retinal astrocytes. (**A** and **E**) Whole-mount ISH for *VEGF* (**A**) or *fibronectin* (**E**) in P7 retinas of *Tlx*^{−/−} mice. The lower panels are magnifications of ISH (black) with immunostaining for PDGFRα (white). In contrast to the retained *VEGF* expression over the astrocyte network, *fibronectin* is expressed only in a small subset of astrocytes in *Tlx*^{−/−} retina. OD, optic disc. (**B**) Whole-mount immunostaining for PECAM-1 in P16 *Tlx*^{−/−} retinal cups 48 hours after intraocular injections of control Fc (upper panels) or VEGF Trap (lower panels). Right panels are the higher magnifications of hyaloid vessels shown in left panels. Persistent hyaloid vessels and their sprouts in *Tlx*^{−/−} eyes regress by neutralization of VEGF signals. (**C** and **D**) Immunostaining for PECAM-1 (green) and GFAP (red) in P14 retina of *Tlx*^{−/−} mouse. Ectopic vessels derived from hyaloids target retinal astrocytes (**C**) but are not guided by the pre-existing astrocyte networks (**D**). (**F** and **G**) Immunostaining for fibronectin (green) and PDGFRα (red) in *Tlx*^{+/−} (upper panels) and *Tlx*^{−/−} (lower panels) P7 retinas. In the astrocyte networks of *Tlx*^{−/−} retinas, fibronectin proteins are detected only in a punctate manner (**F**). Note the retention of fibronectin proteins in perinuclear cytoplasmic regions of *Tlx*^{−/−} astrocytes (**G**). Magnification, ×126 (upper panels of **A** and **E**), ×400 (lower panels of **A** and **E**), ×32 (**B**, left panels), ×100 (**B**, right panels), ×150 (**C**), ×630 (**D**), ×200 (**F**), ×1260 (**G**).

(Figure 5C), possibly resulting in part from the loss of Tlx repression on *GFAP* transcription.

Tlx is required for the formation of proangiogenic scaffold through the control of extracellular assembly of fibronectin matrices in retinal astrocytes. As the major known requirement for proangiogenic properties of retinal astrocytes consists of VEGF secretion and extracellular matrix scaffold formation, we investigated whether impairment in either of these properties might account for the angiogenic defects observed in the retinas of *Tlx*^{−/−} mice.

The overall levels of *VEGF* mRNA were maintained in *Tlx*^{−/−} astrocytes (Figure 6A). Possibly due to this sustained VEGF expression, the hyaloid vessels failed to regress and over time became enlarged and gave rise to numerous endothelial sprouts that grew toward the avascular retina (Figure 6B). Indeed, the sprouting of hyaloid vessels was dependent on VEGF, as these neovessels regressed after the intraocular injection of VEGF Trap (Figure 6B). Interestingly,

in *Tlx*^{−/−} mice, new vessels derived from the hyaloid were frequently observed to target astrocyte-rich areas of the retina (Figure 6C), suggesting that they were attracted by VEGF produced by these cells. However, upon reaching the retina, these ectopic vessels grew in an apparently random fashion, rather than using the preexisting astrocyte network as a template, as is the case in normal retinal vascular development (Figure 6D). Taken together, these findings strongly suggest that the failure to initiate retinal angiogenesis in *Tlx*^{−/−} mice is not due to a lack of VEGF per se but rather molecules essential for the extracellular matrix scaffolds that form the template for endothelial migration and the initial assembly of the primitive retinal vascular network.

Therefore, we next examined the expression of fibronectin, as this matrix molecule is a principle component of the astrocyte-derived extracellular scaffold. In *Tlx*^{−/−} mice, only a very small number of retinal astrocytes expressed *fibronectin* mRNA (Figure
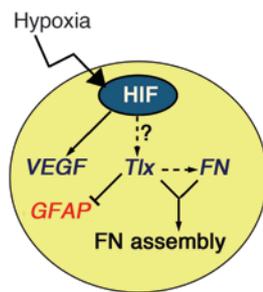
Figure 7
A model for molecular networks regulating the proangiogenic switches in retinal astrocytes. Upon sensing hypoxia, activated HIFs primarily upregulate the transcriptional levels of *VEGF* and possibly *Tlx*. Tlx then controls the secondary cascades involved in the extracellular assembly of fibronectin scaffolds, with concomitant repression of *GFAP*. Once associated with blood vessels, hypoxia-inducible genes supporting the proangiogenic state are downregulated by increasing oxygen concentration. Finally, retinal astrocytes upregulate GFAP and form a blood-retina barrier in cooperation with endothelial cells.

6E). Consistent with this finding, immunostaining revealed that fibronectin protein was scarce in *Tlx*$^{-/-}$ retinas and distributed in a punctate manner (Figure 6F). Intriguingly, in clear contrast to the filamentous and relatively homogenous extracellular deposition of fibronectin around the astrocyte networks in retinas of wild-type and *Tlx*$^{+/-}$ mice, fibronectin protein could not be detected in the extracellular spaces around retinal astrocytes in *Tlx*$^{-/-}$ mice, but was instead retained in the cytoplasm (Figure 6G). Thus, fibronectin-rich matrices are completely absent from the scaffold formed by *Tlx*$^{-/-}$ retinal astrocytes. These observations indicate that Tlx is essential for the formation of proangiogenic scaffolds through regulation of the extracellular assembly of fibronectin matrices.

Discussion

Currently it is well established that astrocytes are the major regulators of angiogenesis in the developing retina (37, 38). Indeed, retinal astrocytes are found only in species that develop vascularized retinas, such as humans and rodents (39, 40). GFAP has been exploited as a representative marker for retinal astrocytes (41, 42), but recent studies demonstrated that GFAP is not expressed at appreciable levels in certain astrocyte populations in the developing retina (22, 31, 43). The GFAP-negative astrocytes have been considered precursors of GFAP-positive astrocytes (43, 44), although the extent to which GFAP expression was a marker for functional heterogeneity remained unclear. In this study, we show that expression of GFAP is inversely correlated with that of 2 major proangiogenic molecules, VEGF and fibronectin. This indicates that GFAP can be exploited as a marker that is negatively correlated with the proangiogenic properties of retinal astrocytes. To identify the molecular mechanisms that regulate the switching of retinal astrocyte phenotype from proangiogenic to angiostatic, we considered the possibility that mechanisms that regulated GFAP expression might also play an integral role in controlling the angiogenic status of retinal astrocytes. Thus, we found that Tlx, a transcriptional repressor of *GFAP* expression, is an essential component of the molecular network involved in switching the angiogenic phenotype of retinal astrocytes.

Astrocytes are frequently associated with blood vessels in other areas of the central nervous system (25), and the process of neural

repair following trauma and the growth of gliomas are characteristically associated with neoangiogenesis. It will therefore be interesting to determine whether GFAP expression is also negatively correlated with the proangiogenic activities of brain astrocytes and whether Tlx is involved in the suppression of GFAP expression in these pathophysiological conditions.

While GFAP is strongly expressed in *Tlx*$^{-/-}$ astrocytes, it remains to be determined whether Tlx repression is the sole mechanism that controls GFAP expression in retinal astrocytes. Despite *Tlx* downregulation after exposure to hyperoxia, *GFAP* expression was not upregulated beyond the vascularized areas of the retina. This finding suggests that efficient expression of GFAP requires some positive signals in addition to relief of Tlx repression. The precise molecular mechanisms controlling GFAP expression in retinal astrocytes require further elucidation.

Regulation of Tlx expression by oxygen concentration. In addition to retinal astrocytes, Tlx is expressed in neural progenitor or stem cells in the developing forebrain and retina, as well as in adult brain (27, 29, 36). Although little is known concerning the functional roles of Tlx, recent studies have indicated that Tlx controls cell cycles in these pluripotent, self-renewing cells (27, 29, 35, 36). To date, the mechanisms regulating Tlx expression have also remained unclear. In the present study, we show for what we believe is the first time that *Tlx* expression is transcriptionally controlled by oxygen concentration in retinal astrocytes, thereby restricting its expression in cells in a proangiogenic state. Together with the decreased mitotic activities of *Tlx*$^{-/-}$ astrocytes, Tlx expression may partly explain the cell-intrinsic molecular mechanisms underlying the recently reported observation that oxygen regulates mitotic activities in retinal astrocytes (22).

While consensus sequences for hypoxia-responsive elements are found in the 5′ flanking region of the *Tlx* gene (at –1277 bp and –2500 bp relative to the transcription start site), we could not detect elevated promoter activities in this region using reporter assays and astrocyte-derived cell lines cultured under hypoxic conditions (mouse cerebellum astrocyte C8-D1A, rat brain astrocyte RCR-1, and human glioma U251, A. Uemura, unpublished observations). Although it remains possible that *Tlx* transcription is controlled directly by HIFs in native retinal astrocytes, it is also possible that changes in oxygen concentration regulate expression of *Tlx* by an alternate mechanism (5). A related important question is whether oxygen concentration regulates Tlx expression in other types of Tlx-expressing cells. We have observed that retinal neurons continue to express *Tlx* mRNA, even under hyperoxic conditions (A. Uemura, unpublished observations). Therefore, it seems likely that oxygen concentration affects the transcriptional levels of *Tlx* only in specific cell types and/or under a restricted set of conditions.

Roles of Tlx in the formation of proangiogenic scaffold by retinal astrocytes. In the processes involved in angiogenesis, a number of signaling molecules that modulate endothelial behavior have been identified (45, 46), but little is known regarding the molecular mechanisms controlling the other angiogenic processes, especially those underlying the formation of proangiogenic matrix scaffolds. The retinal vascular changes induced by blocking of integrin β1 function confirm the importance of endothelial adhesion to matrix scaffolds for the development of the retinal vascular system. Besides providing physical scaffolds for endothelial adhesion and migration, extracellular matrices promote endothelial survival through activating integrin-mediated signal transduction pathways

(47–50) and establish a concentration gradient of matrix-binding VEGF isoforms (VEGF$_{164}$ and VEGF$_{188}$ in mice), which guides the direction of sprouting angiogenesis (19, 32). It has been reported that hypoxia controls several genes involved in matrix degradation, such as *matrix metalloproteinases* (5). However, the means by which hypoxia promoted the assembly of proangiogenic fibronectin matrices remained elusive. In the present study, we show that Tlx acts as the major regulator for proper assembly of fibronectin matrices by proangiogenic astrocytes in the developing retina. The overall reduction in the number of *fibronectin*-expressing astrocytes in *Tlx$^{-/-}$* retinas indicates that Tlx promotes *fibronectin* transcription. Moreover, the cytoplasmic retention of fibronectin protein by *Tlx$^{-/-}$* astrocytes indicates that Tlx is also critically involved in the extracellular transport of fibronectin. That this fibronectin-rich scaffolding is essential to normal retinal vascular development is strongly supported by observations made in *Tlx$^{-/-}$* mice. Here, normal retinal vascular development fails completely, despite the continued expression of VEGF. In these mice, ectopic vessels sprout from the retained hyaloid vasculature and grow toward the retinal surface. However, upon reaching the retinal surface, these new vessels fail to align themselves along the extant astrocyte network, in contrast to the pattern observed during normal retinal vascular development. Deficiencies in the deposition of fibronectin matrices may also affect the proliferation, adhesion, and migration of astrocytes themselves and partly account for the disorganized architecture of the astrocyte network in *Tlx$^{-/-}$* retina.

It should be emphasized, however, that hypoxia is not the sole factor regulating assembly of fibronectin matrices. In fact, fibronectin is involved in multiple processes from early stages of embryogenesis, and there are many situations where fibronectin assembly should not be affected by the fluctuation of oxygen tension. For example, endothelial expression of fibronectin in retinal vessels may not be maintained by hypoxia. Thus, control of fibronectin deposition by Tlx might be a mechanism for linking hypoxia and scaffold formation specifically in retinal astrocytes. Proangiogenic cells in other tissues that guide endothelial sprouting under hypoxia may have their specific cell-intrinsic mechanisms for fibronectin assembly. Hence, we expect that corresponding molecules with similar functions will be identified in each tissue or organ, which will be potentially beneficial for the advance of technologies to manipulate angiogenesis.

To summarize, we present a model of the molecular network that regulates the angiogenic status of retinal astrocytes during postnatal development (Figure 7). Upon sensing hypoxia, transcription levels of *VEGF* and *Tlx* are upregulated, with concomitant upregulation of *fibronectin* transcription in these astrocytes. Tlx then controls secondary molecular cascades required for the assembly of fibronectin matrices and for regulation of astrocyte behavior through the repression of *GFAP* expression. Once associated with blood vessels, oxygenation downregulates the hypoxia-inducible genes in astrocytes, thereby switching off these proangiogenic activities. Subsequently, astrocytes in their nonangiogenic state continue to collaborate with endothelial cells through other signaling molecules and cell-cell contacts to maintain vascular homeostasis, most notably by promoting the formation and maintenance of the blood-retina barrier.

Methods

Mice. ICR mice were purchased from SLC Japan, and GFAP-GFP and Tie2-GFP transgenic mice were purchased from the Jackson Laboratory.

Heterozygous *Tlx* KO mice (26) were crossed to obtain pups that were genotyped by PCR. All animal experiments were approved by the Animal Research Committee of the RIKEN Center for Developmental Biology and were carried out in accordance with the RIKEN guidelines for animal and recombinant DNA experiments.

Histology, immunostaining, and ISH. For whole-mount preparations, retinal cups were isolated and fixed in 4% paraformaldehyde/PBS overnight. Whole-mount immunohistochemistry procedures were performed as previously described (51). For whole-mount ISH, retinas were briefly digested with proteinase K and hybridized with digoxigenin-labeled RNA probes. After hybridization, samples were incubated with alkaline phosphatase-conjugated antidigoxigenin antibodies (Roche Diagnostics Corp.), and signals were visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (Roche Diagnostics Corp.). In combined ISH and immunohistochemistry, the antibody labeling was performed after the ISH protocol was completed. Templates for probes (*VEGF*, 825–1172 of NM_009505: *fibronectin*, 2466–2831 of BC_025521: *Tlx*, 683–1840 of NM_152229) were obtained by reverse transcription of total RNA from 4-day old ICR mouse retinas and PCR amplifications. Template for *GFAP* RNA probe was a gift from James Cohen (King's College London, London, United Kingdom). For cryosections, eyes were frozen in OCT compound (Sakura Finetek and sectioned at a thickness of 10 μm. Nuclear staining was performed using TO-PRO-3 iodide (Invitrogen Corp.).

Abs. Primary Abs for immunohistochemistry were monoclonal anti-GFAP (clone G-A-5, Cy3-conjugated; Sigma-Aldrich), PECAM-1 (clone Mec13.3; BD Biosciences — Pharmingen), integrin α5 (clone 5H10-27; BD Biosciences — Pharmingen), integrin β1 (clone 9EG7; BD Biosciences — Pharmingen), polyclonal anti-PDGFRα (R&D Systems), fibronectin (DakoCytomation), type IV collagen (Cosmo Bio Co.), and GFP (Invitrogen Corp.). Secondary Abs were Alexa 488 fluorescence-conjugated goat or donkey IgGs (Invitrogen Corp.) and Cy3- or Cy5-conjugated donkey IgGs (Jackson ImmunoResearch Laboratories). Biotinylated isolectin B4 (*Bandeiraea simplicifolia*) and Cy3-conjugated antibiotin monoclonal antibody were purchased from Sigma-Aldrich.

Microscopy. Pictures for multilabeling immunohistochemistry were taken with Zeiss laser scanning microscope and LSM510 confocal image software (version 3.2; Zeiss). Pictures for ISH were photographed with Axioplan2 microscope (Zeiss) equipped with a CCD camera (AxioCam HRc; Zeiss) and associated software (AxioVision, version 3.1; Zeiss). In combined ISH immunohistochemistry, photos of the same fields were separately taken in black and white, then signals were colored and merged with Adobe Photoshop 6.0 software.

Intraocular injections. One mg/ml of monoclonal hamster anti-mouse integrin β1 antibody (clone HMβ1-1; BD Biosciences — Pharmingen) or VEGF TrapR1R2 (Regeneron Pharmaceuticals) in sterile buffer was injected into the vitreous humor using glass capillary pipettes with a micromanipulator (Drummond Scientific Co.). Control injections of nonspecific hamster IgG or human IgG Fc fragment (Jackson ImmunoResearch Laboratories) were given into contralateral eyes.

Cell sorting and single-step RT-PCR analysis. Retinal cups from 7-day-old Tie2-GFP transgenic mice were digested into single cells with papain and labeled with biotinylated anti-PDGFRα (APA5) (16) antibody followed by allophycocyanin-conjugated streptavidin (Invitrogen Corp.). After cell sorting by BD FACSAria (BD Biosciences), total RNA was isolated from 1000 sorted cells using RNeasy Micro Kit (QIAGEN), and single-step RT-PCR was performed with OneStep RT-PCR Kit (QIAGEN), using specific primers (Tie2, forward, 5′-TCTTGTGTCTGATGCCGAAAC-3′; Tie2, reverse, 5′-GCAGGTAGGAAGGACGCTTGT-3′; PDGFRα, forward, 5′-AATCCTGCAGACGAGAGCAC-3′; PDGFRα, reverse,

5′-GCCACCAAGGGAAAAGATTT-3′; fibronectin, forward, 5′-CTC-CAGGCGTGGAATACACTTACACCATCC-3′; fibronectin, reverse, 5′-GATCAGCATGGACCACTTCTTCCAGAGAGG-3′; β-actin, forward, 5′-TCGTGCGTGACATCAAAGAG-3′; and β-actin, reverse, 5′-TGGA-CAGTGAGGCCAAGATG-3′).

Detection of cell proliferation. For BrdU incorporation assays, an intraperitoneal injection of BrdU (100 μg/g body weight) was given to P5 neonates 2 hours before enucleation, and the whole-mount retinas were stained by goat anti-PDGFRα polyclonal antibody (R&D Systems), briefly fixed with 4% paraformaldehyde, treated with 2 N HCl containing 0.1% Triton X-100 for 60 minutes at 37°C, and further stained with biotinylated monoclonal antibody for BrdU (clone PRB-1; Chemicon International). BrdU-positive retinal astrocytes at edges of the spreading networks were counted in a fixed area photographed by the ×40 objective. These measurements were performed in 4 random areas and then averaged per retina. For each treatment group, 4 retinas from 4 pups were analyzed. The data were expressed as means ± SD and were statistically analyzed by 2-tailed Student's *t* test.

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