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The deletion of *Math5* disrupts retinal blood vessel and glial development in mice

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Abstract

Retinal vascular development is a complex process that is not yet fully understood. The majority of research in this area has focused on astrocytes and the template they form in the inner retina, which precedes endothelial cells in the mouse retina. In humans and dogs, however, astrocyte migration follows behind development of blood vessels, suggesting that other cell types may guide this process. One such cell type is the ganglion cell, which differentiates before blood vessel formation and lies adjacent to the primary retinal vascular plexus. The present study investigated the potential role played by ganglion cells in vascular development using *Math5*^{-/-} mice. It has previously been reported that *Math5* regulates the differentiation of ganglion cells and *Math5*^{-/-} mice have a 95% reduction in these cells. The development of blood vessels and glia was investigated using *Griffonia simplicifolia* isolectin B4 labeling and GFAP immunohistochemistry, respectively. JB-4 analysis demonstrated that the hyaloid vessels arose from choriovitreal vessels adjacent to the optic nerve area. As previously reported, *Math5*^{-/-} mice had a rudimentary optic nerve. The primary retinal vessels did not develop post-natally in the *Math5*^{-/-} mice, however, branches of the hyaloid vasculature eventually dove into the retina and formed the inner retinal capillary networks. An astrocyte template only formed in some areas of the *Math5*^{-/-} retina. In addition, GFAP⁺ Müller cells were seen throughout the retina that had long processes wrapped around the hyaloid vessels. Transmission electron microscopy confirmed Müller cell abnormalities and revealed disruptions in the inner limiting membrane. The present data demonstrates that the loss of ganglion cells in the *Math5*^{-/-} mice is associated with a lack of retinal vascular development.

Keywords

retina; angiogenesis; persistent fetal vasculature; *Math5*; ganglion cells

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Introduction

The retinal vasculature consists of three connected plexi: the superficial in the nerve fiber or ganglion cell layer, the intermediate in the inner plexiform layer, and the deep in the outer plexiform layer. The complex development of this vascular system is complete by birth in the human (Saint-Geniez and D'Amore 2004) but occurs during the first three post-natal weeks in mice (Dorrell et al. 2002) (Fig. 1). The lens and inner retina are nourished by the hyaloid vascular system prior to the development of retinal vessels. These vessels reside in the vitreous, a gel-like substance between the retina and the lens. The hyaloid system regresses as the retinal vessels form so that it is fully regressed by birth in the human and by 3 weeks of age in mice.

The post-natal development of retinal vessels makes the mouse an ideal model for studying the complicated processes involved in retinal vascular formation. To date, much of the research regarding this process has focused on the astrocyte template which, in the mouse, precedes development of the retinal vasculature (Dorrell et al. 2002). Astrocytes, however, migrate and differentiate behind the primary or superficial retinal vascular plexus in humans (Chan-Ling et al. 2004; McLeod et al. 2006; Hasegawa et al. 2008) and the dog (McLeod et al. 1987). This suggests that other cell types may also provide guidance and assembly signals for developing retinal vessels. The close association of retinal angioblasts and endothelial cells with nerve fibers in the neonatal retina suggests that the ganglion cells and their axons may be important in blood vessel development. Indeed, an intimate relationship has been demonstrated between nerves and blood vessels during development elsewhere in the body (Mukouyama et al. 2002; Carmeliet 2003). In addition, it has been demonstrated that GRP91 produced by ganglion cells is necessary for the initial stages of normal retinal vascular development (Sapieha et al. 2008).

One way to investigate the possible contribution of ganglion cells to retinal blood vessel formation is to look at disease states or animal models in which these cells are lost. Conflicting reports have been obtained from fetal human eyes with anencephaly, which exhibit early ganglion cell loss. In one report, blood vessel and astrocyte development were shown to continue normally despite the loss of ganglion cell axons (Hendrickson et al. 2006). In another study, however, it was reported that the development of blood vessels and astrocytes was attenuated in the anencephalic retina (Kim et al. 2010). These studies suggest a potential role for ganglion cells in human retinal vascular development. *Math5* (*atoh7*; *ath5*) is a gene that has been widely studied and shown to play a key role in the differentiation of retinal ganglion cells (Brown et al. 2001; Wang et al. 2001; Le et al. 2006; Moshiri et al. 2008). Mutations in *MATH5* cause nonsyndromic congenital retinal nonattachment, a condition involving changes to retinal vasculature (Ghiasvand et al. 2011). The deletion of *Math5* in the mouse causes a 95% reduction in ganglion cells (Brown et al. 2001; Wang et al. 2001). Interestingly, this reduction is followed by an increase in photoreceptor cells and Müller glia (Brown et al. 2001; Le et al. 2006; Moshiri et al. 2008). It has been speculated that *Math5* triggers the differentiation of ganglion cells and, in its absence, the ganglion cell progenitors are forced to take on other retinal cell fates.

Math5^{-/-} mice are an ideal model in which to further investigate the influence of ganglion cells on retinal vascular development. While other retinal defects are noted, these are secondary to the loss of ganglion cells and, in many cases, after the primary retinal vasculature normally develops. Brown et al (2001) noted a very small optic stalk and the apparent loss of the central retinal artery in *Math5*^{-/-} mice as well as ectopic vessels in retinal cross sections (Brown et al. 2001). The retinal vasculature in *Math5*^{-/-} mice, however, has yet to be described. The intent of this study was to determine if retinal vessels can develop neonatally in the *Math5*^{-/-} mice. The present study describes the lack of normal

retinal vascular development and the persistence of the hyaloid vasculature in *Math5*^{-/-}(*Atoh7*^{tm1Gan/tm1Gan}; *Math5*^{LaZc/LacZ})mice. In addition, astrocytes and Müller cells were investigated to determine the influence of nerve fibers on their migration and differentiation.

Materials and Methods

Generation of *Math5*^{-/-} mice and genotyping

The *Math5*^{-/-}(*Atoh7*^{tm1Gan})mouse line used in this study has been previously described (Wang et al. 2001). Mice were on a mixed C57BL/6J:129S/SvEV background and heterozygous littermates were used as controls. Mice were maintained and all procedures performed according to ARVO guidelines. Genotyping of these mice was performed by PCR to detect the presence/absence of the *Math5* orf and neo cassette. Mice positive for neo but negative for *Math5* orf were *Math5*^{-/-}, and those positive for both were heterozygous. Primers used for neo were: CAA GGT GAG ATG ACA GGA GA and GAGAGG CTA TTC GGC TAT GA; and primers used for *Math5* orf were ACA AGA AGCTG T CCA AGT AC and CAG GGT CTA CCT GGA GCC TA. These primer sets generated bands of 171 bp (*Math5* orf) and 234 bp (neo). A minimum of 2 eyes (from separate mice) were examined using each technique at each age for both controls and mutants

Tissue collection and immunohistochemistry

Immunofluorescence followed by confocal microscopy was used to investigate the retinal vasculature, astrocytes, and nerve fibers in retinal flatmounts at P4 when blood vessels are forming, at P7 when the primary plexus is complete, at P14 when the secondary or deep retinal plexus is complete, and in the adult (Fig. 1). Enucleated eyes were fixed overnight in 2% PFA in Tris buffered saline (TBS) prior to the dissection of retinas and immunofluorescent labeling. Retinas were blocked with 5% serum in TBS containing 1% Triton X100 (TBST) for 6 hrs followed by overnight incubation with the primary antibody diluted in 2% serum in TBST. After a series of washes, retinas were incubated for 3 hrs in fluorescent conjugated secondary antibodies (1:300; *Jackson ImmunoResearch*) along with *Griffonia simplicifolia* isolectin B4 conjugated to FITC (GS isolectin; 1:200; *Invitrogen*; 121411). For cross section analysis, eyes were cryopreserved and immunohistochemistry was performed as previously described (Baba et al. 2009; Baba et al. 2010). Primary antibodies included: rabbit anti-glial fibrillary acidic protein (GFAP; 1:200; *Dako*; Z0334), rabbit anti-IBA-1 (1:1000; *Wako Chemicals USA*; 019-19741); rat anti-platelet-derived growth factor alpha (PDGFRα; 1:500; CD140a; *R&D*, 558774), rabbit anti-pan laminin (1:750; *Sigma*; L9393), and rabbit anti-neurofilament (1:200; *Abcam*, ab9034). 4',6-diamidino-2-phenylindole (DAPI; 1:1000 *Invitrogen*, D21490) was used for labeling nuclei. Images were captured using a *Zeiss 510 Meta* confocal microscope.

Transmission electron microscopy (TEM) and JB-4

Eyes for TEM and JB-4 methacrylate (*Polyscience*) analysis were fixed in 2.5% PFA/2% glutaraldehyde in 0.1 M cacodylate buffer and processed as previously described (McLeod et al. 1987; Baba et al. 2009; Baba et al. 2010). JB-4 sections were stained with either thionin or periodic acid schiff with hematoxylin.

Results

Developing blood vessels followed ganglion cell nerve fibers in the wild type mouse

The potential relationship between developing blood vessels and ganglion cells was first investigated by labeling post-natal day (P)1 control retinas with *Griffonia simplicifolia* isolectin B4 (GS isolectin) and an antibody against neurofilament to visualize the blood

vessels and nerve fibers, respectively (Fig. 2). Developing blood vessels normally follow the radial pattern of ganglion cell nerve fibers. Furthermore, endothelial cell filopodia could be seen extending along ganglion cell axons (Fig. 2B). These observations suggest that ganglion cells may provide guidance to developing retinal vessels.

***Math5*^{-/-} mice have a drastic reduction in nerve fibers**

The loss of nerve fibers in the *Math5*^{-/-} mice was apparent with immunohistochemistry in neonatal mice. Numerous nerve fibers extended radially from the optic nerve to the periphery in the control retina (Fig. 2A) and they coalesce to form bundles (Fig. 2B). Retinal flatmounts from *Math5*^{-/-} mice labeled with anti-neurofilament, however, contained very few nerve fibers (Fig. 2C). These fibers were thin, individual axons as opposed to the fasciculated axons seen in controls. These tortuous axons overlapped one another in many areas. *Math5*^{-/-} mice appeared to have a rudimentary and off-centered optic nerve head in flatmounts (Fig. 2C). These data confirmed previous observations that *Math5*^{-/-} mice have a drastic reduction in ganglion cells (Brown et al. 2001; Wang et al. 2001; Moshiri et al. 2008).

Retinal vessels did not form in the *Math5*^{-/-} retina despite the presence of astrocytes

The retinal vasculature and glia were investigated by labeling with GS isoelectin [labels blood vessels, hyalocytes (dendritic cells in the vitreous), and microglia] and an antibody against GFAP, respectively. GFAP is predominantly expressed in astrocytes but can also be expressed by Müller cells in diseased states. At P4, the littermate control retinas had a developing primary or superficial retinal vascular plexus in the nerve fiber layer which extended almost half way across the retina behind a complete astrocyte template (Fig. 3A). Hyaloid vessels or the fetal vasculature of vitreous were still present but were easily removed during dissection. In contrast, the retinas of *Math5*^{-/-} mice had astrocytes but no retinal vessels (Fig. 3B–I). In addition, the hyaloid vessels, which were disorganized and emerged from an off center artery, had filopodia that appeared to be forming a capillary network in the vitreous (Fig. 3B, J). Although astrocytes were found throughout the retina, only some areas had the honeycomb-like template observed in controls (Fig. 3B–E). Astrocytes had overpopulated the central *Math5*^{-/-} retina (Fig. 3D), but were sparse in other areas, especially in the periphery (Fig. 3C, F). Many Müller cell processes and endfeet were also GFAP⁺. These were most evident in areas lacking astrocytes. Some astrocytes and Müller cells extended long, thin processes across the retina and even into the vitreous (Fig. 3F). Unfortunately, it was not possible to distinguish the origin of these processes because markers are not available at this neonatal stage to conclusively differentiate these glial cells. Furthermore, Müller cells are known to be mislocalized in the *Math5*^{-/-} retina, with some being found in the nerve fiber layer. These cells appeared to touch vitreal vessels but there was no astrocyte ensheathment of the preretinal blood vessels.

Despite the lack of blood vessels, GS isoelectin⁺ cells were present throughout the *Math5*^{-/-} retina, being most prominent in the periphery (Fig. 3C). In many cases, these cells appeared to align with each other, resembling angioblasts in early vascular cord or tube formation (Fig. 3G–I) (McLeod et al. 1987; McLeod et al. 2006; Hasegawa et al. 2008). It is noteworthy that this tube development was not always in alignment with astrocytes, suggesting that an astrocyte template is not necessary for early vessel assembly. Filopodia also extended from fetal vasculature branches in vitreous but did not always follow an astrocyte template (Fig. 3J).

GS isoelectin was again used to visualize blood vessels in P4 cryosections while anti-PDGFR α and anti-laminin were used to label astrocytes and the inner limiting membrane (ILM) of retina, respectively. The ILM is an extracellular matrix limiting membrane, which

separates the retina from the vitreous, keeping cells within the retina. Therefore, this labeling scheme determined which cells and blood vessels were in the retina and which were in the vitreous. In sections through the optic nerve head, astrocytes and blood vessels extended about midway across the retina from the optic nerve under the laminin⁺ ILM in the P4 littermate control eyes (Fig. 3K). In the mutant eyes, fewer PDGFR α -positive astrocytes and no retinal blood vessels were found on the retinal side of the ILM, confirming flatmount results (Fig. 3L). In some cases, individual GS isolectin⁺ cells were observed on both the vitreal and retinal side of the ILM where breaks in this structure were apparent. It should be noted that hyalocytes, the dendritic cell of the vitreous, also label with GS isolectin.

JB-4 glycol methacrylate serial sections of *Math5*^{-/-} mice stained with periodic acid-schiff and hematoxylin demonstrated hyaloid vessels making close contact with the retina. In addition, astrocyte-like cells were found on the surface of these vessels (Fig. 4A, B). The ultrastructure of the ILM was investigated using transmission electron microscopy (TEM). The control retinas had a double layered ILM on top of Müller cell endfeet across the entire retina (Fig. 4C). In the *Math5*^{-/-} eyes, however, the ILM varied in appearance across the retina (Fig. 4D). While some areas had a normal, double-layered ILM, this was either replaced by a thin, diffuse structure or completely lost in other areas. Many Müller cells did not terminate in endfeet but rather extended long processes across the retina, similar to those seen with anti-GFAP labeling in the flatmount retina (Fig. 3F).

Vascular islands had begun forming in the *Math5*^{-/-} retina at P7

Retinas were next examined at P7 when the primary retinal vasculature was almost complete in the littermate controls (Fig. 5A). In contrast, there was no primary retinal vascular plexus in the *Math5*^{-/-} mice (Fig. 5B). Although there appeared to be more astrocytes in the central retina than was seen at P4, these glial cells were still sparse in the periphery of *Math5*^{-/-} retinas (Fig. 5B). Examination of retinas with the vitreous intact demonstrated GFAP⁺ processes extending around hyaloid vessels as well as a dense astrocyte population in the central retina (Fig. 5C). GS isolectin⁺ cells were again observed across the retina and islands of vessels were beginning to form at P7 (Fig. 5B, D). These vascular islands were not connected to the hyaloid vasculature but rather appeared to have formed by assembly of individual GS isolectin⁺ cells (Fig. 3G–I). GFAP⁺ glial cells underlying these vascular islands formed an extensive, dense network, with many more astrocytes than were seen in control templates (Fig. 5D). In addition, these glial networks had long thin processes radiating from them on which GS isolectin⁺ cells extended filopodia (Fig. 5D). The lack of a normal vascular plexus and the presence of astrocytes within the retina were confirmed in retinal cross sections (Fig. 6). As was observed in the flatmounts at P7 (Fig. 5), individual GS isolectin⁺ cells but no retinal vasculature plexus was observed in the *Math5*^{-/-} retinas. In the central retina, a vascular sprout was observed traversing the ILM (Fig. 6A, B). This could represent an abnormal hyaloid artery or hyaloid vessels making contact with the retina as was observed in other areas (Fig. 6B–D). This vessel, however, was only observed in 1–2 sections making determination of its origin difficult.

GS isolectin is known to label microglia as well as blood vessels. Therefore, the origin of the individual GS isolectin⁺ was investigated by double labeling with GS isolectin and a microglia marker, IBA-1. In littermate control eyes, GS isolectin labeling demonstrated primarily vessels in the nerve fiber layer as well as a number of IBA-1⁺/GS isolectin⁻ cells within the retina (Fig. 6E). The individual GS isolectin⁺ cells in the *Math5*^{-/-} retinas were of a mixed origin with some being GS isolectin⁺/IBA-1⁻, others GS isolectin⁺/IBA-1⁺, and still others positive for only IBA-1 (Fig. 6F–G). This observation suggests that some of these cells were not microglia. The fact that they formed tube-like structures in the retina (Fig. 3G–H) and blood vessel-like islands (Fig. 5B,D) indicates that these cells could be angioblasts or vascular precursor cells.

The hyaloid vessels of *Math5*^{-/-} mice were of choriovitreal origin and sprout to enter the retina rather than regressing

Serial JB-4 whole eye sections taken from P7 mice were analyzed to further investigate the origin of the hyaloid vasculature and the development of the retinal vasculature. In the control, the hyaloid artery extended into the vitreous from the central artery which arose from the optic nerve (Fig. 7A). In the *Math5*^{-/-} eye, a choriovitreal vessel protruded through the retina into the vitreous near but not directly adjacent to a rudimentary optic nerve to form the hyaloid artery (Fig. 7B). Interestingly, this rudimentary optic nerve was only noted in two to three 2.5µm sections, indicating that this structure is very small. These observations confirmed those of previous studies that *Math5*^{-/-} mutants have little or no optic nerve (Brown et al. 2001; Moshiri et al. 2008).

At P14, hyaloid vessels, which are normally well regressed at this age, were observed branching into the retina in JB-4 sections (Fig. 7). These vessels also appeared to be actively proliferating as there were increased preretinal vessels (Fig. 7D). Labeling of *Math5*^{-/-} cryosections with anti-PDGFRα, anti-laminin, and GS isolectin was used to further investigate this finding. One large vessel in the central retina crossed the ILM near where the optic nerve should lie (Fig. 6H). While this structure appeared to be entering the retina, it could also be the abnormal hyaloid artery noted in JB-4 analysis (Fig. 7B). It is of note, however, that adjacent to this vessel traversing the ILM, there were retinal vessels while only individual GS isolectin⁺ cells were found on the opposite side of this retina (Fig. 6H). Labeling sections with anti-GFAP and GS isolectin also demonstrated hyaloid vessels entering the retina where they dove to form the deep vascular plexus (Fig. 6I–J). Examples of diving vessels were observed in the central, mid, and peripheral retina. Anti-GFAP labeling confirmed the presence of astrocytes in the nerve fiber layer and demonstrated the activation of Müller cells throughout the *Math5*^{-/-} retina (Fig. 6J). In addition to expressing GFAP, many Müller cells had tortuous processes that extended along the nerve fiber layer rather than terminating as end feet. In some cases, vessels entering the retina were in between two adjacent Müller cell processes or Müller cell processes wrapped around these vessels (Fig. 6J insert).

Both retinal and vitreal vessels continued to proliferate in the *Math5*^{-/-} retina so that vessels were observed in the outer and inner plexiform layers as well as the nerve fiber layer by three weeks of age (Fig. 8A–C). In Z stacks taken from flatmount retinas, it was apparent that the hyaloid vessels, which had fully regressed in wild type animals, persisted and dove into the retina, giving rise to these inner retinal vessels (Fig. 8A–C). Endothelial cell filopodia were still observed in both the retina and vitreous, indicating that vascular development was still continuing at this age (Fig. 8F). The degree of vascularization and astrocyte template density varied throughout the *Math5*^{-/-} retina (Fig. 8G). Interestingly, there was not always a correlation between astrocyte and blood vessel density. While some peripheral areas completely lacked both vessels and an astrocyte template (Fig. 8E) others had a very dense astrocyte template but no vessels (Fig. 8D, G). Still other areas had three layers of vessels within the retina as well as persistent hyaloid vessels and astrocytes (Fig. 8A–C). In addition to astrocytes, Müller cell endfeet and processes were visible with anti-GFAP labeling (Fig. 8E–F). GFAP⁺ processes could be seen wrapping around some but not all hyaloid vessels (Fig. 8A, D). Unlike many other rodent models with a persistent hyaloid vasculature (Zhang et al. 2005; Edwards et al. 2010), however, not all hyaloid vessels were ensheathed with astrocytes. In fact, not even all retinal vessels were associated with astrocytes. In addition, as was noted at earlier ages, not all filopodia extended along an astrocyte template (Fig. 8F). These observations suggest that the astrocyte-endothelial cell association is impaired in the *Math5*^{-/-} mice and is not necessary to form filopodia or a vasculature.

The diving of hyaloid vessels to form the three layers of retinal vessels was verified by cryopreserving and sectioning of labeled retinal flatmounts (Fig. 8H–K). This also confirmed the variability in vasculature within even a single *Math5*^{-/-} retina. Cross section analysis also demonstrated Müller cell processes around hyaloid vessels both in the vitreous and as they dove into the retina (Fig. 8J, 6J). Blood vessel development in the *Math5*^{-/-} retina is summarized in Figure 1. A hyaloid vasculature formed from an off-center central retinal artery. The vasa hyaloidea propria proliferates and then dives into the retina to form a retinal vasculature.

Discussion

This study clearly demonstrates the absence of a primary retinal vasculature and the persistence of the hyaloid vessels in mice lacking *Math5*. In addition, abnormalities in the ILM of *Math5*^{-/-} mice were observed. Since the primary defect in *Math5*^{-/-} mice is a reduction in ganglion cells, these data suggest that ganglion cells are critical for retinal vascular and ILM development.

One cannot ignore, however, the potential contribution of other retinal changes in the *Math5*^{-/-} retina to the observed vascular abnormalities. Of particular interest are the changes in Müller cells. The positioning of Müller cell endfeet, the extension of Müller cell processes into the vitreous and apparent breaks in the ILM could disrupt the formation of retinal vasculature. While these structural changes are noted throughout the retina, there are areas in which the endfeet and ILM are normal. Mice with little if any ILM have limited retinal vessels in the peripapillary retina (Edwards et al. 2010; Edwards et al. 2011), indicating that defects to this structure would not prevent formation of these vessels. Other neurons, such as amacrine cells, are also affected in the *Math5* mice and their metabolic activity could alter retinal vascular development. It is important to note that these changes are subsequent to the loss of ganglion cells. In fact, Müller cells and some non-ganglion cell neurons are not fully differentiated prior to the initiation of retinal blood vessel development. Therefore, they may contribute to the severity of the phenotype but are not likely to cause the vascular defects.

The fact that ganglion cells are responsible for the vascular defects in the *Math5*^{-/-} mice is supported by studies in transgenic *brn3b* mice, which experience early ganglion cell loss and fail to form a superficial retinal vasculature (Sapieha et al. 2008). While this previous study suggests that ganglion cells are crucial for retinal vascular development, it investigated only the initial stages of retinal vascular development. The current study expanded on this observation by investigating the formation of all three vascular plexi in mice with reduced ganglion cells. Previous research showing that ganglion cell-derived growth factors are important in pathological retinal vascular development provide further support to the idea that ganglion cells are responsible for the vascular defects in the *Math5*^{-/-} retinas (Fukushima et al. 2011; Joyal et al. 2011).

Another possible explanation is that *Math5* itself has pro-angiogenic properties. Indeed, another basic helix-loop-helix protein, TAL-1, is upregulated during in vitro capillary formation (Lazrak et al. 2004). Furthermore, overexpression of TAL-1 increased in vivo angiogenesis (Lazrak et al. 2004). One would expect a gene functioning as a pro-angiogenic factor during retinal development to be highly expressed in the nerve fiber layer in the first post-natal weeks. *Math5*, however, is expressed most abundantly during embryonic stages, with only low levels observed post-natally (Brown et al. 2001; Fu et al. 2009). Furthermore, *Math5* is only observed in the peripheral retina at birth and is completely shut off at P3 (Fu et al. 2009), when retinal vessels are actively forming. Therefore, while *Math5*, as a helix-

loop-helix protein, may have pro-angiogenic properties, its expression pattern does not support such a role in the retina.

Also of note is the formation of vessel-like structures by individual GS isolectin⁺ cells with angioblast-like morphology in the retina. It is particularly interesting that these structures formed in areas lacking astrocytes as well as areas with a dense population of astrocytes. This is the first report of potential angioblasts in the mouse retina during development. Unfortunately, a good angioblast marker is not available for the mouse. Future studies will be directed at determining the identity and origin of these cells.

The migration of astrocytes into the retina despite the drastic reduction in ganglion cells is also a noteworthy observation. This suggests that ganglion cell-derived PDGFAA (Fruttiger et al. 1996) is not the only stimulus for astrocyte migration and demonstrates the need for further studies to identify other stimuli as well as the cells producing them. It would also be interesting to determine how astrocytes enter the retina in the *Math5*^{-/-} mice because Raff and associates have demonstrated that astrocytes are emigrants from the optic nerve, which is rudimentary in these mice (Watanabe and Raff 1988).

Finally, this is the first report to demonstrate an abnormal ILM in the *Math5*^{-/-} mice and suggests that ganglion cells may be important for the formation of this structure as well. While it is beyond the scope of this study to characterize the ILM in these mice, the presence of laminin suggests that the extracellular matrix proteins are present. A detailed developmental study will be required to determine whether the ILM and Müller cell endfeet interface develops normally and then regresses or if the development of this structure is impaired by the loss of *Math5* and ganglion cells. One could hypothesize that Müller cells use the ganglion cell axons as a border and contact between these cells stimulates endfeet formation. The extension of Müller cell processes into the vitreous is known to cause the formation of epiretinal membranes, which can pull on the retina, leading to retinal detachment in humans. Although beyond the scope of the present study, it would be interesting to examine the *Math5*^{-/-} mice at older ages to see if epiretinal membranes form or if retinal detachment occurs. Indeed humans with *MATH5* mutations experience retinal detachment (Ghiasvand et al. 2011). Further investigation is required to fully understand the role of ganglion cells in ILM formation, a process that is not fully understood.

In conclusion, this study supports the use of the retina for elucidating neurovascular relationships and patterning during development. The lack of retinal blood vessels in the neonatal *Math5*^{-/-} mouse clearly suggests that ganglion cells are important for retinal vascular development. This study also demonstrates that retinal vascular development and filopodia associated with it in mouse can occur without an astrocyte template. Finally, the formation of vascular islands in the otherwise avascular *Math5*^{-/-} retina suggests that vasculogenesis can occur in the murine retina.

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Abbreviations

GFAP	Glial fibrillary acidid protein
GS isolectin	<i>Griffonia simplicifolia</i> isolectin B4

ILM	Inner limiting membrane
PDGFRα	Platelet derived growth factor alpha
P	Post-natal day
TEM	Transmission electron microscopy

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Highlights

- *Math5*^{-/-} mice lack a primary retinal vasculature.
- Blood vessel-like islands form in the avascular neonatal *Math5*^{-/-} retina suggesting that vasculogenesis can occur in mouse retina.
- *Math5*^{-/-} astrocytes do not form a normal template.
- *Math5*^{-/-} astrocytes extend into the vitreous and make contact with hyaloid vessels.
- *Math5*^{-/-} Muller cells extend beyond an abnormally thin ILM into the vitreous.
- Hyaloid vessels persists in *Math5*^{-/-} mice and branch into the retina, forming 3 vascular layers
- At 3 weeks, *Math5*^{-/-} retinas have 3 layers of retinal blood vessels in some areas.

Figure 1. A schematic drawing of retinal vascular development

The key points in vascular development of the normal mouse retina (top) and the *Math5*^{-/-} retina (bottom) are diagramed. Solid red lines depict blood vessels either in the retina or vitreous while broken red lines depict the regressing hyaloid vasculature. At P1, vessels have begun forming in the control retina but not in the *Math5*^{-/-} retina. Hyaloid vessels are regressing in the control by P7 and the primary retinal plexus is complete while in the *Math5*^{-/-} retina, hyaloid vessels have undergone some regression but those closest to the retina have proliferated. The retinal vasculature is complete at P21 in the control retina and no hyaloid vessels remain in the vitreous. The hyaloid vessels in the *Math5*^{-/-} retina at P21 persist and have proliferated and formed a retinal vasculature.

Figure 2. Neonatal retina flatmounts labeled with rabbit anti-neurofilament (red)

A drastic reduction in nerve fibers was observed *Math5*^{-/-} mice (C) compared to littermate controls (A). GS isoelectin labeling (green) along with neurofilament (red) showed that blood vessels grow along the nerve fibers in the littermate control (B). Scale bars indicate (A & C: 50 μ m; B: 20 μ m).

Figure 3. Astrocyte and blood vessels at P4

Retinal flatmounts from P4 mice were labeled with GFAP (astrocytes; red) and GS isolectin (blood vessels; green). In a control littermate (A), retinal vessels were forming along an astrocyte template. In contrast, no retinal vessels were observed in the *Math5*^{-/-} retina and the hyaloid vasculature appeared disorganized (B). The GS isolectin⁺ vessels are hyaloid vessels. Individual GS isolectin⁺ cells were, however, observed across the retina (B–F). The astrocyte template was normal in some areas (top B) but attenuated or completely missing in others (asterisk in C). Astrocytes were concentrated in the central retina (D) and were sparse in the mid (E) and peripheral retina (F). GFAP⁺ Müller cells were also observed in areas void of astrocytes (bottom F). In addition, the *Math5*^{-/-} retinas contained unusually long GFAP⁺ processes (double arrow), which could stem from Müller cells or astrocytes. In many areas, the individual GS isolectin⁺ cells were aligned in structures resembling tube formations (E open arrow; G, H). Interestingly, these formations (H, GS lectin only) were formed without an astrocyte template (I, GFAP only)). In some areas, the hyaloid vessels were sprouting with filopodia extending towards the astrocyte template within the retina (J). Hyalocytes in the vitreous were also labeled with GS isolectin. Cross sections labeled with anti-PDGFR α (astrocytes; light blue), anti-laminin (ILM; red), and GS isolectin (blood vessels; green) confirmed that control retinas contained both astrocytes and blood vessels (arrow) (K) while only astrocytes (double arrows) were found in the *Math5*^{-/-} retina (L). Scale bars indicate (A–C, K, L: 40 μ m; D–F: 20 μ m; G–I, J: 10 μ m).

Figure 4. JB-4 and TEM of P4 retinas

(A, B) JB-4 of P4 *Math5*^{-/-} eyes showed that the hyaloid vasculature (arrowheads) was closely associated with the retina. Astrocytes (double arrow) were observed within the retina and on the surface of these vessels. (C) TEM of a control eye demonstrated a double layered ILM (opposing arrows) on top of Müller cell endfeet throughout the retina. Hyaloid vessels (arrowhead; lumen indicated with L) in the vitreous do not make contact with the ILM or retinal cells. (D) In contrast, the ILM in *Math5*^{-/-} retinas varied in thickness across the retina being normal in some areas (opposing arrows) but were completely lacking in others (to right of opposing arrows). Hyaloid vessels (arrowheads) came in close contact with the retina and the ILM was missing beneath these contact points. Astrocytes (double arrows) extended processes (asterisk) toward the hyaloid vessels. Scale bars indicate (A, B: 20 μ m; C, D: 1 μ m).

Figure 5. P7 retinal flatmounts labeled with GFAP (red) and GS isolectin (green)

The almost complete primary vascular plexus is evident in the control retina (A). (B) The *Math5*^{-/-} retinas had no retinal vessels except the islands of blood vessels that had begun forming (top left in B). Individual GS isolectin⁺ cells were again observed across the entire retina. The astrocyte template continued to be more dense in the central retina (left) and sparse in the periphery (right). (C) The hyaloid vessels were very dense and astrocytes condensed in the central retina and GFAP⁺ processes wrapped around the hyaloid vessels. Examination of blood vessel islands demonstrated a dense astrocyte network surrounding and enwrapping these vessels (D). Just beyond these islands, long GFAP⁺ processes were observed which could belong to either astrocytes or Müller cells. Scale bars indicate (A–C: 40 μ m; D: 20 μ m).

Figure 6. Cryosections of *Math5*^{-/-} eyes labeled with anti- PDGFR α (light blue) anti-laminin (red), and GS isolectin (green)
 At P7, only individual GS isolectin⁺ cells (arrows) and astrocytes were observed within the *Math5*^{-/-} retina (A). Near the rudimentary optic nerve area, there was break in the ILM through which vessels appear to be either entering or exiting the retina (A, B). (B, C) When only the blood vessel and laminin channels are shown at higher magnification, the hyaloid vessels (arrowhead from A) and vessels in the inner retina (arrow from A) appear to cross the laminin⁺ ILM. In other areas, hyaloid vessels (arrowheads) made very close contact with the retina (D). The insert in D shows single GS isolectin⁺ cells in the retina. Sections from the same eyes were labeled with IBA-1 to determine whether the individual GS isolectin⁺ cells were microglia. (E) In the control retina, a few individual GS isolectin⁺ cells were observed that were also IBA-1⁺. Arrow indicates an area of colocalization which is shown in higher magnification in the insert. (F) The *Math5*^{-/-} retinas contained single GS isolectin⁺ cells and some IBA-1⁺ cells, indicating that some GS isolectin⁺ cells were not microglia. In addition, some cells were only IBA-1⁺. (G) Where a hyaloid vessel appeared to enter the retina, GS isolectin⁺/IBA-1⁺ cells (yellow) were in both the vitreous and the retina. A similar vitreal-retina branching to that seen at P7 was observed at P14 (H). Hyaloid vessels (arrowhead) and retinal vessels (arrow) were closely associated with the ILM (red). Labeling with anti-GFAP (red) and GS isolectin (green) demonstrated the diving of vitreal vessels (arrows) into the retina as far as the inner plexiform layer (IPL) (I). Many Müller cells (double arrow) were GFAP⁺, indicating their activation (J). Processes from these glial cells wrapped around hyaloid vessels as was evident in both retinal flatmounts (J insert) and sections (J). Scale bars indicate (A, D–F, H–J: 40 μ m; B, C: 5 μ m).

Figure 7. JB-4 sections from P7 and P14 *Math5*^{-/-} eyes

(A) JB-4 sections through a P7 control eye showed the central artery extending through the optic nerve (between arrowheads) and the hyaloid artery forming in the vitreous (black arrowhead). (B) In contrast, the hyaloid artery (black arrowhead) in the P7 *Math5*^{-/-} eyes arose from the choroid near a rudimentary optic nerve (between white arrowheads). (C) By P14, the hyaloid vasculature (arrowhead) had invaded the *Math5*^{-/-} retina. Many hyaloid vessels had astrocytes (double arrow) resting on them. (D) The hyaloid vasculature (arrowhead) appears to have proliferated. Scale bars indicate 20 μ m in all images.

Figure 8. Three week old *Math5*^{-/-} retinal flatmounts labeled with anti-GFAP (red) and GS isolectin (green)

Sections from a Z stack showing (A) the hyaloid vasculature, (B) the intermediate vascular plexus, and (C) the deep retinal vasculature. The degree of vascularization varied within a single retina with some peripheral areas having no vessels in the retina or vitreous (D–G). In some areas, astrocytes extended long processes (double arrow in D) while others were completely void of astrocytes but Müller cell endfeet and processes were GFAP⁺ (E). GS isolectin⁺ filopodia extended into the retina with and without an astrocyte template (F). A retinal flatmount (G) was embedded and cross sectioned to demonstrate the diving of hyaloid vessels and formation of vessels within the retina (H, J, K). GFAP⁺ astrocytes or Müller cell processes extended into the vitreous and wrapped around hyaloid vessels (H–J). (K) Retinal vessels (arrow) appeared to traverse the inner retinal glia. Scale bars indicate (A–F: 20 μ m; G: 200 μ m; H: 20 μ m; I: 10 μ m).