Role of laminins containing beta2 and gamma3 chains in regulating retinal angiogenesis

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Abstract

Retinal vasculature related pathologies account for much of the global blinding disease. Proliferative diabetic retinopathy (PDR) is the main cause of acquired blindness in working adults and retinopathy of prematurity (ROP) is the leader cause of acquired blindness in children. A thorough understanding of the cell intrinsic and extrinsic regulation of vascular development in the retina is required to address such vasculature related pathologies. Unlike other organs, specialized glial cells called astrocytes guide vascular development in the retina. The astrocytes invade the retina around birth and form a template in the retina for endothelial cells to migrate on.

It is well known that an astrocyte-derived template is crucial for vascular development in the retina; however, the factors that guide astrocytes into the retina remain unknown. It is important to know the mechanism by which astrocytes are guided, because models for ROP and diabetic retinopathy (DR) show degeneration of astrocytes. Maintaining astrocytes under such conditions reduces neovascularization events.

In this study, we show that astrocyte migration into the retina is dependent upon laminins deposited at the inner limiting membrane (ILM). Deletion of *Lamc3* gene reduced astrocyte migration rate at early stages and recovers at later stages. Deletion of *Lamb2* and *Lamb2:c3* genes severely affect astrocyte migration and spatial patterning. Astrocytes in these null retinas clump together

and appear like a honeycomb. Interestingly, these *Lamb2* and *Lamb2:c3-* also show a loss of integrin β1 expression specifically in astrocytes.

Using both *in vitro* and *ex vivo* techniques we show that laminins induce astrocyte migration in an isoform specific manner. Exogenous treatment of *Lamb2:c3-* null retinas with EHS-laminin rescues both migration and spatial patterning of astrocytes. Moreover, we show that exogenous laminin restores integrin-β1 in Lamb2:c3- null astrocytes in culture.

Functional blocking of integrin-β1 receptor affects astrocyte migration in the presence of laminin, suggesting that laminin- integrin-β1 interaction is critical for astrocyte migration. In addition, functional blocking of integrin-β1 affects the recruitment of both ILK and FAK, which are downstream kinases that are important for cell migration. siRNA knockdown of FAK affects astrocyte filopodial extension and α-actinin organization during migration. This suggests that laminin likely orchestrates astrocyte migration through integrin-β1- FAK mediated signaling mechanism.

As mentioned above, an astrocyte template is critical for vascular development in the retina. As astrocyte migratory pattern were affected in laminin nulls, we analyzed vascular growth in these nulls. Lamc3- null retinas show a delay in vascular growth progression at early stages with increased vascular branches at the leading edges. At later stages vascular growth was complete and appeared as similar to WT. Deletion of Lamb2 and Lamb2:c3- genes however, severely affected vascular growth with persistent hyaloid vessels (commonly observed in

ROP). These nulls also show abnormal astrocyte-endothelial interactions, which resulted in leaky vasculature, analyzed by fluorescein angiography.

In summary, our study clearly suggests that laminin mediated signaling mechanism is critical for astrocyte migration and spatial organization and subsequent vascular growth.

INTRODUCTION

The Cell-Matrix Interface A Possible Target For Treating Retinal Vascular Related Pathologies

Gopalan Gnanaguru and William J. Brunken.

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Introduction

Retinal angiogenesis is a complex process, which involves cell-cell, and cell-matrix interactions. Any abnormalities associated with the retinal vasculature during development or at later stages of life will lead to severe pathologies resulting in blindness. For example, retinopathy of prematurity (ROP) is a retinal vasculature associated pathology that occurs in prematurely born infants (Chen and Smith 2012; Chen et al., 2011). Whereas, proliferative diabetic retinopathy (PDR) occurs in working age adults (Gariano and Gardner., 2005) and wet age related macular degeneration affects older adults (Chen and Smith., 2012) Together, these retinal blood vessel associated pathologies are major causes of global blindness. To treat or to find a novel target for these complex pathologies it is necessary to carefully understand both cell intrinsic and extrinsic players, which regulate the development and function of retinal blood vessels. The goal of this study is to understand the role of ECM-cell interactions during development and correlate their contributions to the pathobiologic events of neovascularization. This will finally help us to identify potential therapeutic candidates to control disease progression.

Embryonic Blood Supply

Early in development, the inner portion of the eye is nourished by the hyaloid vascular system (HVS), which is a transient intraocular arterial supply attached to the posterior pole of the lens (Fruttiger., 2007). After birth, myeloid cells mediate a gradual regression of the HVS (Diez-Roux and Lang 1997; Lee et al., 2009). In human clinical conditions -- such as ROP, Familial exudative vitreoretinopathy and Pearson's syndrome -- regression of the HVS is altered, resulting in persistent hyaloid vessels (Bredrup et al., 2008; Nissenkorn et al., 1988; Chang-Godlinich et al., 1999). Mouse models in which various laminin chains are deleted also show persistent hyaloid vessels (Edwards et al., 2008; Gnanaguru et al., 2012) as do mouse mutants with in which laminin binding is disrupted (Lee et al., 2005; Takahashi et al., 2011) and other basement membrane mutants (Hurskainen et al 2005).

Laminins regulation of hyaloid vessels regression is mediated via integrin $\alpha6\beta1$. Integrin activation results in changes in chemokines such as TNF, INF-α, and IFN-γ (Milner and Campbell., 2002) and the chemokines in turn may interact with microglia. Thus, the effects of laminin deletion are likely to be the result of the failure to activate or recruit microglia during hyaloid vessel regression. It is of interest to analyze clinical samples where persistent fetal vessels are observed for alterations in ECM pathways, particularly integrin-mediated pathways. If these pathways are deficient, it may then be possible to transiently activate microglia to promote HSV regression.

Retinal Vascular Development

The retinal vascular system consists of three interconnected vascular layers: superficial, intermediate and deep. The development of the superficial layer is the initial step in retinal angiogenesis and is dependent upon astrocytes (Fruttiger., 2007). Once the superficial vasculature reaches the periphery of the retina, the formation of the deep and intermediate plexiform layers occurs; Muller cells regulate these later stages of retinal vascularization (Stone et al., 1987) (Figure1).

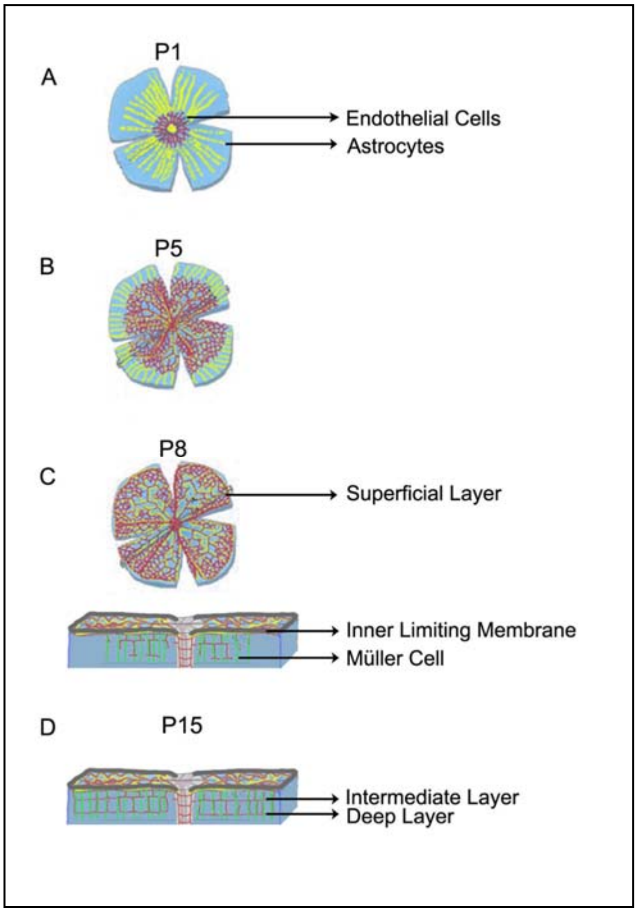

Figure 1: Astrocytes and Müller cells guide endothelial cells to form superficial and deep layer blood vessels respectively. Modified from Gerhardt et al., 2003.

(A) At P1, astrocytes (in yellow) migrate into the retina through the optic nerve head and make a template for endothelial cells to migrate.
(B) By P5, endothelial cells cover more than half of the retinal surface.

(C) By P8, superficial blood vessel formation is completed and then Müller cells (in green) attract the endothelial cells start to form deep and intermediate vascular layers. This process completes around P15.

Astrocyte Migration into the Retina:

Astrocytes are star-shaped glial cells that regulate a wide variety of functions in the central nervous system (Wang and Bordey., 2008). One of their most well characterized functions is the regulation of retinal angiogenesis and the participation in the blood-retinal barrier (Gardner et al., 1997). Retinas are only vascularized in mammalian species in association with retinal astrocytes. Retinas lacking astrocytes, are avascular and those in which astrocyte distribution are limited have spatially limited vascular development. For example, in adult possums, astrocytes are distributed only around the optic disc, and the optic disc remains the only vascularized region of the possum retina (Stone and Dreher., 1987). In human and non-human primate retinas, the foveal region, which is avascular, also lacks astrocytes (Provis and Hendrickson., 2008; Provis et al., 2000). These data suggest that retinal blood vessel formation is strictly dependent upon astrocytes.

Retinal astrocytes are generated from astrocyte precursor cells outside of the retina in the optic nerve. The astrocyte precursors express Pax-2 transcription factor (Chan-Ling et al., 2009), which is critical for astrocyte determination and differentiation (Chan-Ling et al., 2009; Sehgal et al., 2008; Soukkarieh et al., 2007). In order to populate the retina, differentiating astrocytes enter the retina through the optic nerve head (Watanabe and Raff., 1988) and migrate over the vitreal surface.

Astrocytes invade the mouse retina around embryonic day 17 – 18 (Chan-Ling et al., 2009; Uemura et al., 2006). Upon entering the retina, a proliferating population of astrocytes spreads toward the periphery in a centrifugal fashion (Fruttiger., 2007) (Figure 1). The proliferation of astrocytes during this migration is regulated by platelet-derived growth factor (PDGF)-A secreted by ganglion cells (Fruttiger et al 1996; Fruttiger., 2007). Over expression of PDGF-A or neutralization of the PDGF receptor, PDGFR-α, affects astrocyte proliferation and network formation (Fruttiger et al 1996). These results demonstrate that PDGF-A signaling is crucial for astrocyte proliferation.

In addition, the mouse homolog of drosophila tailless, Tlx (Nr2e1), is critical for astrocyte morphogenesis. Mice lacking the Tlx gene display abnormal astrocyte morphology, poor astrocyte scaffold formation and a complete failure of assembly of extracellular matrices (Uemura et al., 2006; Miyawaki et al., 2004). Although these studies have provided valuable information regarding the regulation of astrocyte proliferation and differentiation, the factors that guide astrocytes into the retina remain unknown.

Regulation of Astrocyte Migration by Laminins

Basement membranes (BM) are acellular extracellular matrices, which separate tissue compartments and serve as key regulators of differentiation and migration in a variety of tissues including the retina. Classically these structures are composed of laminin, collagen type IV, nidogen and heparin sulfate protoglycans (Yurchenco., 2011). Laminin deposition is the primary and key step in BM formation; the laminin family is a group of heterotrimeric glycoproteins. A laminin isoform is composed of a α chain, a β chain, and a γ chain. There are five alpha, three beta and three gamma chains identified with a total of 16 laminin isoforms that have been isolated and confirmed (Yurchenco., 2011). Interactions between laminins and integrins are isoform specific and regulate migration of a variety of cells in various tissues including retina (Yurchenco., 2011; Armstrong et al., 1990; Fujiwara et al., 2004; Desban et al., 2006). The specificity of laminin's biological activity is also presumed to arise from their differential expression, spatially, in adult tissues and, temporally, during development.

Our studies have shown that deletion of the *Lamb2* gene (encoding the laminin β2 chain) alone, as well as in combination with the *Lamc3* gene (encoding the laminin γ3 chain), affects the assembly of one BM in the retina, the inner limiting membrane (ILM) [Pinzon-Duarte et al., 2010]. Importantly, deletion of these laminin genes, singly or in combination also affects astrocyte migration and patterning (Gnanaguru et al., 2012). A similar defect in astrocyte patterning and vascular growth is present in *Lama1* gene (encoding the laiminin α1 chain) mutant retinas (Edwards et al., 2010). These results suggest that laminins in the ILM are guiding and regulating astrocyte migration. Several ex vivo experiments support this hypothesis. Specifically, the addition of exogenous laminin onto the retinal surface of cultured *Lamb2:c3* compound null retinae rescued both astrocyte migration and spatial patterning (Gnanaguru et al., 2012). Astrocyte spatial patterning is critical for subsequent vascular growth because the astrocyte-derived template guides endothelial migration and patterning (Dorrell et al., 2010; Gerhardt et al., 2003).

Tip Cell Selection and Endothelial Cell Migration

It has been suggested that the astrocyte template secretes a fibronectin-rich matrix, which sequesters VEGF and subsequently guides endothelial cells (Uemura et al., 2006, Gerhardt et al., 2003). However, astrocyte-specific deletion of fibronectin only delays vascular growth progression and does not completely abolish endothelial growth (Stenzel et al., 2011) suggesting that astrocyte-derived fibronectin is not necessary for endothelial development. A critical role for other matrix molecules is likely since the canonical matrix receptors, integrin β1, is expressed by endothelial cells and they have been shown to regulate vascular growth and branching (Malan et al., 2010; Tanjore et al., 2008). Given the data discussed above regarding laminins, and the fact that β1integrins are strong laminin binding molecules, it is likely that interaction between ligand (laminin) and receptor (integrins) play a key step in endothelial patterning and make an attractive therapeutic target.

The elongating vascular tube consists of two endothelial cell types: leading tip cells that perform sensory function responded to cues, and the lagging stalk cells that form the more stable wall of the vessel. Thus, the regulation of the number and behavior of both tip and stalk cells determines the rate of vascular growth and the density of branching. Initially, the formation of tip cells is induced by astrocyte-derived VEGF-A gradient (Gerhardt et al., 2003). These tip cells express the vascular endothelial growth factor receptor (VEGFR)-2 (Gerhardt et al., 2003), which allow tip cells to respond to the astrocyte-derived VEGF-A gradient. In addition to this VEGF-A-VEGFR2 signaling pathway, the VEGF-C-VEGR3 pathway also regulates tip-stalk cell formation in the retina (Tammela et al. 2011; Benedito e al., 2012).

Vascular density is regulated by regulating tip cell production; specifically the number of tip cells determines the branch points in the elongating vessel. Tip cell selection is regulated by a notch-Dll4 signaling mechanism (Suchting et al., 2007; Hellstrom et al., 2007). Deletion of one of the alleles for Dll4 and endothelial specific deletion of notch-1 increases tip cell number (Hellstrom et al., 2007). Together, these results suggest that the activation of the notch-Dll4 pathway is critical for controlled vascular growth. Moreover, laminin-integrin mediated signaling is thought to modulate endothelial tip cell selection through a notch-delta like ligand-4 (Dll4) signaling pathway (Estrach et al., 2011) suggesting that the matrix plays an important role in modifying vascular patterning and thereby density.

In summary, communication between cell extrinsic and intrinsic molecules is necessary to achieve proper vascular growth and patterning. An imbalance in the interactions between cell extrinsic and intrinsic signaling will affect vascular development. Moreover, it is likely that neovascularization events are the result of disruptions anywhere in these pathways. Thus, the most effective treatment of vascular related pathologies will result from a careful manipulation of the complex signaling pathways involved. Thus, the role of the clinician scientist will be to carefully define the etiology of the vascular defects and construct a rational therapeutic strategy.

Vascular Specification and Branching

Other critical events that occur during vascular growth progression include the specification of arteries and veins and their branching. The exact mechanism by which some endothelial cells take up arterial and venous fate is not completely understood. However, it has been suggested that ephrins and Eph receptors play important roles. The differential expression of ephrin-B2 ligand and the EphB4 receptor segregate endothelial cells from each other. The endothelial cells in arteries express ephrin-B2, whereas endothelial cells in veins express EphB4

(Adams et al., 1999; Gerety et al., 1999; Uemura et al., 2006). These data suggest that specific ephrins including ephrin-B2 play a role in specifying arterial fate, and specific Ephs including EphB4 play a role in specifying venous fate.

The combinatorial action of the specific VEGF-A isoforms 188, 164, and 120 also regulates vascular specification and branching (Ruhrberg et al., 2002; Stalmans et al., 2002). For instance, mice solely expressing the heparan sulfate binding isoform, VEGF188 ($VEGF^{188/188}$), show excessive branching and impaired arterial specification (Stalmans et al., 2002). On the other hand, mice expressing only the diffusible VEGF120 ($VEGF^{120/120}$) show severe defects in vascular branching as well as impairment of artery specification (Ruhrberg et al., 2002; Soukkarieh et al., 2007). The hypothesis is that matrix bound VEGF serves to orient tip cell extension and thereby vascular branching (Ruhrberg et al., 2002); the loss of the matrix bound form disorients this process. Importantly, when VEGF188 was ectopically expressed, in $VEGF^{120/120}$ animals, vascular defects were rescued (Ruhrberg et al., 2002). Mice expressing only VEGF164, which has both diffusible and heparan sulfate binding characteristics, display no vascular branching or specification defect (Stalmans et al., 2002). Taken together these results suggest that both matrix-bound and diffusible VEGFs are required for vascular growth progression, specification and branching.

Recruitment of Smooth Muscle Actin Expressing Cells (SMCs) and Blood Vessel Maturation

Vessel branching and elongation are the initial stages of angiogenesis, following this step the vessel wall must be stabilized. This stabilization is achieved during vascular growth when endothelial cells attract a class of smooth muscle actin expressing cells, the pericyte into the vascular wall. Endothelial cells are thought to secreting platelet derived growth factor (PDGF) B, the incorporation of pericytes as mural cells leads a stabilization of the vascular basement membrane (Uemura et al., 2006) and subsequently the vascular wall. Intraperitoneal injection of monoclonal antibodies directed against PDGFR-β affects pericyte recruitment and blood vessel integrity (Uemura et al., 2002), leading to vascular leakage.

The migration of the SMCs over the vascular wall is modulated by interactions between the cellular environment and the cell surface receptors including integrins containing the integrin $\beta 1$ subunit (Abraham et al., 2008). For example, cell specific deletion of integrin $\beta 1$ affects SMC migration, and integration into the vascular wall and destabilizes overall vascular integrity (Abraham et al., 2008). Once SMCs are recruited into the vascular wall, they in turn secrete stabilizing factors such as angiopoetin-1 (Ang-1) and transforming growth factor-β (TGF-β). Both Ang-1 and TBG-β are critical for endothelial stabilization of the vascular wall including deposition of basement membrane molecules over the vascular wall (Suri et al., 1996; Uemura et al., 2002; Ramsauer and D'Amore., 2007). Thus, interaction of SMCs with the vascular wall is critical for vascular stability.

Moreover, the loss of mural cells such as the pericyte is a key step in destabilizing an existing vessel. Interference with pericyte mural adhesion results in the death of the pericyte by anoikis (Liu et al., 2008). These studies suggest that manipulations of the mural cell – ECM interactions are likely to be important therapeutically (Hasan et al., 2011; Caballero et al., 2011).

Formation of Deeper Vascular Plexus

After the formation of the superficial vascular plexus is complete, deep and intermediate vascular plexiform layers start to develop. The development of these layers is complete at approximately postnatal day 15 in mice. These differentiation events occur through several signaling pathways, including those involving Wnts and VEGF.

Wnts expressed by microglia suppress excessive vascular branching through non-canonical Wnt-Flt1 pathway (Stefater et al., 2011). Microglial specific deletion of the Wnt ligand transporter, Wntless, results in excessive vascular branching in the deep vascular layer (Stefater et al., 2011). Thus, the Wnt pathway is a component during the formation of the deep vascular plexus.

The high metabolic demand caused by the maturation of cells in the outer retina (particularly the on-set of functional synapses), Müller cells secrete VEGF (Stone et al., 1995; Fruttiger., 2007). In response to this burst of extracellular VEGF, the endothelial cells of the veins and venules of the superficial vascular layer extend along Müller cell processes (Fruttiger., 2007) and form the deep and intermediate vascular layers. Unlike the superficial capillary branching, which was regulated by astrocyte-endothelial interactions, during the formation of these deeper beds, microglial cells regulate vascular branching of the deep and intermediate vascular layers (Stefater et al., 2011). These studies on the formation of the deep vascular plexus have provided valuable insights into vascular regulation but further more studies is required to know the involvement of other factors such as delta and notch signaling in this process.

Overall, a major question is how to tackle the complex process of vascular remodeling under pathological conditions, i.e., during neovascularization. As demonstrated above, the molecular processes of angiogenesis during development respond to environmental factors such as metabolic demand to increase vascular supply. These mechanisms remain active throughout adulthood, albeit without the temporal regulation exerted during normal development.

One of the leading causes of neovascularization is hypoxia-driven VEGF expression. Hypoxia-induced, VEGF-mediated signaling during pathogenesis promotes abnormal new blood vessel formation (Chen and Smith., 2007). However, anti-VEGF therapy may not appropriate for all conditions, because VEGF-A is an important factor for neuronal survival under ischemia (Nishijima et al., 2007): this likely to be particularly important in the premature infant. Thus, finding new targets to stabilize the vasculature would generate a better method to treat pathological neovascularization. The cell-matrix interface is a profoundly important target. Indeed, recent studies report abnormalities at the cell-matrix interface during pathological conditions (summarized below). And indeed some initial steps have been made to in using mural cell-ECM interactions as therapies (above). These successes provide an impetus for further therapeutic developments.

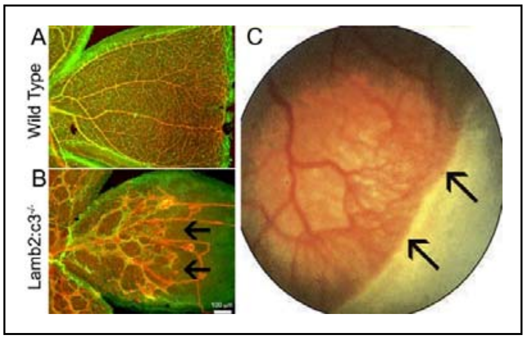

Figure 2: Vascular growth in peripheral retinal is affected in laminin nulls as well as infants with ROP.

(A) Postnatal day 15 wild type retina was immunostained for GFAP (green) to label astrocytes and CD31 (Red) to label blood vessels.
(B) Postnatal day 15 Lamb2:c3 null retina was immunostained for GFAP (green) to label astrocytes and CD31 (Red) to label blood vessels. Notice abnormal astrocyte patterning and vascular growth. Astrocyte patterning and subsequent vascular growth was affected in the peripheral retina (black arrows).
(C) Black arrows at the peripheral retina show no vascular growth in infant with ROP **(Data in A-B are from the author's laboratory; data reprinted in C are from Gariano and Gardner, 2005)**.

Changes in Cell-Matrix Interactions During Retinal Vasculature During Pathologies related to Retinopathy of Prematurity (ROP)

ROP occurs in two phases: in the first phase, vascular growth attenuates; in the second phase, neovascular sprouts develop in response to VEGF secretion (Chen and Smith, 2007). Both phases are likely dependent on cell-matrix interactions. Blood vessel growth in the peripheral retina is severely affected in infants with ROP (Figure 2C) (Gariano and Gardner., 2005). In ROP, this defective growth could be a result of defective astrocyte migration and template formation in the peripheral retina. As detailed above the template on which astrocytes migrate and vessels form is comprised, in part, of laminins. Deletion of laminin chains contributes to defective astrocyte patterning and blood vessel formation (Edwards et al., 2010; Gnanaguru et al., 2012). Astrocyte distribution is severely affected in *Lamb2* null retinae (data not shown) and *Lamb2:c3* double null retinae (Figure 2B), This disruption of astrocyte distribution in laminin null animals is particularly marked in the peripheral regions of the retina, where there is poor or no blood vessel formation (Gnanaguru et al., 2012). In addition, infants with ROP also show signs of vascular leakage (Figure 2B) [Nissenkorn et al., 1988], a featured shared with *Lamb2* nulls and *Lamb2:c3* double null retinae (Figure 3C and D). Because of this similarity, it is of interest to analyze whether the expression and distribution of laminins are affected in ROP models. If there were a loss of laminins in ROP models, a rescue of laminin expression may help astrocytes to migrate and pattern properly. This rescue would then allow endothelial cells to re-vascularize in a normal fashion.

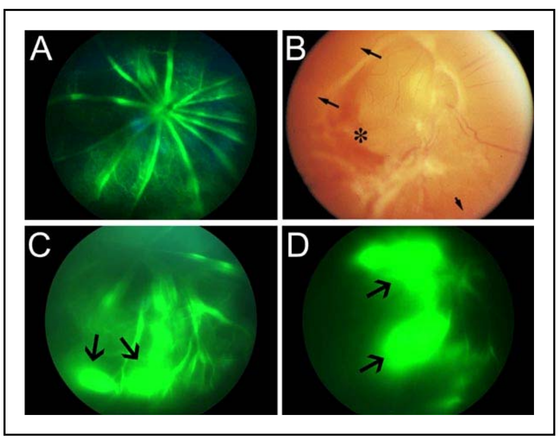

Figure 3: Defective vascular integrity in laminin null retinas and advanced stage 4 ROP.

(A) Fluorescein angiography in wild type P15 retina shows no signs of vascular leakage approximately 120 sec after intra peritoneal injection of sodium fluorescein.
(B) Advanced stage 4 ROP reveals hemorrhage (asterisk) and avascular peripheral retina (black arrows)
(C) Fluorescein angiography in Lamb2 null retina shows vascular leakage approximately 120 sec after intra peritoneal injection of sodium fluorescein (black arrow).
(D) Fluorescein angiography in Lamb2:c3 null retina shows vascular leakage approximately 120 sec after intra peritoneal injection of sodium fluorescein (black arrows).
(Data in A, C and D are from the author's laboratory; data reprinted in B is from Saint-Geniez and D'Amore, 2004).

The defective vascular growth observed in infants with ROP could also be due to degeneration of astrocytes under hyper/hypoxic conditions. In experimental animal models, astrocytes degenerate under hypoxic conditions

(Stone et al., 1996, Dorrell et al., 2010). In addition, brain derived astrocytes lose integrin (α1, α6, β1) and dystroglycan receptors under ischemic conditions (Tagaya et al., 2001, Milner et al., 2008).

It is likely that the loss of contact with the BM is causing astrocyte degeneration under ROP. It is also possible that remodeling of BM during cyclic oxygen condition is affecting the astrocyte-BM interaction resulting in cell death, because hypoxia has shown to activate matrix metalloproteinases (MMPs) (Bauer et al., 2010). Activation of MMPs affects cellular interaction and causes vascular leakage (Bauer et al., 2010). Maintaining the astrocyte population under hypoxic conditions reduces neovascularization (Dorrell et al., 2010). Thus, it is possible that stabilizing astrocyte and endothelial interactions with the BM during ROP might reduce the risk of neovascularization in ROP.

Changes in Cell-Matrix Interactions in Diabetic Retinopathy

One of the key clinical features of diabetic retinopathy is basement membrane thickening, with a marked increase in collagen IV deposition (Roy et al., 1994). Rat models of diabetic retinopathy also have increased levels of fibronectin and experimental down-regulation of fibronectin in these models improves vascular lesions (Roy et al., 2011). Moreover, laminin β2 chain synthesis is decreased by elevated glucose in both experimental animal models and in-vitro studies in the kidney (Abrass et al., 1997; Schaeffer et al., 2010).

Laminins are critical for astrocyte and Muller cell interaction with the ILM in the retina (Gnanaguru et al., 2012, Hirrlinger et al., 2011). In addition, mutations in the LAMB2 gene lead to Pierson syndrome (Bredrup et al., 2008; Mohney et al., 2011) and animals with mutations in this gene phenocopy many aspects of the human disease (Libby et al., 2000; Pinzon-Duarte et al., 2010). It will be of interest to analyze whether the expression of collagens, fibronectin and laminins are altered in hyperglycemic retina, as hyperglycemia has been shown to cause astrocyte degeneration and induce GFAP expression in Müller cells, which is suggestive of reactive gliosis (Figure 4) (Ly et al., 2011; Barber et al., 200). These abnormalities with the glial cells could be due to remodeling of the matrix or to changes involving cell surface receptors. In addition, to changes in the glial cells, hyperglycemia also causes loss of mural pericytes, which leads to mural instability, vascular leakage and subsequent neovascualrization (Hammes et al., 2002). As noted above mural cell adhesion to the vascular wall is mediated through integrins (Abraham et al., 2008).

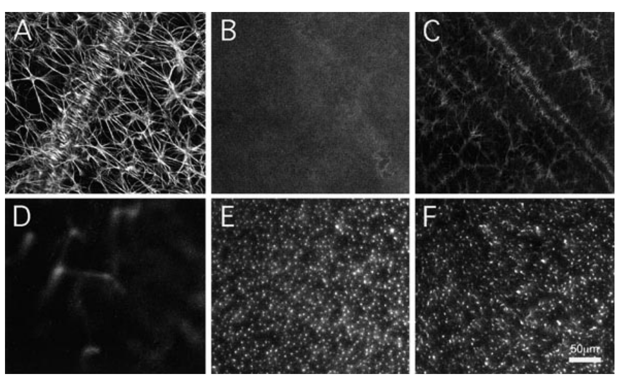

Figure 4: Experimental rat model for diabetes shows loss of GFAP expression in astrocytes and reactive gliosis of Müller cells **(Data are reprinted from Barber et al., 2000, reference 5)**.

(A) Control flat-mount retina showing GFAP expression in astrocytes.
(B) STZ-diabetic rats show loss of GFAP expression in astrocytes after 4 months of induction of diabetes.
(C) Insulin treatment for 48 hr of STZ-diabetic rats shows partial recovery of GFAP expression in astrocytes.
(D) Control rats show no GFAP immunoreactivity in Müller cells (Image focused on the outer plexiform layer).
(E) STZ-diabetic rats shows GFAP expression in Müller cells after 4 months of induction of diabetes (Image focused on the outer plexiform layer).
(F) Insulin treatment for 48 hr of STZ-diabetic rats shows partial reduction of GFAP in Müller cells (Image focused on the outer plexiform layer).

In summary, the data above suggest that coordinated signaling between cell extrinsic ECM molecules and cell intrinsic molecules are necessary for proper retinal vascular development and functioning. As mentioned above the key step in the development of retinal angiogenesis is the migration of astrocytes, which guides the subsequent vascular growth. The goal of this study is to understand the mechanism by which the ECM (particularly laminin) derived cues regulate astrocyte migration, spatial patterning and subsequent angiogenesis in the retina.

CHAPTER 2

Laminins Containing the β2 and γ3 Chains Regulate Astrocyte Migration And Angiogenesis In the Retina

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Introduction

Retinal vascular development is a complex process regulated by various cell extrinsic and intrinsic signaling mechanisms. A thorough understanding of this process is necessary because diseases of the retinal vasculature in either children (retinopathy of prematurity, ROP) or adults (diabetic retinopathy) are among the major causes of blindness in the developed world (Chen and Smith, 2007; Sapieha et al., 2010; Kempen et al., 2004). A detailed understanding of the molecular mechanisms of retinal vascularization could unravel novel therapeutic targets.

During retinal angiogenesis, the retinal vasculature develops three interconnected capillary beds or layers: the superficial, intermediate, and deep plexiform layers (Fruttiger, 2007). The first step in this development – the formation of the superficial layer – is wholly dependent on the presence of astrocytes. For example, mammalian retinas that lack astrocytes never become vascularized; moreover, in humans and other primates, the fovea, which lacks astrocytes, remains avascular (Schnitzer, 1988; Provis and Hendrickson, 2008; Provis et al., 2000).

In retinas that are vascularized, astrocytes enter the retina through the optic nerve head (ONH) (Watanabe and Raff, 1988). Upon entry, astrocyte proliferation is stimulated by platelet-derived growth factor-A (PDGF-A) produced by ganglion cells (Fruttiger et al., 1996; Fruttiger et al., 2000). As they proliferate, astrocytes spread across the entire surface of the retina in a centrifugal fashion (Fruttiger, 2007). During their migration to the periphery, astrocytes secrete vascular growth factors such as vascular endothelial growth factor (VEGF) and serve as a template for endothelial cells to follow thereby guiding the formation of the superficial vasculature (Gerhardt et al., 2003; Dorrell et al., 2002).

Because astrocytes are critical for development of vascular patterning, disruption of astrocyte patterning in the retina severely affects vascular growth (Uemura et al., 2006). In addition, under pathological conditions such as oxygen-induced retinopathy (OIR), an experimental condition produced by a prolonged period of hyperoxic preconditioning followed by a return to atmospheric oxygen levels, astrocytes degenerate, leading to a breakdown of the blood retinal barrier (BRB) and pathological neovascularization (Stone et al., 1996). Rescue of astrocytes from degeneration in OIR reduces pathological neovascularization (Dorrell et al., 2010), suggesting that stabilization of retinal astrocytes is a promising target to prevent pathological neovascularization. Given the strong correlation between astrocytes development and retinal angiogenesis, it is incumbent for the development of effective therapies to explore all the factors that regulate astrocyte migration and development. Here, we provide evidence that extracellular matrix components – laminins – guide astrocytes into the retina, promote at least one aspect of the maturation and regulate subsequent blood vessel development.

Laminins are key glycoprotein components of basement membranes (BMs), composed of three chains: alpha, beta, and gamma (Yurchenco, 2011). 5 alpha, 3 beta, and 3 gamma chains have been identified. Sixteen different heterotrimeric isoforms have been isolated in their native state (Aumailley et al., 2005; Macdonald et al., 2010), and these laminin isoforms have overlapping yet distinct tissue distribution and function (Colognato and Yurchenco 2000, Durbeej, 2010).

One of the known functions of laminins and their receptors is induction and regulation of cell migration (Suh and Han, 2010; Fujiwara et al., 2004; Desban et al., 2006; Echtermeyer et al., 1996). Notably, laminins induce migration of brain astrocytes in culture (Armstrong et al., 1990). Because laminins have known guidance properties and laminins are present in the retina, we hypothesized that laminins regulate both astrocyte migration and spatial organization in the retina.

We have previously reported that distinct laminin heterotrimers containing the laminin β2 and γ3 chains are expressed in retinal BMs (Libby et al., 2000). Deletion of the *Lamb2* gene alone partially disrupted formation of the inner limiting membrane (ILM) of the retina, and deletion of *Lamb2:c3* genes together severely disrupted ILM formation (Pinzon-Duarte et al., 2010).

To determine whether the disruption of the ILM in *Lamb2* and *Lamb2:c3* null animals affects astrocyte migration, patterning, and subsequent blood vessel formation, we examined these events in *Lamb2* and *Lamb2:c3* nulls. Our analyses demonstrate that laminins containing the β2 and γ3 chains are indispensable for astrocyte migration, spatial distribution, and subsequent vascular growth *in vivo*. *In vitro* and *ex vivo* studies demonstrate that laminins regulate astrocyte migration by acting as haptotactic factors in an isoform-specific manner. Further, treatment with exogenous laminins rescues astrocyte migration and spatial patterning in laminin-null retinal cultures. Finally, we demonstrate that deletion of the laminin β2 and γ3 chains affects retinal vascularization and vascular stability.

Methods:

Mice:

Animal procedures were performed in accordance with the institutional animal care and use committee of SUNY Downstate Medical Center (DLAR). Targeted deletion of *Lamb2 and Lamc3* genes and the production of the *Lamb2-/-Lamc3-/-* animals have been described previously (Noakes et al 1995; Li et al., 2012, Denes et al., 2007; Pinzon-Duarte et al., 2010). The Lamb2 null animals are maintained as a heterozygous line, the Lamc3 nulls are maintained as homozygous null line. Both of those lines used in this study were backcrossed over 9 generations to C57bl/6J. The *Lamb2:c3* and *Lamc*3 nulls were created by mating *Lamb2*+/- and *Lamc3*-/- animals. This line was on a mixed genetic background of C57bl/6J and 129Sv/J; genetic background characterization was performed by Taconic, Inc (Germantown, NY) using a 377 SNP panel and demonstrated that the *Lamb2*+/-:*Lamc3*-/- line matched more than 83% of C57bl/6J recipient genome. From here forward the following notations are used in the text and figures for genotypes: β2 deletion, *Lamb2*-null or *Lamb2*$^{-/-}$; γ3 deletion, *Lamc3*-null or *Lamc3*$^{-/-}$; for compound deletion of β2 and γ3, *Lamb2;c3*—null or *Lamb2:c3*$^{-/-}$.

Whole Mount Retinal Preparation and Immunostaining:

Whole mounts were prepared as described previously (Li et al., 2012). In brief, eyes were enucleated and fixed in 2% paraformaldehyde (PFA) prepared in PBS (see below) for 5 to 10 min. The anterior chamber was removed and retinas were dissected out in PBS. Retinas were flat mounted by making four radial cuts. The flat mounts were treated with absolute methanol at -20°C for 10 min. The flat mount retinas were washed in PBS. The whole mount retinas were incubated in blocking buffer (15% goat or donkey serum, and 0.03% triton-X 100 prepared in PBS) overnight at 4°C. The whole mounts were then incubated with primary antibodies (diluted in 5% goat or donkey serum, and 0.01% triton-X 100 prepared in PBS) for 48 h at 4°C. The flat mounts were washed and incubated with respective secondary antibodies, overnight at 4°C. After incubation period the samples were washed in PBS, cover slipped using ProLong gold (Invitrogen, Grand Island, NY), and sealed.

Preparation of Retinal Sections:

Eyes were enucleated and fixed for 5 minutes with ice cold 2% PFA by making a small incision near the ora serrata. The anterior chamber was removed in PBS and the eyecup was further fixed with ice cold 2% PFA for 15 min. The tissue was cryoprotected with serial 10%, 20%, and 30% sucrose treatment before embedding in OCT (Sakura Finetek, Torrance, CA). 20 μm-thick sections were prepared. The sections were washed in PBS, then blocked for 2 hr at room temperature (RT). After blocking, the samples were treated with appropriate primary antibodies overnight at 4° C. After washes with PBS, the samples were incubated with respective secondary antibodies for 2-3 hr RT. The samples were then washed in PBS, cover slipped using Vectashield (Vector Laboratories, Burlingame, CA) and sealed.

Reagents:

Hank’s Balanced Salt Solution (HBSS) and DMEM (Life Technologies, Grand Island, NY), 0.25% Trypsin and anti-laminin (Sigma, St. Louis, MO). Antibodies: β2 laminin (gift of Dr. Sasaki, Max-Plank-Institut für Biochemie), γ3 laminin (R96, our laboratory), PDGFR-α (BD Biosciences, San Jose, CA), rabbit anti-GFAP (Chemicon, Billerica, MA), CD31 (Millipore, Billerica, MA), Perlecan (Santa Cruz Biotechnology, Santa Cruz, CA), and integrin β1 (Millipore, Billerica, MA). Goat anti-rabbit 488 and 568 and, Goat anti-rat 488 and 568, Donkey anti-rabbit 488 and 594, and Donkey anti-rat 488 and 594 (Life Technologies, Grand Island, NY). EHS-laminin (Life Technologies, Grand Island, NY), Laminin 5 (Gift From Dr. Manuel Koch, Universität zu Köln). Low-melting Agarose (Boston Bioproducts, Ashland, MA), Collagenase (cls 1) (Worthington Biochemical Corporation, Lakewood, NJ), Papain (Sigma, St. Louis, MO).

Measurements And Statistics:

Astrocyte migration and vascular growth were measured using Improvision Volocity™ software, v. 5.4.1 (Perkin-Elmer, Waltham, MA). Astrocyte migration and blood vessel growth occur in a centrifugal fashion to reach the periphery of the retina (Fruttiger, 2007). To measure mean migration rate per quadrant, the distance covered by astrocytes from the optic nerve head was measured using Volocity software. Measurements were made in all the quadrants and averaged to get the migratory rate per quadrant. Vascular growth (labeled with CD31) was also measured in a similar fashion. For tip cell quantification, the number of CD31 positive tip cells at the leading edges was marked using Improvision Volocity™ software, v. 5.4.1 and counted in all the quadrants across a 150 μm region per quadrant. The mean value was calculated by averaging the number of tip cells in all four quadrants. To determine whether the slower astrocyte migration and vascular growth rates observed in *Lamc3* nulls were statistically significant, a Student's t-test was performed. A p-value less than 0.05 was considered statistically significant; specific p-values are given for various experiments.

Isolation Of Retinal Astrocytes:

Retinal astrocytes were isolated from P0-P3 WT and laminin nulls, as described previously (West et al., 2005). In brief, retinas were treated with 1% collagenase type-1 (in DMEM) for 30min at 37°C and then treated with papain (0.5 mg/ml) at 37°C with 5% CO2. The retinas were gently triturated in DMEM containing 10% FBS using pipette. The dissociated cells were spun and plated in 6 well culture plates (Corning Life Sciences, Tewksbury, MA). After 6 to 8hr of culture, the plates were shaken at regular intervals to remove other contaminating cells. The cells were grown for 12 days, detached using 0.25% trypsin and re-plated. The obtained cells were more than 90% astrocytes, which was confirmed by immunostaining for GFAP and DAPI (data not shown). These cells were used for further experiments.

Agarose Drop Migration Assay:

The astrocyte migration assay was adapted and modified as described previously (Frost et al., 2000). In brief, sterile coverslips were coated with PBS (control), laminin-332 (10μg/ml) and EHS-laminin (10μg/ml), respectively. The coated coverslips were placed in a 24 well chamber cell culture plate (Corning). The retinal astrocytes (isolated from WT) were trypsinized as stated above and mixed in a DMEM- low melting agarose mix (agarose final concentration: 0.3%, cell density: approximately 2×10^6 cells per ml). 2μl of agarose-cell suspension was placed on top of substrate-coated area and immediately kept at 4°C for 10 min for the agarose to solidify. 500μl of media containing DMEM and 10% heat inactivated FBS was carefully added to the sides of the culture plate (Corning). The samples were brought back to 37°C and maintained with 5% CO_2. Migration of astrocytes was observed under the light microscope every 6 h. In the EHS-laminin coated coverslips, migration of astrocytes through the agarose was noticed within 48 h. The experiment was stopped after 3 and 6 days by fixing the agarose drop for 5 min using 4% PFA and immunostained for GFAP expression. The represented data are from 6 day cultures. The number of GFAP expressing astrocytes that migrated through the agarose and elaborated on the substrate-coated area were counted and quantified. This experiment was performed in duplicate and repeated.

Optic Nerve Head (ONH) Explant Preparation:

Eyes were carefully enucleated from P7 WT animals the optic nerve intact. The ONH was dissected under a microscope in sterile Hank's Balanced Salt Solution

(HBSS; Sigma). The ONH was then carefully placed on a 0.4μm Transwell membrane insert (Corning) and the bottom chamber was filled with DMEM containing 10% heat inactivated FBS. The explants were incubated at 37°C with 5% CO_2. After 6 hrs, the ONH was treated with exogenous laminin-332 (10μg/ml) or EHS-laminin (10μg/ml) or PBS (control) and maintained for 36 hrs. The membrane with the ONH was fixed with 4% PFA for 5 min. The membrane was carefully detached without disturbing the ONH. The ONH was blocked for 1 hr at RT and immunostaining was performed for GFAP as described above. This experiment was repeated using P10 and P14 WT ONH.

Integrin Restoration and Function Blocking Experiments:

Retinal astrocytes were isolated from P2 WT and *Lamb2:c3*–null animals. After trypsinization, single cell suspensions were plated on uncoated or laminin-coated coverslips at low densities (approximately 100 to 250 cells per coverslip) and incubated at 37°C with 5% CO2. After 24hr of incubation, the cells were fixed in 4% PFA for 5 min. The cells were then immunostained for GFAP and β1 integrin. This experiment was performed in duplicate.

To test whether astrocyte migration in the retina was regulated by laminin-integrin β1 interaction, we performed functional blocking of integrin β1 receptors. Retinas were isolated from WT P0-P2 pups and dissected around the ONH as described above. The explants were placed on EHS-laminin (10-25 μg/ml) coated coverslips and culture in DMEM containing 10% heat-inactivated FBS. After 36 hr of culture, one explant was incubated with DMEM containing 10%

heat-inactivated FBS + 10-25 μg/ml non-specific control IgG (Jackson Immunoresearch Laboratory, West Grove, PA). The other explant prepared from the other eye was incubated with DMEM containing 10% heat inactivated FBS + 10-25 μg/ml integrin β1 antibody (BD Biosciences, San Jose, CA). After 48 hr of antibody treatment, the explants were fixed with 4% paraformaldehyde for 5 min on ice. The samples were washed in PBS and blocked for 1-2 hr at room temperature in 1X PBS containing 10% fetal bovine serum (FBS), 0.05% triton-X 100 and 0.001% sodium azide. The samples were washed in PBS and incubated with GFAP antibody prepared in 1X PBS containing 2% FBS, 0.01% triton-X 100 overnight at 4°C. Following the primary antibody incubation, the samples were incubated with respective RITC or FITC tagged secondary antibodies for 2-4 hr at room temperature. After washes in PBS, the samples were coverslipped using Prolong Gold and sealed.

Inner limiting membrane preparation:

ILMs were stripped according to published methods (Hu et al., 2010) with minor changes. In brief, sterile coverslips were coated with 0.01% poly-D-lysine (PDL) (Sigma Aldrich, USA). For ILM stripping animals aged P5-P15 were used. The retinas were isolated in sterile PBS (ice cold). The isolated retinas were used as a whole or cut into two halves. The dissected retinal tissues were spread on a sterile nitrocellulose paper, photoreceptor side facing the nitrocellulose paper. The retinal tissues attached to nitrocellulose paper were then placed on a PDL coated coverslips (vitreal side of the retina facing the coverslip). After attachment, the nitrocellulose paper was carefully peeled, which left the ILM attached to the coverslip. The coverslips were incubated in 2% triton X-100 at 4°C overnight to remove the cell debris from the ILM. The ILMs were washed extensively in sterile dH2O and then in PBS. The stripped ILMs were used for either immunostaining or migration assays.

Migration assay on stripped ILM:

ONH samples were prepared as mentioned above. In order to test if ILM guides astrocyte migration, the ONH explants were carefully placed on the stripped ILM and cultured for 2-3 days in DMEM containing 10% heat inactivated FBS at 37°C with 5% CO2. The explants were analyzed for astrocyte migration by immunostaining for GFAP. This experiment was repeated using P7 ON and P3 ONH explants.

Retinal Culture:

P0 or P1 *Lamb2:c3* null retinas were dissected in HBSS under sterile conditions. Four radial cuts were made and each retina was carefully placed on a Transwell membrane (0.4μm, Corning) with the photoreceptor side facing down. DMEM containing 10% FBS was slowly added to the bottom chamber until the medium touched the membrane (approximately 500μl). The retinas were incubated overnight at 37°C with 5% CO_2. After overnight incubation, EHS-laminin (0.5 mg/ml) + DMEM was added to one of the retinas; the other retina received DMEM only. The retinas were fixed with 4% PFA for 10 min. The membrane containing the retina was carefully cut out and analyzed for GFAP and integrin $\beta1$ expression by immunostaining.

Fluorescein Angiography:

Fluorescein angiographies were performed in P15 mice (n=3) as described previously (Lai et al., 2005). In brief, animals were anesthetized using intraperitoneal injection of ketamine (100mg/kg body wt) and xylazine (10mg/kg body wt) and the pupils were dilated with topical 1% tropicamide and 2.5% phenylephrine hydrochloride 10 min before imaging. A drop of hydroxypropyl methylcellulose demulcent solution (2.5%) was placed on each eye. The animals were then administered with 0.1ml of 10% sodium fluorescein by intra peritoneal injection. Live recording was performed at different time scales using a Micron-II imaging system (Phoenix Research Laboratories, Pleasanton, CA). Snapshots at different time scales were created from the live recordings to analyze.

Results

Astrocytes Migrate Along Laminins Containing the β2 chain

At postnatal day (P) 15, the laminin β2 chain is expressed in Bruch's membrane (BM), the ILM and retinal vascular basement membrane (Fig. 1A), with high expression in arteries and microvasculature (Fig. 1B,C). The laminin γ3 chain is expressed in Bruch's membrane and weakly in the ILM (Fig. 1D). Interestingly, in the vascular BM, γ3 laminin chain expression was restricted to only veins and microvessels (Fig. 1E, F).

As astrocytes migrate over the retinal surface, we asked if this migration took place in a laminin rich compartment. To do this we examined the spatial organization of laminin deposition in the ILM at the same time we visualized astrocytes. WT P0 retinal sections were co-labeled with laminin β2 chain reactive serum (Fig. 1G), and with a marker of astrocytes anti-PDGFR-α (Fig. 1H). The overlay clearly demonstrates that astrocytes are wholly contained within the laminin β2-rich compartment, i.e., the ILM (Fig. 1I). We also examined whether astrocytes express cognate laminin receptors. As integrin β1 is well known to affect brain astrocyte polarization, process extension, and migration in culture (Peng et al., 2008); we first determined whether laminin-astrocyte interaction by this class of receptors. To accomplish this, we co-localized another marker of astrocytes, GFAP (Fig. 1J) with integrin β1 expression (Fig. 1K) at P0. The overlay clearly demonstrates that GFAP-expressing astrocytes, migrating through the ILM, express the integrin β1 subunit (Fig. 1L). Next, we analyzed whether astrocyte migration and the expression of the integrin β1 subunit are affected in laminin null retinas.

Deletion of *Lamb2* And *Lamc3* Genes Affect Astrocyte Migration And Spatial Patterning In the Retina

We have previously shown that the ILM is somewhat disrupted in *Lamb2*-null and grossly disrupted in *Lamb2:c3*–null animals (Pinzon-Duarte et al., 2010). Here, we compared the integrity of the ILM to distribution of astrocytes in P15 WT and in laminin-null retinae. We observed that, in both *Lamb2-* and *Lamb2:c3*–nulls, GFAP-expressing astrocytes were found only in the regions ILM remained relatively intact as judged by perlecan deposition (Fig. S1). This spatial linkage suggested that laminin-rich substrates were critical for astrocyte migration. Thus, we analyzed the temporal progression of astrocyte migration during development in WT, *Lamb2*, *Lamc3*, and *Lamb2:c3* nulls. Because astrocyte migration largely takes place during the first postnatal week, we examined retinae at P0, P1, P3 and P5 to examine the progress of migration in situ in each of the genotypes. There is considerable remodeling of the astrocyte template and vascular bed during the second post-natal week so we examined retinae at P15 and older to examine astrocyte patterning.

GFAP, a commonly used marker for astrocytes, is expressed at low levels in astrocytes in the early postnatal (P0-P5) retina; thus we used a more robust marker, PDGFR-α, to identify astrocytes during this period. At P0, astrocytes in the WT retina had migrated well over half of the retinal surface advancing to 239.49 +/- 2.01 μm (Mean +/- SEM) from the head of the optic nerve (Fig. 2A,E).

Remarkably, although the ILM remains morphologically intact through development in the *Lamc3*-null retina (Fig. S1), astrocyte migration rate was significantly slower from P0-P3 than the wild type (Fig. 2B, E). At P3, astrocytes in the WT had migrated 345.97 +/- 5.23 μm (Mean +/- SEM), whereas astrocytes in the *Lamc3*-null retina had covered a statistically significant smaller distance of 257.86 +/- 5.86 μm (Mean +/- SEM) (Fig. 2E). By P5, astrocytes in the *Lamc3*-null retina catch up to wild type controls and reached the retinal periphery (Fig. 2E).

Migration of astrocytes in the *Lamb2*–null retina demonstrated a more significantly retarded migration throughout all ages of vascular development (Fig. 2C,E). At P0, astrocytes in *Lamb2* null had migrated only a distance of 99.4 +/- 3.22 μm (Mean +/- SEM); three days later at P3, the astrocyte front has extended modestly to 176.72 +/- 9.31μm (Mean +/- SEM). By P5 when the astrocyte front has reached the retinal margin in the wild type and *Lamc3*-null retinae, in the *Lamb2*-null it has advanced only 213.99 +/- 14.6 μm (Mean +/- SEM) from the optic nerve head. Thus, the astrocyte migration is halted prior to the full coverage of the retina.

Deletion of both *Lamb2* and *Lamc3* genes virtually halted astrocyte migration around the head of the optic nerve (Fig 2D). At P0, astrocytes in *Lamb2:c3*–null migrated only distance of 35.71 +/- 1.08 μm (Mean +/- SEM) from the optic nerve head, and they advanced a mere 30 μm in the next three days, as the wave front was located at a distance of 61.4 +/- 3.26μm (Mean +/- SEM). As in the Lamb2 null, astrocytes in the *Lamb2:c3*–null animals were unable to reach the retinal periphery by P5 having advanced only 106.46 +/- 8.38 μm (Mean +/- SEM) from the optic nerve head. This position is significantly retarded migration compared with the wild type retinae, in which astrocytes have advanced over twice that distance to 240 μm. Thus, the deletion of laminins from the ILM significantly slows the migration of astrocytes during the first post-natal week.

Next, we examined the spatial distribution of astrocytes in the more mature retina and found that in contrast to migration, spatial patterning is only disrupted un the Lamb2 nulls. By P15 in the wild type retina, astrocytes have assumed their adult distribution and are seen as a single sheet of cells covering the retinal surface. Despite their slowed migration, the spatial distributions of astrocytes in Lamc3-null retinae were virtually identical to WT retinae (Fig. 2F,G). In contrast, in the *Lamb2*-null animals, the distribution of astrocytes was severely affected. In these nulls, few GFAP-expressing astrocytes were present at the retinal periphery at P15; however, the distribution pattern of astrocytes was abnormal, and appeared as a net-like pattern, with some regions completely devoid of astrocytes (Fig. 2H) forming holes in the sheet of astrocytes. Moreover, the star-like appearance of the cells was lost and the cells maintained a fusiform shape. In the *Lamb2:c3*–nulls at P15, the astrocytes were largely confined to central retina and were distributed in patches or cords of cells (Fig. 2I). Additionally, in *Lamb2-* and *Lamb2:c3*–nulls, astrocytes migrated abnormally over the surface of the hyaloid vessels (data not shown). These data strongly suggest that astrocyte migration and spatial patterning are dependent on laminins or other cues deposited in the ILM.

Astrocyte Migration Is Laminin-Dependent and Exogenous Laminin Restores Migration in Laminin Nulls.

These *in vivo* data demonstrate that astrocytes migrate in a laminin-rich ILM niche and that deletion of either *Lamb2* or *Lamc3* slows astrocyte migration while deletion of Lamb2 disrupts spatial patterning and maturation. We next tested whether the laminin-rich ILM niche induces astrocyte migration. To test this hypothesis, we first isolated ILM from P10-15 WT and laminin null retina and analyzed for laminin expression by immunostaining. Laminin was deposited homogenously in the WT and *Lamc3-* null ILMs (Fig. 2J,K) whereas laminin distribution appeared like a honeycomb with large holes in *Lamb2* and *Lamb2:c3-* null ILMs (Fig. 2L,M) (Hirrlinger et al., 2011). As astrocytes enter the retina through the optic nerve, we dissected the optic nerve head from WT P10 and placed on the stripped WT ILM and cultured for 2-3 days. The cultures were examined for astrocyte migration by immunostaining for GFAP and perlecan. Both GFAP and pan-laminin antibodies were raised in rabbit; therefore we used perlecan (raised in rat) instead of laminin to reveal the ILM. The analysis demonstrates that astrocytes show robust migration over the ILM (Fig. 2). Interestingly, astrocytes preferred to stay within the ILM, suggesting that ILM indeed is acting as a substrate for astrocyte migration. Thus, the defective ILM assembly observed in Lamb2 and Lamb2:c3 nulls is likely the cause of abnormal astrocyte migration and patterning.

We next used several migration assays to determine whether laminins act as haptotactic factors guiding astrocyte migration. Retinal astrocytes, isolated from WT P2 retinae, were suspended in an agarose drop that was placed on laminin-coated substrates. Astrocytes that penetrated through the agarose and elaborated processes on the coated substrates were counted and quantified (Fig. 3A-D). On uncoated or laminin-332-coated coverslips, only a few astrocytes penetrated and elaborated processes on the coverslip (Fig. 3A,B). In contrast, placing the astrocyte-agarose suspension on EHS-laminin-coated coverslips resulted in large numbers of astrocytes penetrating through the agarose and elaborating processes on the coverslips (Fig. 3C). Although there was only a modest increase in penetration when astrocytes were plated on laminin-332, there was approximately 4-fold increase in penetration when astrocytes were plated on EHS-laminin (Fig. 3D). This suggests that laminins induce astrocyte migration in an isoform-specific manner.

The previous assay used dissociated cells on an artificial surface, an admittedly artificial set of conditions. Thus, to further approximate the *in vivo* situation, we developed an *ex vivo* explant culture which allowed us to track the normal migratory behavior of astrocytes. We prepared optic nerve head (ONH) explants from 1-week old WT retinas that were then left untreated or treated with exogenous laminin. There was little astrocyte migration from untreated control ONH and laminin-332 treated ONH explants on to the transwell membrane (Fig. 3E,F), whereas EHS-laminin treated ONH showed robust induction of astrocyte migration (Fig. 3G). This data clearly suggests that laminins induce astrocyte migration in an isoform-specific manner (Fig. 3H).

The ultimate restoration of function would be to treat the whole retina, with laminin to rescue the astrocyte phenotype. This experiment is not readily accomplished because of the large size of laminin and limited diffusion. Thus, we chose an alternative. We asked if exogenous laminins would restore astrocyte migration in laminin-null retinae in organotypic cultures of the retina. For this experiment, we chose *Lamb2:c3*-null retina, because the combined null displayed severe astrocyte migration defects (Fig. 2). Organotypic cultures of whole retinal were prepared from P0 or P1 *Lamb2:c3*–nulls; they were treated for 4 days with exogenous EHS-laminin *ex vivo*. The reason for choosing EHS-laminin is because our *in vitro* and *ex vivo* migration assays shows that EHS-laminin induced robust migration of astrocytes, whereas laminin 332 did not induce migration. It suggests that EHS-laminin (contains mostly laminin 111) is capable of inducing selective receptors that astrocytes use to migrate, whereas laminin 332 is not capable of inducing specific receptors that astrocytes use to migrate. Therefore, we decided to use EHS-laminin to rescue astrocyte migration in *Lamb2:c3*–nulls. In untreated *Lamb2:c3*–null retinal explants, GFAP-expressing astrocytes were present mostly clumped around the ONH (Fig. 3I,J), consistent with our observations of *Lamb2:c3*–null retinas *in vivo*. Strikingly, exogenous addition of EHS-laminin to the *Lamb2:c3*–null retinal culture restored astrocyte migration and patterning (Fig. 3K,L). The rescue of astrocyte migration by laminin treatment was statistically significant (Fig. 3M; control, n=4 and treated, n=5, P<0.002). These *ex vivo* experiments clearly demonstrate that laminins are key for astrocyte migration and patterning in the retina.

Loss Of Laminin Affects β1 Integrin Expression Specifically In Astrocytes.

As we have noted above, β1 integrins are expressed by astrocytes in the laminin-rich ILM niche (Fig 1) and are necessary for their migration *in vitro* (Peng et al., 2008). Thus, we were interested to determine if the genetic ablation of laminins affects β1 integrin expression in these cells. Therefore, we localized β1 integrin and GFAP in P5 WT and laminin null retinas. In WT and *Lamc3*-null P5 retinas, β1 integrin was localized around the surface of GFAP- expressing astrocytes (Fig. 4A-C;Fig. 4D-F). Strikingly, deletion of *Lamb2* and combined deletion of *Lamb2* and *Lamc3* genes markedly affected β1 integrin expression in astrocytes (Fig. 4G-L). In these nulls, β1 integrin immunoreactivity was completely absent surrounding the GFAP-expressing astrocytes' cell bodies, with the exception of a few astrocyte processes adjacent to the hyaloid vessels (Fig. 4G-I; Fig. 4J-L). This suggests that the deletion of laminins from the astrocytes' substrate affected integrin receptor localization in astrocytes. We calculated the Pierson coefficient for co-localization of the two markers; these data are given for each of boxed areas in the overlay images in the bar graphs under each image. The experiment has been repeated in wholemount retinae as well as in radial sections.

To test specifically whether the loss of integrin expression in the absence of laminins is a direct consequence of the loss of an interaction with extracellular laminins, we isolated astrocytes from WT and *Lamb2:c3*–null P2 retinas and analyzed β1 integrin expression in the presence or absence of exogenous laminins after 24hr in culture. In the absence of exogenous laminins, WT retinal astrocytes formed a few processes, with β1 integrin localized at the process extensions (Fig. 5A-C) suggesting that these cells produced a matrix and was adhering to it. In contrast, *Lamb2:c3*–null astrocytes platted on uncoated coverslips were small, rounded with few if any processes or integrin β1 expression (Fig. 5D-F). WT retinal astrocytes cultured on EHS-laminin-coated coverslips produced abundant processes, with integrin localization at well-organized, focal adhesion points (Fig. 5G-I) in which FAK and ILK are expressed (not shown). Strikingly in contrast to their behavior on uncoated glass, *Lamb2:c3*–null retinal astrocytes grown on EHS laminin-coated coverslips behaved identically to wild type astrocytes and extended numerous processes with β1 integrin localized at the focal adhesion points (Fig. 5J-L). These data demonstrate that exogenous laminin stimulates rapid process formation and integrin localization in WT retinal astrocytes, a critical first step for cell spreading. Moreover, they demonstrate that the loss of integrin-β1 expression in *Lamb2:c3*–null cells is cell non-autonomous. That is the expression of the laminin ligand is necessary and sufficient to induce or stabilize the expression of the receptor regardless of the genotype of the cells.

Deletion of Lamb2 and Lamb2:c3 genes specifically affect integrin b1 expression in astrocytes. When Lamb2:c3 null astrocytes were grown on EHS-laminin coated coverslips, astrocyte process formation and integrin b1 expression at the focal points was restored, suggesting that laminin helps integrin localization.

Next, we asked whether the interaction of integrin β1 receptor with laminin is required for astrocyte migration. To test this, we dissected WT-P0 retinas around the optic nerve head and placed the vitreal side down on the EHS-laminin coated coverslips. After 36-48hr of culture, astrocytes started to extend their processes out of the tissue. At this stage, the explants were either treated with control IgG or integrin b1 antibody. Control antibody treatment did not affect astrocyte migration and they migrated a distance of 205.4+/- 20.9mm out of the tissue (Fig 5 M, N). In addition, astrocytes in the controls showed elongated process formation over the laminin coated surface, suggestive of polarized migration (5N). Interestingly, integrin β1 antibody treatment affected their astrocyte migration, process elongation and cytoskeletal organization (Fig 5O,P). These cells had migrated only a distance of 24.6+/-7.5mm (Fig 5Q).

Laminin Deletion Affects Retinal Vascular Growth

Retinal vascular growth is dependent on astrocyte migration and patterning (Schnitzer, 1988; Uemura et al., 2006). Because the migration and patterning of astrocytes were affected in laminin-null retinas, we next analyzed whether vascular growth was also affected in laminin-null retinas. Retinas of P1, P3, P5, and P15 from WT and laminin nulls were analyzed for vascular growth with the vascular endothelial specific marker, CD31. Blood vessel formation in the WT retinas covered a distance of 306.47 +/- 9.03 µm (Mean +/- SEM) at P5 (Fig. 6A,E). In contrast, in the *Lamc3*-null retinas, the growth of blood vessel formation was significantly delayed ($P<0.001$) and covered only a distance of 223.14 +/-

11.85 μm (Mean +/- SEM) at P5 (Fig. 6B, E). Because vascular growth was delayed in *Lamc3*-null retinas, we determined whether the tip cell number was affected at the leading edges. At P5, we found that the number of tip cells in the *Lamc3*-null retina was significantly reduced ($P<0.001$) at the leading front when compared to WT (Fig. 6J, Fig. S3C, D).

In *Lamc3*-null retinas, an abnormal vascular branching is also present at the leading edge of the vascular front (cf. Fig. 6A-6B and Fig. S4E-S4F). In the γ3 null, the advancing vascular front is more heavily branched. To understand further whether the laminin γ3 chain regulates vascular branching, we co-localized CD31 and the laminin γ3 chain in WT P5 retinas and found that the laminin γ3 chain was predominantly deposited at the notches of the growing vascular branches (Fig. S3A, B).

To address why the deletion of laminin γ3 chain increases vascular branching and reduces vascular growth rate, we analyzed microglia interaction with the growing vasculature. This is because microglia has been shown to regulate endothelial cell anastomosis through notch signaling (Outtz et al., 2011). Our analysis shows that the deletion of laminin γ3 chain recruits more microglia to the vascular branch points (Fig S4). The recruitment of microglia is likely the cause of increased vascular branching, which is probably affecting vascular growth progression. These results suggest that laminin γ3 chain negatively regulates microglia interaction with the growing vasculature during vascular development.

At P15 in the *Lamc3*-null retina, blood vessel growth and patterning appeared normal (Fig. 6G) consistent with the finding above that astrocyte migration recovered albeit two days late. It has been noted elsewhere, that there was an increase in the density of the deep capillary bed in the outer retina (Li et al., 2012). In the *Lamb2*-null retina, a few endothelial sprouts entered the retina at P1 around the head of the optic nerve (data not shown), but these sprouts were unable to migrate further at P5 (Fig. 6C, E). By P15, blood vessel formation was incomplete in the *Lamb2*-null animal. Importantly, surface capillary network was entirely absent in the most peripheral regions of the retina (Fig. 6H) and surviving hyaloid vessels were obvious. In the *Lamb2:c3*–null retina, vascular growth was severely affected at all stages (Fig. 6D, E, I). At P5, there is no endothelial wave front visible and only the loose networks of adherent hyaloid vessels are obvious. By P15 a small network of microvessels had formed in the central most regions of the retina. Persistent hyaloid vessels were present, and in most cases they sprouted into the retina (data not shown).

Laminins are Critical for Maintaining Proper Astrocyte-Endothelial Interaction and Vascular Integrity

Astrocytes are not only important for retinal vascular development, they are also important for maintaining the blood-retinal barrier. Thus, we analyzed the integrity of the vascular wall in laminin-null retinae. We tested whether astrocyte-endothelial interactions were affected in the retinas in the absence of laminins as well as permeability. In WT and *Lamc3*-null retinas at P15, astrocytes were spatially organized and the processes of astrocytes neatly wrapped around the blood vessels (Fig. 7A, B). On the other hand, in P15 *Lamb2*-null and *Lamb2:c3*–null retinas, the spatial organization of astrocytes was compromised: astrocytes clumped over the vascular BM and did not wrap around the blood vessels as in the WT (Fig. 7C, D).

Fluorescein angiography was used to detect signs of vascular leakage in these mice. In WT and *Lamc3*-null retinas 120 sec after dye injection, there was no vascular leakage, and the fluorescein dye was well retained within the blood vessels (Fig. 7E, F). In contrast, severe vascular leakage was observed in the *Lamb2*-null and *Lamb2:c3*–null retinas (Fig. 7G, H, arrows). These studies demonstrate that laminins containing the β2 and γ3 chains are critical for maintaining astrocyte-endothelial interactions and the stability of the vascular wall.

Discussion:

Astrocyte Migration Is Regulated By Laminins Present In The ILM.

An astrocyte-derived template is critical for the development of retinal angiogenesis (Dorrell et al., 2002; Uemura et al., 2006). In adults, astrocytes play a crucial role in maintaining the blood retinal barrier (BRB). To date, the factors that guide astrocytes into the retina are not well characterized. In this study, using both *in vivo* and *in vitro* assays, we provide evidence that laminins at the ILM regulate astrocyte migration, spatial organization, and subsequent vascular growth.

One of the molecules that may regulate astrocyte proliferation and migration is PDGF-A (Fruttiger et al., 1996). Overexpression of PDGF-A results in hyper-proliferation of astrocytes (Fruttiger et al., 1996). Similarly, neutralization of PDGFR-α, a receptor for PDGF-A, results in reduced astrocyte network elaboration in the retina (Fruttiger et al., 1996). This suggests that such molecules are likely regulating astrocyte proliferation. Other studies have shown that astrocytes isolated from the rat brain migrate toward laminin (Armstrong et al., 1990), suggesting a possible role of laminin in inducing astrocyte migration.

In the retina, laminins have been shown to be critical for ILM formation (Pinzon-Duarte et al., 2010; Hirrlinger et al., 2011; Halfter et al., 2005; Edwards et al., 2010). Similarly, mutations in α-dystroglycan (a laminin receptor) and in an enzyme that participates in glycosylation of α-dystroglycan (POMGnT1) also produce defective deposition of laminin at the ILM, resulting in defective ILM formation (Lee et al., 2005; Takahashi et al., 2011). In addition to ILM disruption, laminin, dystroglycan, and POMGnT1 mutants also display abnormal astrocyte distribution and blood vessel formation (Edwards et al., 2010; Lee et al., 2005; Takahashi et al., 2011). Thus, these studies demonstrate that an intact ILM is necessary for normal astrocyte distribution in the retina. Moreover, our study shows that deletion of *Lamb2* and *Lamb2:c3*- genes results in defective ILM assembly with large holes (Fig. 2). Laminin β2 chain is the major laminin β subunit present in the ILM (Balasubramani et al., 2010). Because laminin β2 chain is the predominant subunit, other laminin β subunits fail to compensate for its loss. This suggests the laminin β2 chain is necessary for ILM formation.

In these previous studies, it was unclear whether the abnormal astrocyte migration and distribution was due directly to the loss of laminin or due to the disruption of the ILM. Data from our in-vivo and in-vitro studies clearly suggest that laminin itself is indeed regulating astrocyte migration. At P0, PDGFR-a expressing astrocytes that are migrating into the retina interact with laminins containing β2 chain at the ILM. This in-vivo observation suggests that laminins containing the β2 chain provide a substrate for astrocytes to enter into the retina.

Our analysis of the *Lamb2*-null supports our hypothesis that laminins containing the β2 chain provide a substrate for astrocytes entering the retina. The deletion of the laminin β2 chain disrupted astrocyte migration and spatial patterning. These data suggests that the presence of laminins containing β2 chain might regulate astrocyte migration and patterning on its own, or the presence of laminins containing β2 chain in the ILM stabilizes other cues that attracts astrocytes to migrate and attain its spatial positioning.

On the other hand, the deletion of the laminin γ3 chain did not severely affect astrocyte patterning, but its deletion did reduced the rate of migration at early postnatal days. This suggests that the laminin γ3 chain may regulate astrocyte-ILM interactions during migration.

The C-terminus of the laminin γ3 chain lacks a critical Glu residue, which prevents it from binding to integrins (Ido et al., 2008). It is possible that deletion of the laminin γ3 chain increases integrin-BM interaction, which results in slower migration. The combined deletion of β2 and γ3 chains had a severe effect on astrocyte migration and patterning, which could be due to the severe loss of the substrate. In both *Lamb2* and *Lamb2:c3* nulls, in addition to their defective distribution, astrocytes were clumped together in a net-like pattern. These data demonstrate that laminins containing β2 chain do not simply act as substrates; laminins containing β2 chain also seem to provide cues for migration and spatial organization of astrocytes.

The clustering of astrocytes in *Lamb2*— and *Lamb2:c3*—nulls could also be due to the loss of integrin β1 receptors. It has been reported that differentiated integrin β1-null neurons exhibit clustering properties in culture similar to that in *Lamb2* and *Lamb2:c3*—nulls (Andressen et al., 1998).

Our *in vivo* analysis of laminin nulls suggests that particular laminins in the ILM may act as haptotactic factors and guide astrocytes over the retinal surface. This was further confirm by ONH was placed over the ILM, which induced robust astrocyte migration (Fig. 2). In addition, our *in vitro* and ONH explant migration assays clearly demonstrate that astrocytes prefer to migrate on EHS-laminin coated substrates compared to laminin-332 coated or uncoated substrates. Laminin-332 has unique integrin receptor interactions (strong binder of integrin $\alpha6\beta4$). We have shown that astrocyte migration is dependent on integrin $\beta1$ expression and which are not good laminin-332 binders. All of this is consistent with the failure of laminin 332 to induce astrocyte migration. The preferential migration of astrocytes on an EHS-laminin substrate (which contains largely laminin-111) shows that astrocyte migration is laminin-isoform dependent.

In addition, treatment of *Lamb2:c3*—null retinal explants with EHS-laminin rescued astrocyte migration and their spatial patterning (Fig. 3). These data demonstrate that, besides providing substrate for migration, laminin also helps the astrocytes to attain their spatial positioning. This is because, proteomic study on stripped ILM shows that $\alpha5$, $\alpha1$, $\beta2$ and $\gamma1$ chains are the predominant laminin subunits present in the ILM (Balasubramani et al., 2010) and $\beta2$ chain partners with both $\alpha5$ and $\alpha1$ chains. The treatment of *Lamb2:c3*—null retinal explants with EHS-Laminin (contains mostly laminin 111) is likely allowing astrocytes to express appropriate integrin receptor, which helps them to migrate and establish their spatial positioning.

Taken together, our data clearly demonstrate that laminins are indispensible for astrocyte migration and patterning in the retina. A key observation in animal models of ROP is the degeneration of astrocytes and subsequent neovascularization (Stone et al., 1996). Recent studies demonstrate that stabilization of astrocytes in models of ROP improves normal revascularization (Dorrell et al., 2010). Whether or not laminin expression is lost during ROP remains unexplored. The loss of laminins under such conditions may cause astrocyte degeneration, and exogenous laminin treatment may aid astrocyte re-patterning and normal revascularization.

Laminins Are Required For Integrin Expression In Astrocytes.

Cell migration is a critical event that occurs during development. Some of the key steps that take place during cell migration are polarization, cytoskeletal reorientation, and lamellipodial extension (Ridley et al., 2003; Huttenlocher, 2005). These events are mediated, in part, by the interaction of extracellular matrix molecules including laminins with integrins. For example, in keratinocytes, laminin-integrin interactions have been shown to regulate FAK/Src, which in turn activates Rac-1, resulting in keratinocyte polarization, lamellipodia extension, and induction of migration (Choma et al., 2007). Similarly, laminin-integrin interactions have been shown to regulate embryonic stem cell migration via Epac1/Rap1 and Rac1/Cdc42 (Suh and Han, 2010).

Laminin-integrin interactions also affect astrocytes. Activation of integrins during astrocyte migration recruits Cdc42, which in turn activates the mPar6/PKCζ complex, resulting in astrocyte polarization and process extension (Etienne-Manneville and Hall, 2001). Conversely, inhibition of integrins or Cdc42 in astrocytes affects polarization and process extension (Etienne-Manneville and Hall, 2001). Integrin β1-null astrocytes in culture also demonstrate defects in polarization, process extension, and abnormal migration (Peng et al., 2008).

Here, in *Lamb2* and *Lamb2:c3*—null retinae, we show that expression of integrin β1 is specifically affected in astrocytes, suggesting the necessity of laminins for integrin receptor localization. Previous *in vitro* culture models also show that cells deposit laminins and use them as substrates on which to polarize and migrate (Frank and Carter, 2004). Astrocytes isolated from *Lamb2* and *Lamb2:c3*—null brains demonstrated defective laminin secretion in culture. Laminin immunostaining was observed mostly intracellular rather extracellular (Fig S2). When *Lamb2:c3*—null astrocytes were grown on glass coverslips for 24 hr, most cells failed to extend processes and were unable to localize integrin β1, which could be due to the lack of laminin secretion. On the other hand, when *Lamb2:c3*—null astrocytes were grown on EHS-laminin, they rapidly induced numerous process extensions, with focal localization of integrin β1. These results clearly demonstrate that laminin is critical for focal localization of integrins, which is necessary for cell adhesion and migration.

We further confirmed that laminin-integrin β1 interaction is crucial for astrocyte migration by functional blocking assay. Functional blocking of integrin β1 receptor not only affected astrocyte migration; it also affected filopodia like process extension in the presence of laminin (Fig.5). This data clearly indicate that laminin-integrin β1 interaction necessary for laminin directed astrocyte migration. It will be interesting to further investigate the downstream signaling events that were disrupted in the laminin null astrocytes during migration.

Laminins Are Necessary For Retinal Vascular Development

Endothelial tip cells follow a template laid down by astrocytes in the retina (Gerhardt et al., 2003; Dorrell et al., 2002). Disruption of astrocyte template formation affects vascular growth progression in retina (Uemura et al., 2006), suggesting the importance of astrocytes during vascular development in the retina. Previous studies have suggested that astrocyte-derived vascular endothelial growth factor (VEGF) and fibronectin guide blood vessel formation in the retina (Gerhardt et al., 2003; Uemura et al., 2006). However, astrocyte-specific deletion of VEGF or fibronectin does not affect vascular growth (Scott et al., 2010; Stenzel et al., 2011). These results suggest that molecules other than VEGF and fibronectin regulate endothelial migration over the astrocyte-derived matrix.

It is possible that astrocytes make a laminin-rich matrix, which acts as a substrate for endothelial cells to migrate. A recent study has shown a laminin-integrin $\alpha6\beta1$ interaction in endothelial tip cell selection (Estrach et al., 2011), which leads the growing vasculature. Our study shows that the deletion of laminin chains has differential effect on endothelial migration over an astrocyte template. Analysis of *Lamc3* null retinas showed slower vascular growth progression at early postnatal days. The effect could be due to the delay in astrocyte patterning. Interestingly, in these *Lamc3* nulls, we observed increased vascular branching at the leading edges of the growing vasculature. These results suggest that the deposition of laminin $\gamma3$ chains at vascular branch points negatively regulates angiogenesis by preventing excessive branching during retinal vascular development.

In both *Lamb2*— and *Lamb2:c3*—nulls, vascular growth was severely affected in both early and late postnatal days. This effect could be due to a lack of proper astrocyte template formation. Astrocytes isolated from *Lamb2* and *Lamb2:c3*—

null brains fail to secrete laminin (Fig. S2). This result suggests that the lack astrocyte derived laminin in *Lamb2* and *Lamb2:c3*—null brains possibly affected endothelial migration over the astrocyte template. Similarly, defective vascular growth was observed in *Lama1* mutant retinas as well (Edwards et al 2010). It is possible that astrocyte derived $\alpha1$ and $\beta2$ containing laminin trimers are regulating endothelial migration.

In addition to defective vascular growth, we observed persistent hyaloid vessels in both *Lamb2* and *Lamb2:c3*—null brains. This defective vascular regression is similar to that seen in ROP and Familial Exudative VitreoRetinopathy (FEVR) (Nissenkorn et al., 1988; Errais et al., 2008). The normal regression of HV is mediated by macrophage lineage cells (Lee et al., 2009). Although we detected a reduction in myeloid lineage cells in *Lamb2:c3*—null HV (data not shown), the defect in recruitment of macrophages in *Lamb2:c3*—null animals during HV regression has not been determined, and requires further investigation. One possible explanation for the reduced vascular regression could be the presence of astrocytes over the HV, inhibiting HV regression.

In summary, our analyses clearly show that laminins containing the $\beta2$ and $\gamma3$ chains play important and distinctive roles during retinal angiogenesis. In this regard, a multigenerational mennonite family carrying a mutation of LAMB2 were reported to have chorioretinal pigmentary changes (Mohney et al., 2011). Although the authors did not comment on it, the presented data also suggest the possiblility of abnormal retinal vascular development. It requires further investigation to understand how the requirement of the LAMB2 gene regulates normal retinal vascular growth. Recent reports also suggest a possibility of the role of various genetic mutations in other genes such as Familial Exudative Vitreoretinopathy and ROP (Dickinson et al., 2006). It will be worthwhile to verify any possibilities of mutations in laminin genes and these other genes in proliferative retinopathies to better understand the pathology in these diseases.

Laminins Maintain Vascular Stability.

Astrocytes play a crucial role in maintaining the blood-brain barrier (Abbott et al., 2006). Cell culture models demonstrate that astrocytes induce properties of the blood-brain barrier in endothelial cells (Wolburg et al., 1994; Gardner et al., 1997). In several ischemic models, loss of laminin-binding integrin and dystroglycan receptors at the astrocyte-vascular interface occurs (Wagner et al., 1997; Tagaya et al., 2001; Milner et al., 2008). This suggests that laminins mediate astrocyte and endothelial interactions, thereby maintaining vascular stability.

Our analysis of *Lamb2* and *Lamb2:c3*—null retinas demonstrates that the interaction of astrocytes with the vascular BM is abnormal. Most astrocytes in *Lamb2* and *Lamb2:c3*—null retinas clumped abnormally over the vascular BM, wrapping around the vascular wall. Fluorescein angiography of *Lamb2* and *Lamb2:c3*—nulls demonstrates that abnormal interaction of astrocytes with the vascular BM may have resulted in vascular leakage. It will be interesting to further analyze the *Lamb2*— and *Lamb2:c3*—null retinas for the distribution of the laminin receptors such as integrins containing $\alpha6$, $\alpha1$, and $\beta4$ subunits, and dystroglycan receptors, which are expressed at the astrocyte-vascular BM interface. It is possible that the loss of laminins may affect receptor localization at the astrocyte-vascular BM interface. Together, our in vivo analyses indicate that the laminin β2 and γ3 chains are necessary for astrocyte-endothelial interactions and vascular integrity maintenance.

In conclusion, our in vivo and in vitro studies show that laminins are indispensable for astrocyte migration and subsequent blood vessel formation in the retina. Deletion of the laminin β2 and γ3 chains disrupts astrocyte migration, spatial patterning, and subsequent blood vessel formation in the retina.

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Figures

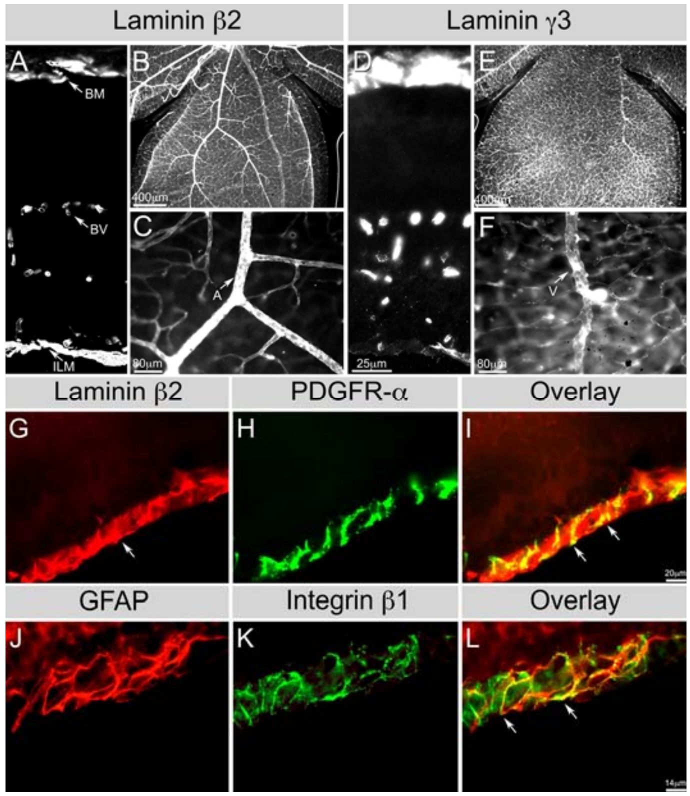

Fig.1. Astrocytes Migrate Within β2-containing Laminin Rich Niche.
(A-C) WT P15 retinal section (A) and wholemount retinae (B, C) were reacted with anti-laminin β2 chain antibodies and imaged using fluorescent microscopy.
(D-F) WT P15 retinal section (D) and wholemount retinae (E, F) were reacted with anti-laminin γ3 chain antibodies and imaged using fluorescent microscopy.
(G, H) WT P0 retinal section was immunostained for laminin β2 chain (G, red) and PDGFR-α to label astrocytes (H, green). I is the merge of G and H. Arrow in G represents the expression of laminin β2 chain at the ILM. Arrows in I shows the PDGFR-α labeled astrocytes interaction with β2 chain containing laminins at the surface of the ILM.
(J, K) Immunostaining of WT P0 retinal section using anti-GFAP (J, red) and anti- β1 integrin (K, green) antibodies. L represents the merge of J and K. The arrows in L point to co-localization of β1 integrin in GFAP positive astrocytes at the ILM.

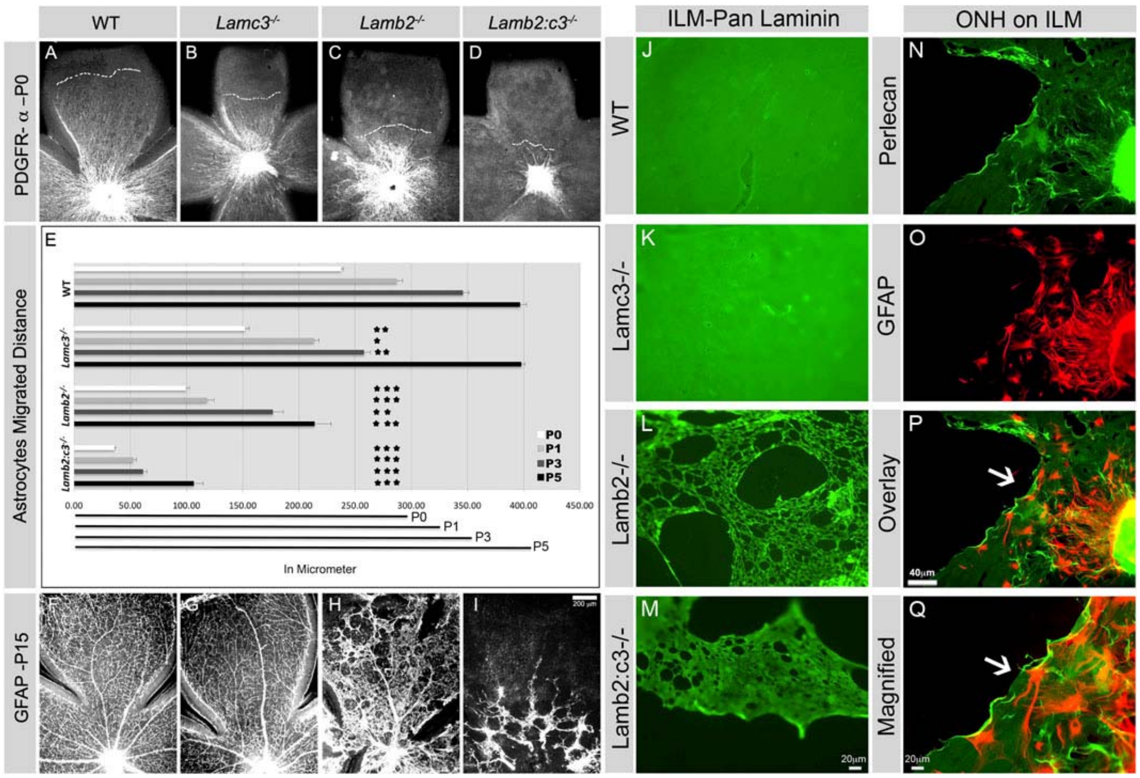

Fig.2. Astrocyte migration and spatial organization is dependent on laminin at the ILM.

(A-D) Astrocyte distribution analyzed with PDGFR-α expression in P0 whole-mount retinae from WT (A), *Lamc3*$^{-/-}$ (B), *Lamb2*$^{-/-}$ (C), and *Lamb2:c3*$^{-/-}$ (D) animals. The dashed white lines in panels A-D mark the boundaries of the distance astrocytes had migrated.

(E) Distance covered by astrocytes from P0 to P5. Retinae were cut into quadrants, and the average was calculated for the distance covered by astrocytes in each quadrant (n=3, except *Lamb2*$^{-/-}$ P5, n= 2). Error bar: SEM. The delay in astrocyte migration seen in *Lamc3* nulls was statistically significant. The horizontal black lines at the bottom of the chart represent the average length of retinal quadrants at represented ages. P values are indicated as follows: $p \leq 0.001$ (□□□); $p \leq 0.003$ (□□); $p \leq 0.01$ (□).

(F-I) Astrocyte distribution analyzed with GFAP expression in P15 whole-mount retinas from WT (F), *Lamc3*$^{-/-}$ (G), *Lamb2*$^{-/-}$ (H), and *Lamb2:c3*$^{-/-}$ (I) animals.

(J-M) ILM were stripped from P15 WT and Laminin nulls and analyzed for laminin expression by immunostaining for anti-laminin. *Lamb2*$^{-/-}$ (L), and *Lamb2:c3*$^{-/-}$ (M) display a defective laminin deposition at the ILM with the presence of large holes in the ILM and appears like a honeycomb.

(N-Q) WT P10 ONH cultured on WT P10 ILM and analyzed for perlecan (green) and GFAP (red).

(N) Immunostaining for perlecan reveals WT P10 ILM.

(O) Immunostaining for GFAP reveals astrocytes migrating out of ONH.

(P) Overlay of (N) and (Q).

(Q) Is the higher magnification of (P), arrow.

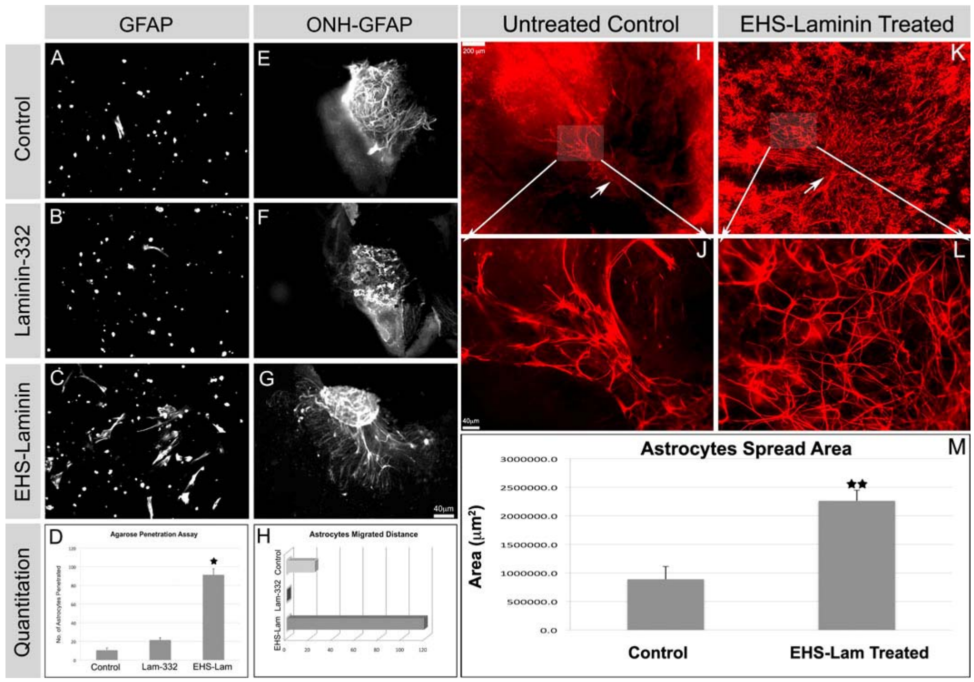

Fig.3. Exogenous laminin treatment rescues astrocyte migration in laminin-null retinal explants.

(A-C) Migration of astrocytes from agarose suspension onto coated coverslips. Coverslips were coated with PBS (A), laminin-332 (10µg/ml) (B) or EHS-laminin (10µg/ml) (C). Astrocyte-agarose suspensions were placed on the substrate-coated area and allowed to solidify. Astrocytes that had migrated through the agarose and elaborated on the substrate-coated areas after 6 days were analyzed for GFAP expression.
(D) Quantification of number of astrocytes that migrated through the agarose and elaborated on the substrate-coated area. Error bar: SD (□, $P<0.04$).
(E-G) ONH explants prepared from one week-old mice. Explants were untreated (E) or treated with laminin-332 (10µg/ml) (F) or EHS-laminin (10µg/ml) (G). After 36 hr., astrocytes that migrated out of the ONH were analyzed for GFAP expression.
(H) The area covered by astrocytes that migrated from ONH explants was measured and quantified using Volocity™ software (v. 5.4.1).
(I, J) Untreated P1 *Lamb2:c3*$^{-/-}$ organotypic retina grown for 4 days in culture and analyzed for GFAP expression. Arrow points to the head of the optic nerve.
(J) is a magnified portion of (I) indicated by shaded box and lines.
(K, L) Laminin-treated P1 *Lamb2:c3*$^{-/-}$ retinal explant grown for 4 days in culture from P1 and analyzed for GFAP expression. Arrow at center points to the head of the optic nerve.
(L) is a magnified portion of (K) indicated by shaded box and lines.
(M) Area over which astrocytes spread, quantified using Volocity™ software (v. 5.4.1). Error bar: SD

Scale bar in I, 200µm for I and K, 40 µm for J and L. (□□, P < 0.002).

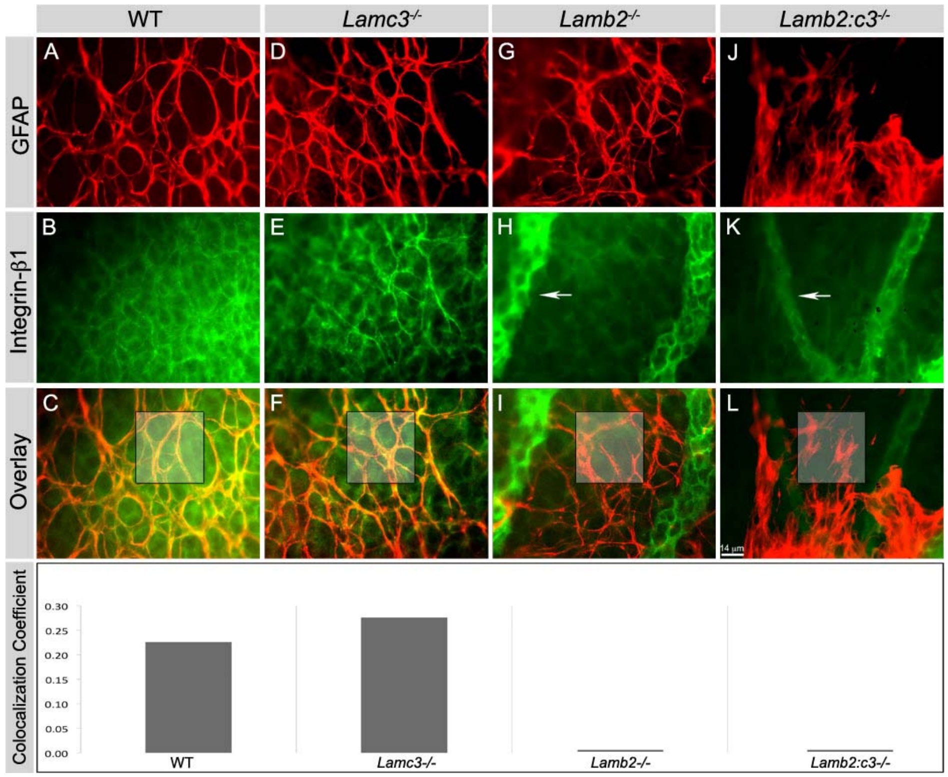

Fig.4. Deletion of laminins affects expression of β1 integrin in astrocytes.

(A-C) P5 whole-mount WT retina analyzed for GFAP (A, red) and β1 integrin (B, green). (C): overlay of (A) and (B).
(D-F) P5 whole-mount *Lamc3*$^{-/-}$ retina analyzed for GFAP (D, red) and β1 integrin (E, green). (F): overlay of (D) and (E).
(G-I) P5 whole-mount *Lamb2*$^{-/-}$ retina analyzed for GFAP (G, red) and β1 integrin (h, green). (I): overlay of (G) and (H).
(J-L) P5 whole-mount *Lamb2:c3*$^{-/-}$ retina analyzed for GFAP (J, red) and β1 integrin (K, green). (L): overlay of (J) and (K).
(M) Pearson's colocalization coefficient was calculated for the boxed areas in images C (WT), F (*Lamc3*$^{-/-}$), I (*Lamb2*$^{-/-}$), and L (*Lamb2:c3*$^{-/-}$) using Volocity™ software (v. 5.4.1).
Arrows in H and K point to β1 integrin expression in hyaloid vessels.
Scale bar in L, 14µm for A-L.

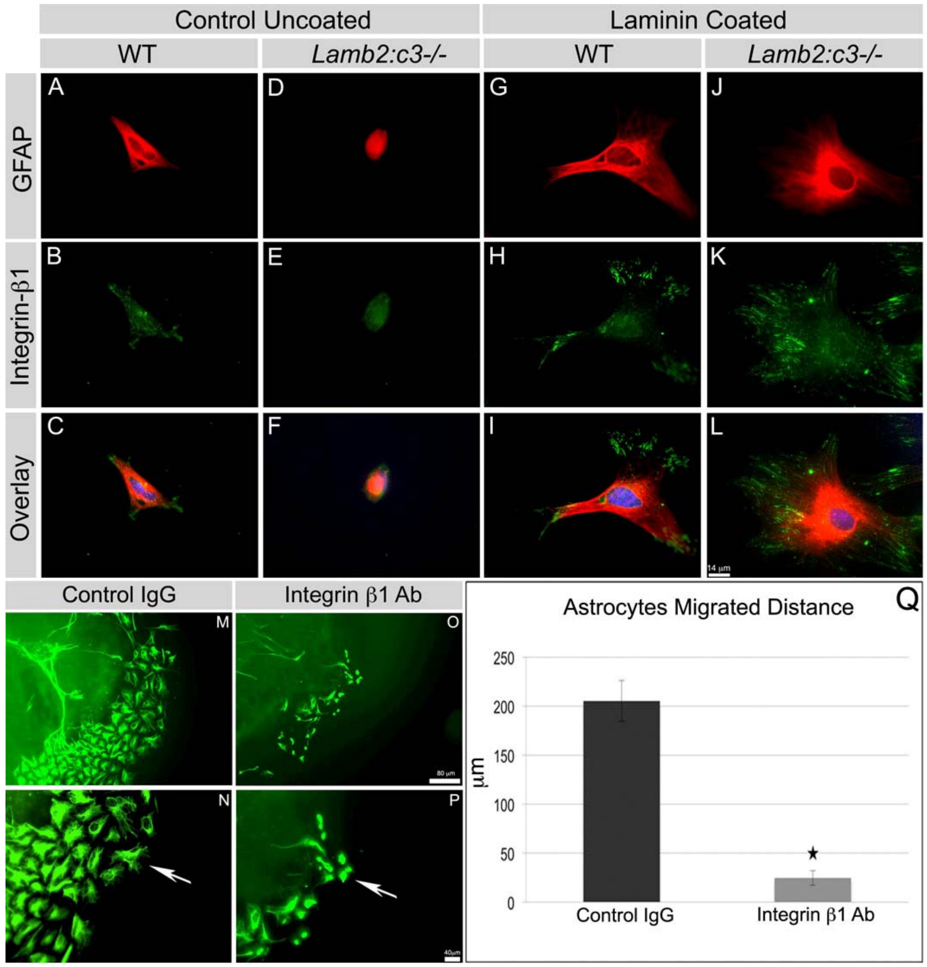

Fig.5. Laminin restores focal localization of β1 integrin in laminin-null astrocytes.

(A-C) Astrocytes isolated from WT P2 retinae plated on an uncoated coverslip were analyzed for GFAP (A, red) and β1 integrin (B, green) expression within 24hr of culture. (C): overlay of A and B (blue, DAPI staining of nucleus).
(D-F) Astrocytes isolated from *Lamb2:c3*$^{-/-}$ retinae plated on an uncoated coverslip were analyzed for GFAP (D, red) and β1 integrin (E, green) within 24hr of culture. (F): overlay of D and E (blue, DAPI staining of nucleus).
(G-I) Astrocytes isolated from WT P2 retinae plated on an EHS laminin-coated coverslip were analyzed for GFAP (G, red) and β1 (H, green) integrin expression within 24hr of culture. (I): overlay of G and H (blue, DAPI staining of nucleus).
(J-L) Astrocytes isolated from *Lamb2:c3*$^{-/-}$ retinae plated on an EHS laminin-coated coverslip were analyzed for GFAP (J, red) and β1 integrin (K, green) within 24hr of culture. (L): overlay of J and K (blue, DAPI staining of nucleus).
(M-P) ONH explants were cultured on EHS-Laminin coated coverslips and treated control IgG (M,N) or integrin- β1 antibody (O,P) for 48hr and analyzed for GFAP (green). EHS-laminin induced robust astrocyte migration and control IgG treatment did not affect this process (M). Migrating cells showed elongated filapodia, arrow in (N).

Function blocking of integrin- β1 receptor affects astrocyte migration (O) and filapodial elongation even in the presence of laminin (O).
(Q) Quantitation of astrocytes migrated distance away from the explants. Error bar: SEM Scale bar: A-L 14µm, M,O 80µm, and N,P 40µm. (□ P< 0.01).

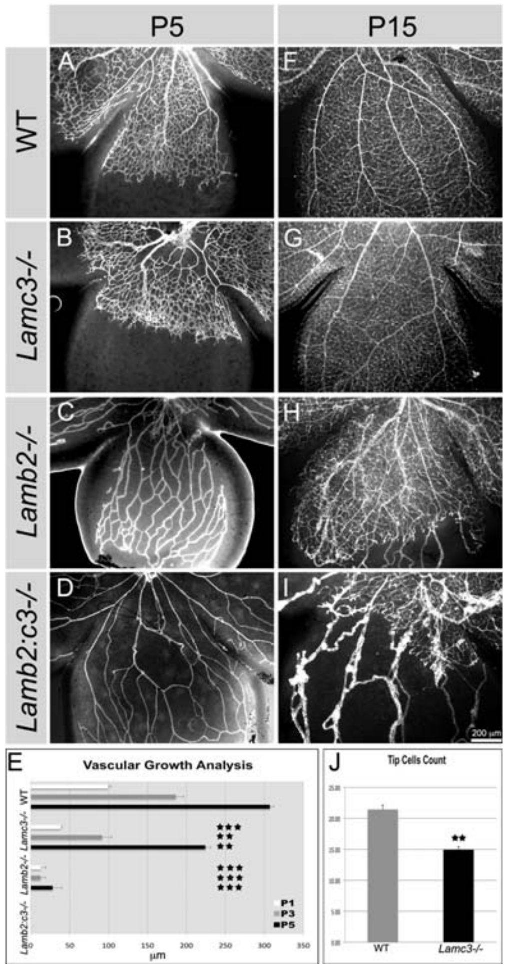

Fig.6. Laminins are necessary for vascular growth.

(A-D) Vascular growth assayed with CD31 expression in P5 whole-mount WT (A), *Lamc3*$^{-/-}$ (B), *Lamb2*$^{-/-}$ (C), and *Lamb2:c3*$^{-/-}$ (D) retinae.
(E) Vascular growth (labeled for CD31) in P1, P3 and P5 of WT, *Lamc3*$^{-/-}$, *Lamb2*$^{-/-}$ and *Lamb2:c3*$^{-/-}$ retinas (n = 3 and 4). Error bar: SEM. The delay in vascular growth in *Lamc3*$^{-/-}$ retinae was statistically significant. No outgrowth of endothelial cells were seen in the *Lamb2:c3*$^{-/-}$ animal from P1 to P5.
(F-I) Vascular growth assayed with CD31 expression in P15 whole-mount WT (F), *Lamc3*$^{-/-}$ (G), *Lamb2*$^{-/-}$ (H), and *Lamb2:c3*$^{-/-}$ (I) retinae.
(J) Tip cell quantitation at P5 in WT and *Lamc3*$^{-/-}$ retinae in a 150µm^2 region per quadrant (n=4). Error bar: SD
P values were ≤ 0.0002 (□□□) and ≤ 0.001 (□□)

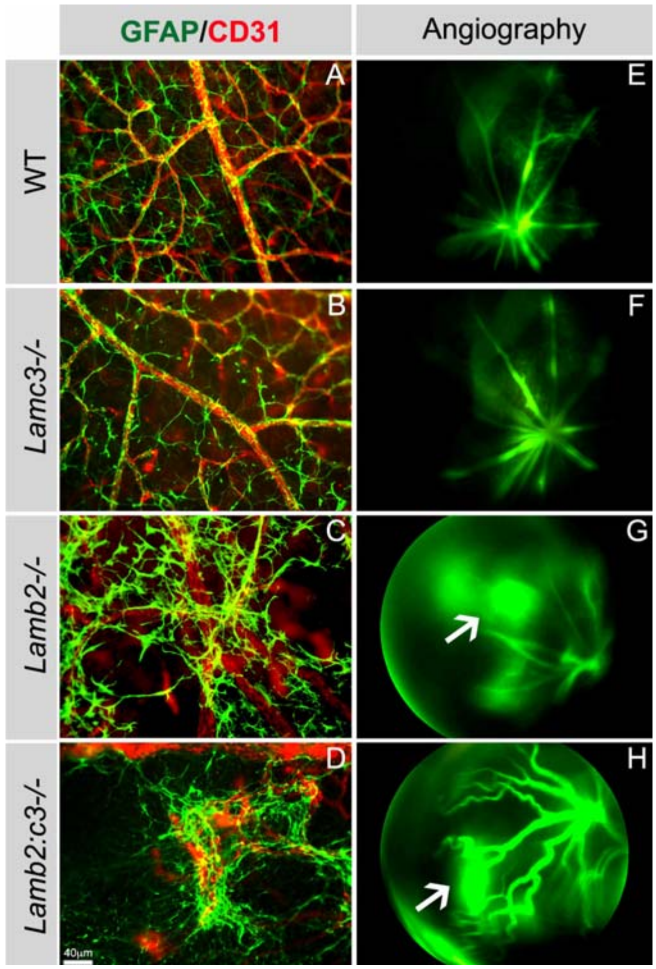

Fig.7. Deletion of laminins affects vascular integrity.

(A-D) The interaction between the GFAP-expressing astrocytes and CD31-expressing blood vessels was examined in P15 retinas from WT (A), *Lamc3*$^{-/-}$ (B), *Lamb2*$^{-/-}$ (C), and *Lamb2:c3*$^{-/-}$ (D) animals. Scale bar: 40 μm.

(E-H) Vascular integrity was examined with fluorescein angiography approximately 120 sec after dye injection in P15 WT (E), *Lamc3*$^{-/-}$ (F) *Lamb2*$^{-/-}$ (G), and *Lamb2:c3*$^{-/-}$ (H) animals. Arrows in G and H show vascular leakage in *Lamb2*$^{-/-}$ and *Lamb2:c3*$^{-/-}$ animals.

Supplementary Figures

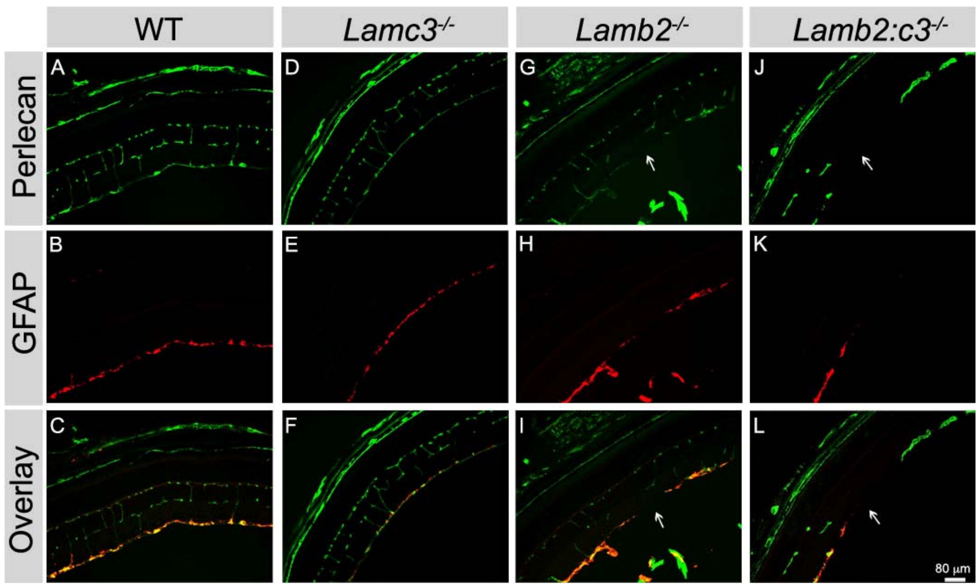

Fig. S1. Astrocytes are present only in retinal regions where the ILM is relatively intact.

(A-C) WT P15 retinal section was reacted with antibodies to perlecan, a component of basement membranes (A) and GFAP, expressed by astrocytes (B). (C): overlay of (A) and (B).

(D-F) *Lamc3*$^{-/-}$ P15 retinal section was reacted with antibodies to perlecan (D) and GFAP (E). (F): overlay of (D) and (E).

(G-I) *Lamb2*$^{-/-}$ P15 retinal section was reacted with antibodies to perlecan (G) and GFAP (H). (I): overlay of (G) and (H). The ILM is disrupted in the *Lamb2*$^{-/-}$ retinal section (arrows).

(J-L) *Lamb2:c3*$^{-/-}$ P15 retinal section was reacted with antibodies to perlecan (J) and GFAP (K). (L): overlay of (J) and (K). The ILM is severely disrupted in the *Lamb2:c3*$^{-/-}$ retinal section (arrow).

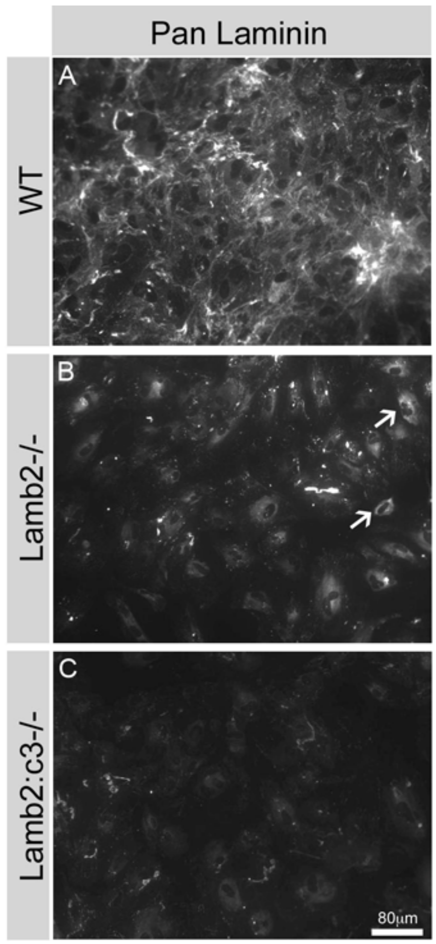

Fig. S2. Deletion of Lamb2 and Lamb2:c3 genes affect laminin secretion.
(A-C) P2 retinal astrocytes isolated from WT (A), Lamb2-/- (B) and Lamb2:c3-/- (C) shows laminin expression after 6 days in culture.
(A) WT astrocytes secrete laminin, which was observed by anti-laminin immunostaining over the cell surface.
(B) Lamb2-/- retinal astrocytes failed to secrete laminin and mostly appeared intracellular (arrow).
(C) Similar to (B) Lamb2:c3-/- retinal astrocytes also failed to secrete laminin and mostly appeared intracellular (arrow).

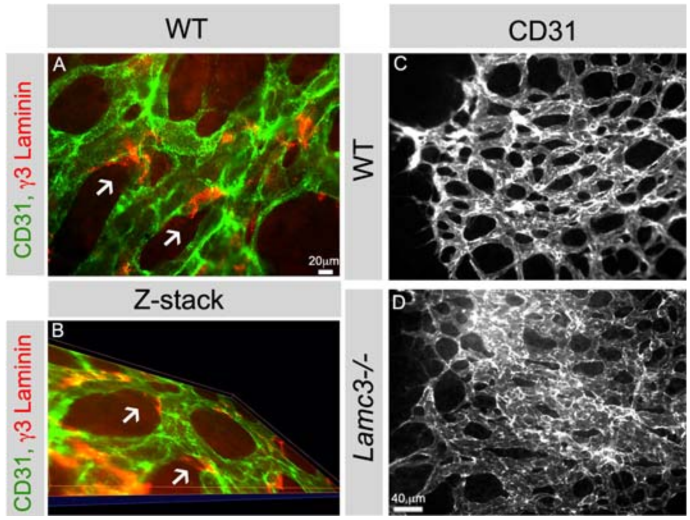

Fig. S3. Deletion of laminin γ3 chain affects vascular branching pattern during angiogenesis.

(A) WT P5 wholemount retina was reacted with antibodies to laminin γ3 chain (red) and CD31 (green). The laminin γ3 chain is prominent at vascular branch points (arrows). This image was captured using fluorescent microscopy and deconvolved using Volocity™ software (v. 5.4.1). Scale bar: 20μm.

(B) WT P5 wholemount retina was reacted with antibodies to laminin γ3 chain (red) and CD31 (green). A z-stack was created from 0.5 μm steps using Volocity™ software (v. 5.4.1) and a 3-dimensional image was created in Volocity and rotated to reveal the expression laminin γ3 chain around the vascular branch points (arrows).

(C) WT P7 wholemount retina was reacted with antibodies to CD31 to analyze the vascular branching pattern. A regular branching array is present in the wild-type.

(D) *Lamc3*$^{-/-}$ P7 wholemount retina was reacted with antibodies to CD31 to analyze the vascular branching pattern. The branching array is disrupted in the absence of the laminin γ3 chain. Scale bar: 40μm.

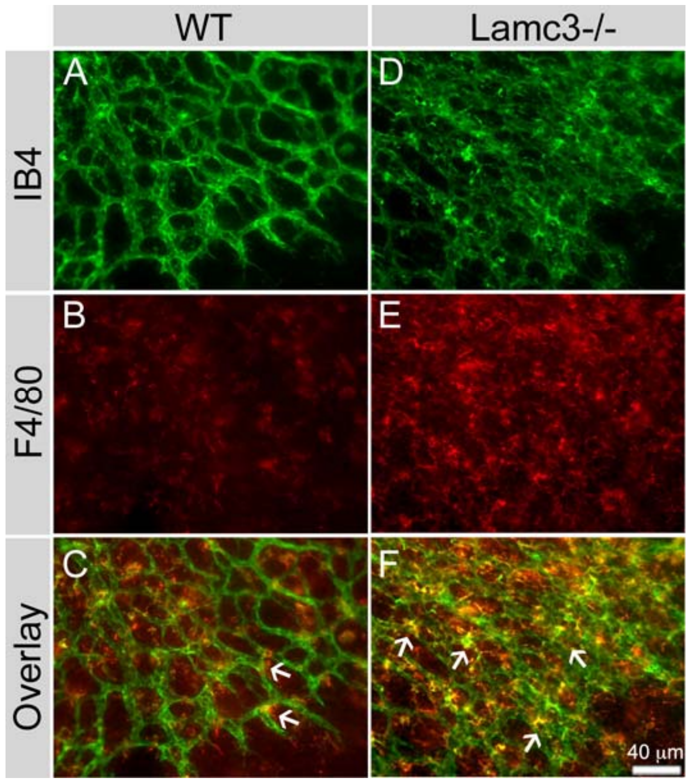

Fig. S4: Laminin γ3 chain regulates microglia-vasculature interactions.
(A-F) P3 wholemount retinae from WT and Lamc3-/- were immunostained for isolection B4 (IB4, green) and F4/80 (microglia specific marker, red) to reveal blood vessel and microglia interactions.
(A), (D) are showing blood vessels as well as microglia in green.
(B), (E) are showing only microglia in red.
(C) is the overlay of (A) and (B) showing a few microglia at the vascular branch points (represented by arrows).
(F) is the overlay of (D) and (E) showing more microglial association with the blood vessels at branch points (represented by arrows).

Chapter 3

Laminin mediates astrocyte migration through an integrin β1-FAK mediated signaling mechanism

Introduction

Astrocytes are abundantly found glial cells in the central nervous system (CNS) that regulate a wide variety of functions (Ullian et al 2004; Wang 2008). It is well established that in the retina, astrocytes are crucial for blood vessel formation. They provide a template over which endothelial cells to migrate (Dorrell et al., 2002) and they enseath mature blood vessels contributing to the blood retinal barrier (Gardner et al 1997).

Despite their importance in retinal vascularization, the mechanism of astrocyte migration and spatial organization in the retina remains poorly understood. A more complete understanding of the mechanisms of astrocyte migration would contribute not only to developmental biology of the retina but also to our understanding the pathobiology of retinal neovascular disease, as experimental models for retinopathy of prematurity (Stone et al., 1996; Dorrell et al., 2010) and diabetic retinopathy (DR) show degeneration of astrocytes (Barber et al., 2000; Ly et al., 2011). Preventing the loss of astrocytes has been shown to prevent the neovascularization that accompanies hypoxia in animal models of ROP (Dorrell et al., 2010).

Our studies as well as others have shown that the genetic deletion of laminin chains and their binding partners, dystroglycans, affect inner limiting membrane formation (ILM) (Pinzon-Duarte et al., 2010; Halfter et al 2005; Edwards et al., 2010; Lee et al 2005; Takahashi et al 2011). The studies have also shown that disruption in ILM formation leads to abnormal spatial distribution of astrocytes and defective angiogenesis (Edwards et al., 2010; Gnanaguru et al., 2012). We have shown that placing an optic nerve head on stripped ILM induced astrocyte migration, confirming that ILM has cell guidance properties (Gnanaguru et al., 2012). Further, using in-vitro and ex-vivo experiments, we have shown that laminin regulates astrocyte migration through integrin β1 receptor. These data suggest that laminins in the ILM are likely regulating astrocyte migration. In this study, using optic nerve head explants, we have further characterized the mechanism by which laminin regulates astrocyte migration.

The two key kinases that are immediately downstream of integrin activation are: integrin-linked kinase (ILK), a serine/threonine protein kinase; and focal adhesion kinase (FAK), a non-receptor tyrosine kinase. Both these kinases interact with integrin β1 at the cytoplasmic tail, and activate further downstream signaling molecules, leading to actin rearrangement and cell migration (Hannigan et al., 1996; Hannigan et al., 2005; Wennerberg et al., 2000; Mitra et al 2005). As integrin β1 interacts with both ILK and FAK, we analyzed both ILK and FAK involvement in astrocyte migration.

Our data shows that WT astrocytes during migration express ILK and FAK. Interestingly, deletion of Lamb2 and Lamb2:c3 genes downregulates both ILK and FAK expression specifically in retinal astrocytes. Furthermore, using optic nerve head cultures, we show that laminin induces astrocytes filopodial elongation with focal localization of ILK and FAK at the tip of the processes. Functional blocking of integrin β1 affects the recruitment of both ILK and FAK by astrocytes in the presence of laminin, and retards the astrocyte filopodial extension. siRNA silencing of FAK expression affects astrocyte filopodial extension more than silencing of ILK in the presence of laminin. The analysis shows that laminin communicates to the cell through integrin β1, and mediates astrocyte migration through FAK.

Methods

Mice

Animal procedures were performed in accordance with the institutional animal care and use committee of SUNY Downstate Medical Center (DLAR). Targeted deletion of *Lamb2 and Lamc3* genes and the production of the *Lamb2-/-Lamc3-/-* animals have been described previously (Noakes et al 1995; Li et al., 2012, Denes et al., 2007; Pinzon-Duarte et al., 2010). The Lamb2 null animals are maintained as a heterozygous line, the Lamc3 nulls are maintained as homozygous null line. Both of those lines used in this study were backcrossed over 9 generations to C57bl/6J. The *Lamb2:c3* and *Lamc*3 nulls were created by mating *Lamb2*+/- and *Lamc3*-/- animals. This line was on a mixed genetic background of C57bl/6J and 129Sv/J; genetic background characterization was performed by Taconic, Inc (Germantown, NY) using a 377 SNP panel and demonstrated that the *Lamb2*+/-:*Lamc3*-/- line matched more than 83% of C57bl/6J recipient genome. From here forward the following notations are used in the text and figures for genotypes: β2 deletion, *Lamb2*-null or $Lamb2^{-/-}$; γ3 deletion, *Lamc3*-null or *Lamc3*$^{-/-}$; for compound deletion of β2 and γ3, *Lamb2;c3*—null or *Lamb2:c3*$^{-/-}$.

Preparation of Retinal Sections

Eyes were enucleated and fixed for 5 minutes with ice cold 2% PFA by making a small incision near the ora serrata. The anterior chamber was removed in PBS and the eyecup was further fixed with ice cold 2% PFA for 15 min. The tissue was cryoprotected with serial 10%, 20%, and 30% sucrose treatment before embedding in OCT (Sakura Finetek, Torrance, CA). 15-20 μm-thick sections were prepared. The sections were washed in PBS, then blocked for 2 hr at room temperature (RT). After blocking, the samples were treated with appropriate primary antibodies overnight at 4° C. After washes with PBS, the samples were incubated with respective secondary antibodies for 2-3 hr RT. The samples were then washed in PBS, cover slipped using Vectashield (Vector Laboratories, Burlingame, CA) and sealed.

Whole Mount Retinal Preparation and Immunostaining

Whole mounts were prepared as described previously (Li et al., 2012). In brief, eyes were enucleated and fixed in 2% paraformaldehyde (PFA) prepared in PBS (see below) for 5 to 10 min. The anterior chamber was removed and retinas were dissected out in PBS. Retinas were flat mounted by making four radial cuts. The flat mounts were treated with absolute methanol at -20°C for 10 min. The flat mount retinas were washed in PBS. The whole mount retinas were incubated in blocking buffer (15% goat or donkey serum, and 0.03% triton-X 100 prepared in PBS) overnight at 4°C. The whole mounts were then incubated with primary antibodies (diluted in 5% goat or donkey serum, and 0.01% triton-X 100 prepared in PBS) for 48 h at 4°C. The flat mounts were washed and incubated with respective secondary antibodies, overnight at 4°C. After incubation period the samples were washed in PBS, cover slipped using ProLong gold (Invitrogen, Grand Island, NY), and sealed.

Preparation of retinal explants for astrocyte migration assays

To study laminin-mediated astrocyte migration, retinal explants were prepared from WT P0-P3 animals. The retinas were isolated in sterile ice-cold serum-free DMEM in a tissue culture hood. The retinal tissue was dissected around the head of the optic nerve head with the aid of a dissecting microscope. The tissue was then placed on a glass coverslip (vitreal side facing the coverslip), which was previously coated with approximately 20μg/ml of EHS laminin (prepared in sterile PBS (137mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.4)). The coverslips were then placed in a 24-well plate. To each well, 250 μl of media (DMEM containing 10% FBS) was carefully added to the sides and the plate was maintained at 37° C with 5% CO_2. Extension of astrocyte processes out of the explant generally occurred at 24-36 hr of culture. These cultures were used to study astrocyte migration using function blocking antibodies or siRNAs.

Antibody blocking assay

To test whether astrocyte migration in the retina was regulated by laminin-integrin β1 interaction, we performed functional blocking of integrin β1 receptors. Retinal explants were prepared from WT P0-P2 pups, as described above. After 36 hr of culture, one explant was incubated with DMEM containing 10% heat-inactivated FBS + 10-25 μg/ml non-specific control IgG (Jackson Immunoresearch Laboratory, West Grove, PA). The other explant prepared from the other eye was incubated with DMEM containing 10% heat inactivated FBS + 10-25 μg/ml integrin β1 antibody (BD Biosciences, San Jose, CA). After 48 hr of antibody treatment, the explants were fixed with 4% paraformaldehyde for 5 min on ice. The samples were washed in PBS and blocked for 1-2 hr at room temperature in 1X PBS containing 10% fetal bovine serum (FBS), 0.05% triton-X 100 and 0.001% sodium azide. The samples were washed in PBS and incubated with GFAP antibody (Chemicon, Billerica, MA) prepared in 1X PBS containing 2% FBS, 0.01% triton-X 100 overnight at 4°C. Following the primary antibody incubation, the samples were incubated with respective RITC or FITC tagged secondary antibodies (Life Technologies, Grand Island, NY) for 2-4 hr at room temperature. After washes in PBS, the samples were coverslipped using Prolong Gold (Life Technologies, Grand Island, NY) and sealed.

siRNA Knockdown experiment

The siRNA-transfection reagent complexes were prepared per the manufacturer's instructions (Mirus Bio LLC, Madison, WI). Control (Cell Signaling Technology, Boston), ILK and FAK (Upstate Cell Signaling Solutions, Temecula, CA) siRNAs were used at final concentrations of 50 nM. The retinal explants were prepared as described above and cultured on EHS-laminin coated surfaces for 24-36 hr. The explants were then treated with control, ILK and FAK siRNAs for 48 hr in Opti-MEM (Life Technologies, Grand Island, NY). The samples were gently washed in PBS and then fixed for 2-5 min in 4% paraformaldehyde. The samples were gently washed a few times in 1X PBS. The samples were then blocked in PBS containing 10% goat or donkey serum and 0.03% triton X-100 for 2 hr at room temperature. In each experimental condition, one explant treated with control siRNA and one explant treated with ILK siRNA was analyzed for ILK and α-actinin expression, by probing for anti-ILK and anti- α-actinin (Chemicon, Billerica, MA). The other explants treated with control and ILK siRNAs were probed for anti- α-actinin. Similarly, one explant treated with control siRNA and one explant treated with FAK siRNA was analyzed for FAK and α-actinin expression by probing for anti-FAK (Cell Signaling Technology, Boston) and anti- α-actinin. The other explants treated with control and FAK siRNAs were probed for anti- α-actinin. These experiments were performed in at least one ONH prepared from 3-4 animals for each condition.

Results

Deletion of laminins leads to a reduction of ILK and FAK expression in astrocytes

Ligand binding to integrin β1 leads to an interaction of the cytoplasmic tail of integrin β1 with ILK and FAK (Hannigan et al., 1996; Hannigan et al., 2005; Wennerberg et al., 2000; Mitra et al 2005). This interaction recruits several downstream signaling events leading to cell migration. Integrin localization in astrocytes may be modulated by laminins: we have shown that integrin β1 expression is decreased in astrocytes in *Lamb2* and *Lamb2:c3*- nulls (Gnanaguru et al., 2012). Thus we tested whether the loss of integrin β1 localization observed in *Lamb2* and *Lamb2:c3* nulls (Gnanaguru et al., 2012) affects ILK and FAK recruitment in astrocytes during migration into the retina.

We localized ILK and FAK in WT P0 retinal sections at the same time we selectively labeled astrocytes with the marker PDGFR-α. Both ILK and FAK are expressed by WT astrocytes at P0 during migration into the retina (Fig. 1A-F). Both WT and *Lamc3*-null astrocytes express ILK and FAK (Fig. 2A-F and 3A-F), whereas deletion of *Lamb2* (Fig. 2G-I and 3G-I) and *Lamb2:c3* genes (Fig. 4 D, E and F, J, K and L) affect ILK and FAK expression, specifically in astrocytes. This suggests that loss of laminins affects integrin β1 localization in astrocytes, leading to a consequent down-regulation of ILK and FAK.

Laminin-integrin interaction recruits ILK and FAK to a filopodia-like structure during astrocyte migration

Integrin linked kinase (ILK) and focal adhesion kinase (FAK) regulate cell migration through integrin-mediated signaling mechanisms (Esfandiarei et al., 2010; Suh and Han 2010). We have previously shown that astrocytes in the retina express integrin β1 and deletion of *Lamb2* and *Lamb3:c3* genes leads to a decrease in β1 expression in astrocytes (Gnanaguru et al., 2012). Thus, we hypothesized that the loss of laminin-integrin interactions affects further activation of downstream events that induce cell migration such as kinase activation.

To test this, we asked whether laminin-integrin interaction recruits ILK and FAK during astrocyte migration into the retina. WT optic nerve head explants prepared from P0-P2 mice were cultured on EHS-laminin coated surfaces, and recruitment of ILK and FAK during astrocyte outgrowth was examined after 48 to 72 hours. Astrocytes migrating over laminin-coated surfaces extend processes and recruit ILK at the tip of those processes. These events were unaffected in the presence of control IgG (Fig. 5A). Blocking of integrin β1 receptors affected both process extension and ILK recruitment (Fig. 5B).

We also tested whether FAK is recruited during astrocyte migration. On laminin-coated surface, astrocytes showed elongated processes with FAK localization (Fig. 5C). Similar to our results with ILK recruitment, FAK recruitment was affected when the samples were treated with function-blocking integrin β1 antibodies (Fig. 5D). These results suggest that integrins regulate astrocyte migration through FAK and ILK. Our hypothesis is supported by previous studies where FAK was shown to regulate cell migration through integrins (Sieg et al., 1999).

In summary, laminin-integrin β1 interaction recruits both ILK and FAK, and this recruitment likely activate further downstream signaling events during astrocyte migration.

Reduction of FAK expression inhibits astrocyte process formation in the presence of laminin

Our data reported above suggests that integrin β1 may regulate astrocyte migration through ILK and FAK. The regulation of migration through these kinases may be through alterations of the cytoskeleton. Both ILK and FAK regulate actin cytoskeleton rearrangement during cell migration via the Rho GTPases, rac/cdc42 (Filipenko et al., 2005; Chang et al., 2007). To investigate further whether ILK and FAK are direct targets of laminin-integrin interactions, we performed siRNA-mediated knockdown of ILK and FAK in the presence of laminin. Optic nerve head cultures from WT P0-P2 animals were grown on laminin-coated coverslips. The cultures were treated with siRNAs for 48 hr and analyzed for astrocyte process extensions. Control siRNA-treated astrocytes displayed elongated filopodia, in which α-actinin was organized towards the filopodia extensions (Fig 6B). Moreover, in the control siRNA-treated samples, ILK was localized at the tip of the filopodia (Fig 6F & I). ILK siRNA treatment affected its localization at the tip of the filopodial structures (6O & 6R). However, although ILK recruitment was disrupted, α-actinin organization was not drastically altered upon ILK siRNA treatment (6K, N & Q).

In contrast to control and ILK siRNA treatments, silencing of FAK affected the formation of filopodia in astrocytes (Fig. 7A & B). The leading astrocytes appeared blunted, with no filopodial projections (Fig 7B). Moreover, in the control siRNA treatment samples focal localization of FAK was observed (7D & E), whereas FAK siRNA treatment led to an apparent decrease in FAK levels and less localization at the elongating processes. In addition, upon FAK siRNA treatment, α-actinin was organized in a parallel position instead of orienting toward the filopodia (Fig. 5B). These data suggest that FAK may be involved in the process by which astrocytes extend filopodia and rearrange α-actinin toward a laminin-directed migratory path.

Discussion

Astrocyte migration into the retina is crucial for the development of retinal angiogenesis. During pathological conditions such as retinopathy of prematurity and diabetic retinopathy, astrocytes degenerate, leading to vascular instability and neovascualrization. Understanding the mechanism of astrocyte migration and spatial organization will help us find a better way to stabilize astrocytes and perhaps reduce degeneration during such pathologies. In this study we report that laminin mediates astrocyte migration through integrin-mediated signaling mechanism. These results may help to elucidate the mechanism of astrocyte migration in the retina.

Astrocytes are distributed throughout the CNS; recent studies are unraveling the importance of astrocytes in regulating various functions of the CNS. Some key functions that astrocytes regulate are: synaptogenesis, synaptic activity, retinal angiogenesis, and maintaining the blood-neural barrier (Tsai et al 2012; Pfrieger and Barres 1997; Abbott et al 2006). Astrocytes are not only involved in maintaining normal CNS function, but are also involved in several pathologies of the CNS (Pekny and Nilsson 2005).

With respect to the retina in particular, astrocytes tend to respond to physiological changes, which could result in deleterious effects. For example, animal models for retinopathy of prematurity and diabetic retinopathy suggest that astrocytes degenerate during hypoxic or hyperoxic as well as hyperglycemic conditions (Stone et al., 1996; Dorrell et al., 2010; Barber et al., 2000; Ly et al., 2011).

Degeneration of astrocytes during development might retard vascular growth and cause neovascularization. This can be correlated with retinopathy of prematurity, where prematurely born infants show poor vascular development and neovascularization (Chen and Smith 2007; Gariano and Gardner 2005). Degeneration of astrocytes in adulthood might affect vascular integrity and cause neovascularization, as is seen in diabetic retinopathy (Barber et al 2000; Gariano and Gardner 2005). Because models for ischemia also show losses of integrins and dystroglycan receptors in astrocytes at the vascular wall interface (Tagaya et al 2001; Milner et al., 2008), stabilizing the ECM-cell surface receptor interaction could potentially reduce disease progression.

During cell migration the events that take place are: polarization of cells, formation of protrusions or filopodia at the leading edge, stabilization of ECM interaction through cell surface receptors, and formation of focal adhesion complexes, which finally results in migration by actin rearrangement (Ridley et al., 2003). Integrins are cell surface receptors that link the ECM and cytoskeleton through their cytoplasmic tails (Huttenlocher and Horwitz 2011). Both ILK and FAK are known to interact with the cytoplasmic tail of integrin β1 receptors (Hannigan et al., 1996; Hannigan et al., 2005; Wennerberg et al., 2000; Mitra et al 2005). This interaction leads to the activation of Rac/Cdc 42 (Filipenko et al 2005; Suh and Han., 2010), resulting in actin rearrangement and cell migration.

Laminin has also shown to induce cell migration through ILK (Chun et al., 2003) and FAK upregulation, leading to Rac1/cdc42 activation (Suh and Han., 2010), which suggests that integrins bridge extrinsic and intrinsic signaling pathways during migration.

The question we asked was whether a similar signaling mechanism occurs during astrocyte migration in the retina. We observed that the loss of laminin chains affects astrocyte migration and patterning with a concomitant loss of integrin β1 expression (Gnanaguru et al., 2012). Moreover, we have also shown that laminin regulates astrocyte migration through integrin β1 receptors (Gnanaguru et al., 2012), and others have reported that integrin β1 regulates oriented astrocyte migration (Peng et al., 2008). These results suggest that a laminin-integrin β1 mediated signaling pathway likely regulates astrocyte migration in the retina.

In this study, we provide evidence that laminin-integrin β1 interaction signals FAK to orient astrocyte filopodia and organize α-actinin towards the migratory path. As mentioned earlier, laminin-integrin interaction regulates cell migration through ILK and FAK activation (Chun et al 2003; Desban et al., 2006; Suh and Han., 2010). To understand whether these kinases are involved in mediating astrocyte migration in the retina, we analyzed their expressions in WT P0 retinae during the active astrocyte migration phase. Our analysis shows that astrocytes express both ILK and FAK (Fig. 1), which suggests that one of these kinases regulate astrocyte migration. In order to verify this hypothesis, we analyzed ILK and FAK expression in laminin-null astrocytes. Interestingly, deletion of *Lamb2* and

Lamb2:c3 genes downregulates the recruitment of ILK and FAK specifically in astrocytes (Fig 2 and 3). The results may be due to the loss of integrin β1 (Gnanaguru et al., 2012).

We have previously shown that functional blocking of integrin β1 receptors affects astrocyte migration in the presence of laminin, suggesting that integrin β1-laminin interactions are critical for astrocyte migration (Gnanaguru et al 2012). To further test whether laminin-integrin β1 interaction recruits ILK and FAK in astrocytes, we performed additional functional blocking assays. Retinal tissue from WT P0-P2 animals were dissected around the head of the optic nerve and cultured on laminin-coated coverslips. Control astrocytes showed elongated filopodial extensions on the laminin-coated area. At the tip of these extensions, both ILK and FAK were recruited (Fig 4). Interestingly, functional blocking of integrin β1 receptor affected both ILK and FAK recruitment at these tips (Fig 4). This suggests that ILK and FAK are likely involved in regulating astrocyte migration in the retina.

To understand the role of ILK and FAK during astrocyte migration, we performed transient knockdown experiments using siRNAs. The optic nerve head cultures were treated with control, ILK or FAK siRNAs for 48 hr. Filopodial extension formation at the leading astrocytes was analyzed after siRNA treatment by immunostaining for α-actinin. α-actinin is known to orient itself towards the migratory path and crosslinks the actin cytoskeleton to cell surface receptors by interacting with several adaptor molecules (Vicente-Manzanares et al., 2009). Our data demonstrated that knockdown of ILK did not affect astrocyte filopodial extension and α-actinin organization, suggesting that ILK may play a role in other functions such as cell maturation. Alternatively, knockdown of ILK may result in upregulation of other molecules such as FAK, which may rescue astrocyte filopodial extension and α-actinin orientation.

In contrast to the knockdown of ILK, the knockdown of FAK had a dramatic effect on astrocyte filopodial extension. Knockdown of FAK affected a-actinin organization; moreover, the majority of migrating astrocytes often clumped and the cells had blunt ends with no filopodial extensions (Fig 5).

It is possible that knockdown of FAK has affected astrocyte filopodial orientation during migration by affecting the activation of Cdc42. FAK is known to activate Cdc42 (Mitra et al., 2005) and Cdc42 has been shown to regulate astrocyte polarity during migration (Etienne-Maaneville and Hall., 2001). Another possibility for the affect of the knockdown of FAK is a disruption of focal adhesion complex formation. Formation of adhesion complexes at the leading front of a migrating cell is one of the hallmarks of cell migration (Hu et al., 2007), and FAK is known to interact with some of the adhesion complex molecules such as paxillin and talin (Scheswohl et al., 2008; Chen et al., 1995).

The knockdown of FAK could have affected the interaction of FAK with focal adhesion complex molecules. The latter is highly likely because α-actinin has been shown to interact with focal adhesion complex molecules (Bois et al., 2006). Our data demonstrates that knockdown of FAK affects α-actinin organization in migrating astrocytes, our data suggest that FAK is one of the key regulators of astrocyte migration, and our data suggest that this FAK-mediated regulation is activated through laminin-integrin interactions.

In conclusion, our data suggest that FAK is critical for laminin-mediated astrocyte migration. Laminin-integrin β1 interaction recruits FAK and ILK during astrocyte migration. Deletion of the *Lamb2* gene affects astrocyte migration, concomitant with a loss of integrin β1 (Gnanaguru et al., 2012), ILK, and FAK expression. This study, for the first time, describes the mechanism of laminin-mediated astrocyte migration in the retina. The results obtained from this study can be used to correlate with several astrocyte-endothelial related pathologies in the retina for a more effective prognosis.

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Figures:

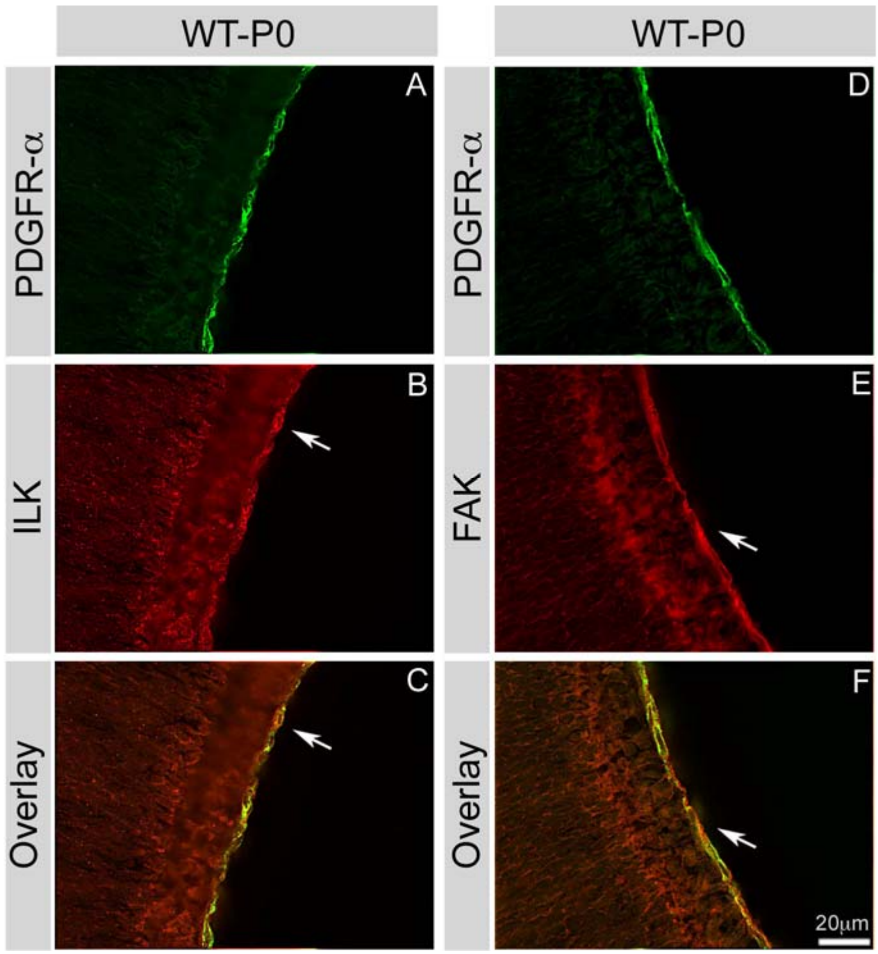

Fig.1. Migrating astrocytes express ILK and FAK.

(A-C) WT P0 retinal section was reacted with anti-PDGFR-a and anti-ILK antibodies and imaged using fluorescent microscopy and deconvolved using volocity™ software 5.5.1. The arrows in (B) and (C) show the localization of ILK in astrocytes.

(D-E) WT P0 retinal sections were reacted with anti-PDGFR-a and anti-FAK antibodies and imaged using fluorescent microscopy and deconvolved using volocity™ software 5.5.1. The arrows in (B) and (C) show the localization of FAK in astrocytes.

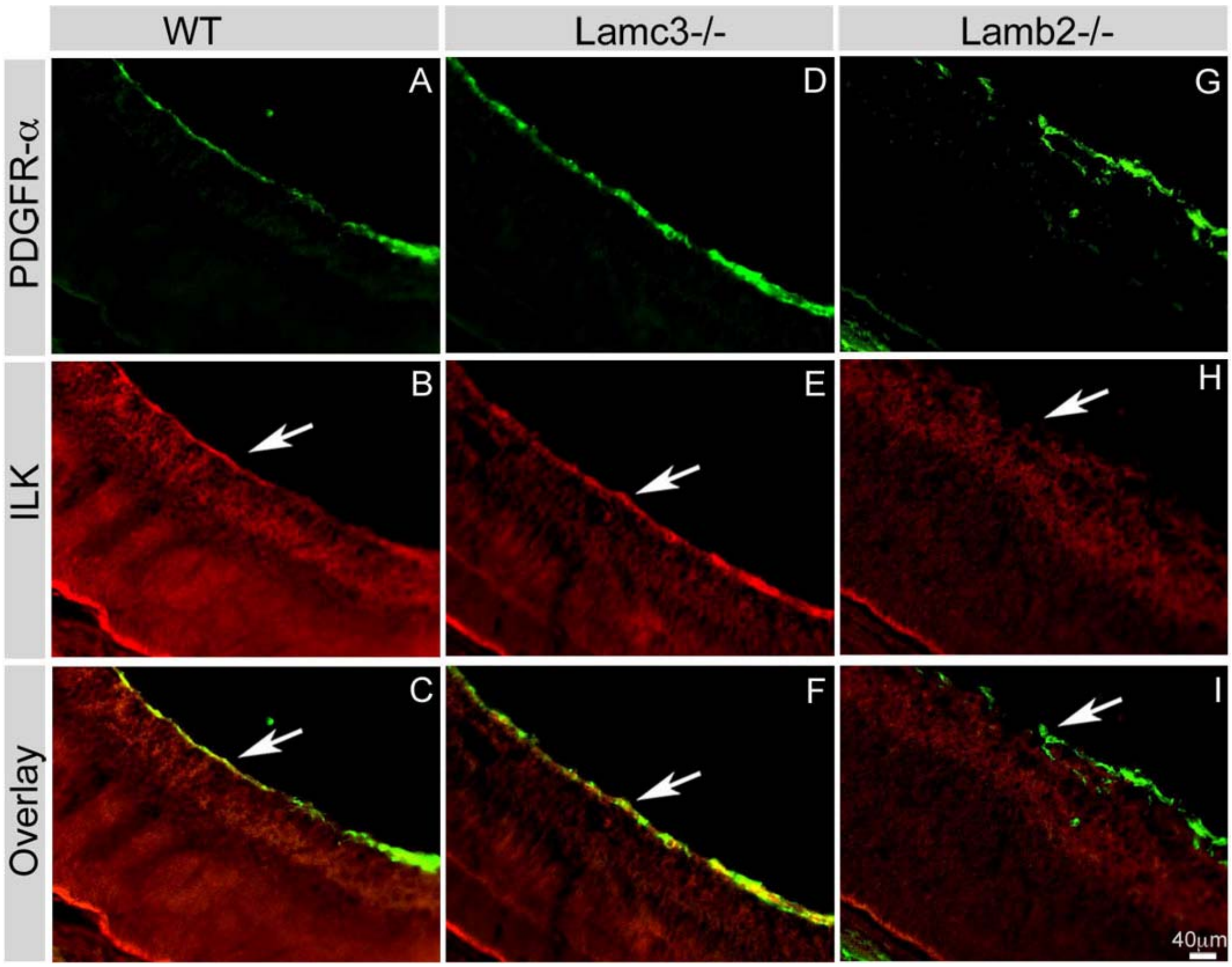

Fig.2. Loss of Lamb2 gene affects ILK expression in astrocytes.

(A-C) WT P5 retinal section was reacted with anti-PDGFR-a and anti-ILK antibodies and imaged using fluorescent microscopy. The arrows in (B) and (C) show the localization of ILK in astrocytes.
(D-F) Lamc3-/- P5 retinal section was reacted with anti-PDGFR-a and anti-ILK antibodies and imaged using fluorescent microscopy. The arrows in (B) and (C) show the localization of ILK in astrocytes.
(G-I) Lamb2-/- P5 retinal section was reacted with anti-PDGFR-a and anti-ILK antibodies and imaged using fluorescent microscopy. The arrows in (B) and (C) shows the loss of ILK localization in astrocytes.

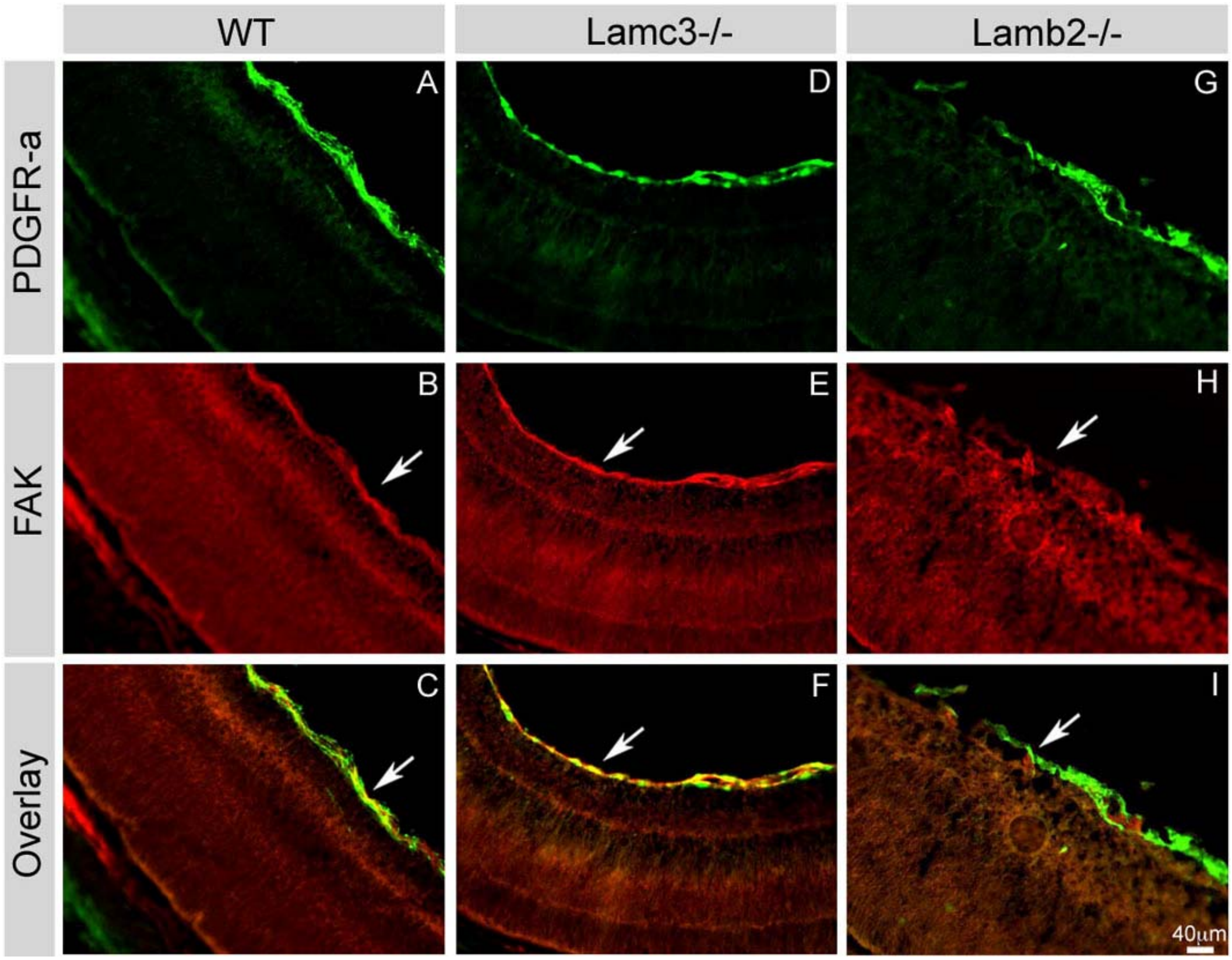

Fig.3. Loss of Lamb2 gene affects FAK expression in astrocytes.

(A-C) WT P5 retinal section was reacted with anti-PDGFR-a and anti-FAK antibodies and imaged using fluorescent microscopy. The arrows in (B) and (C) show the localization of FAK in astrocytes.
(D-F) Lamc3-/- P5 retinal section was reacted with anti-PDGFR-a and anti-FAK antibodies and imaged using fluorescent microscopy. The arrows in (B) and (C) show the localization of FAK in astrocytes.
(G-I) Lamb2-/- P5 retinal section was reacted with anti-PDGFR-a and anti-FAK antibodies and imaged using fluorescent microscopy. The arrows in (B) and (C) shows the loss of FAK localization in astrocytes.

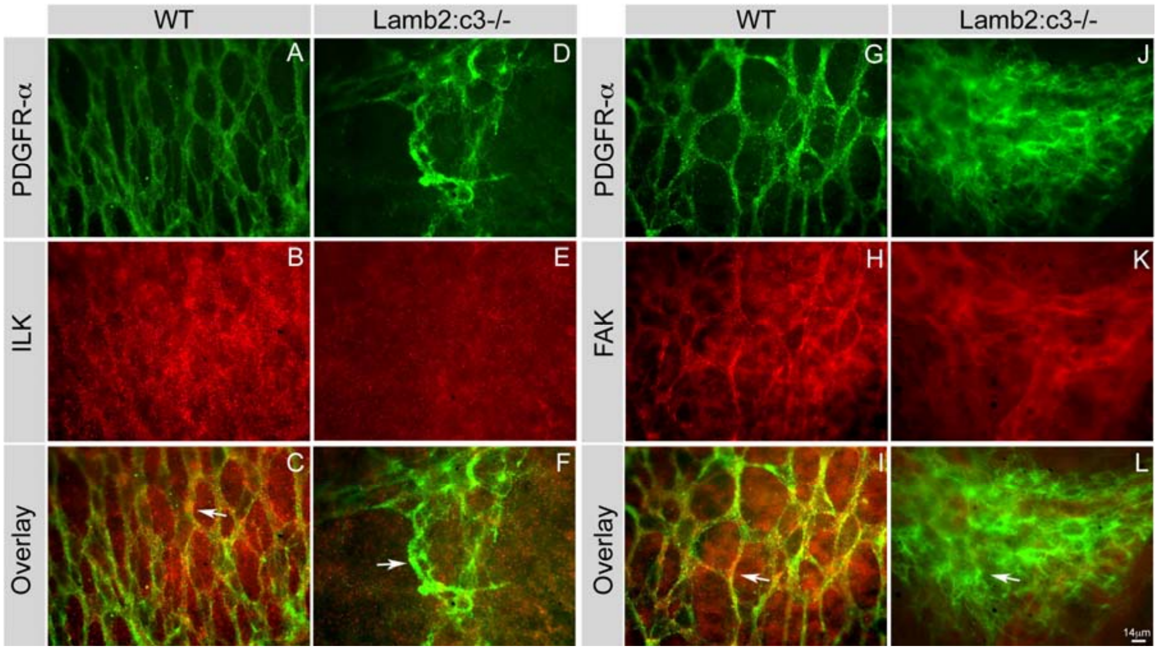

Fig. 4: Deletion of Lamb2:c3 genes affect ILK and FAK expression in astrocytes.

(A-C) WT P3 wholemount retina was analyzed for PDGFR-α (A) (marker for astrocytes) and ILK (B) by immuno reacting for anti-ILK and anti-PDGFR-α. (C) is the overlay of (A) and (B). Arrow in (C) shows ILK expressing astrocytes.
(D-F) *Lamb2:c3-/-* wholemount retina was analyzed for PDGFR-α (D) (marker for astrocytes) and ILK (E) by immuno reacting for anti-ILK and anti-PDGFR-α. (F) is the overlay of (D) and (E). Arrow in (F) shows loss of ILK expression in astrocytes.
(G-I) WT P3 wholemount retina was analyzed for PDGFR-α (H) (marker for astrocytes) and FAK (H) by immuno reacting for anti-FAK and anti-PDGFR-α. (G) is the overlay of (G) and (H). Arrow in (I) shows FAK expressing astrocytes.
(J-L) *Lamb2:c3-/-* wholemount retina was analyzed for PDGFR-α (J) (marker for astrocytes) and ILK (K) by immuno reacting for anti-ILK and anti-PDGFR-α. (L) is the overlay of (J) and (K). Arrow in (L) shows loss of ILK expression in astrocytes.

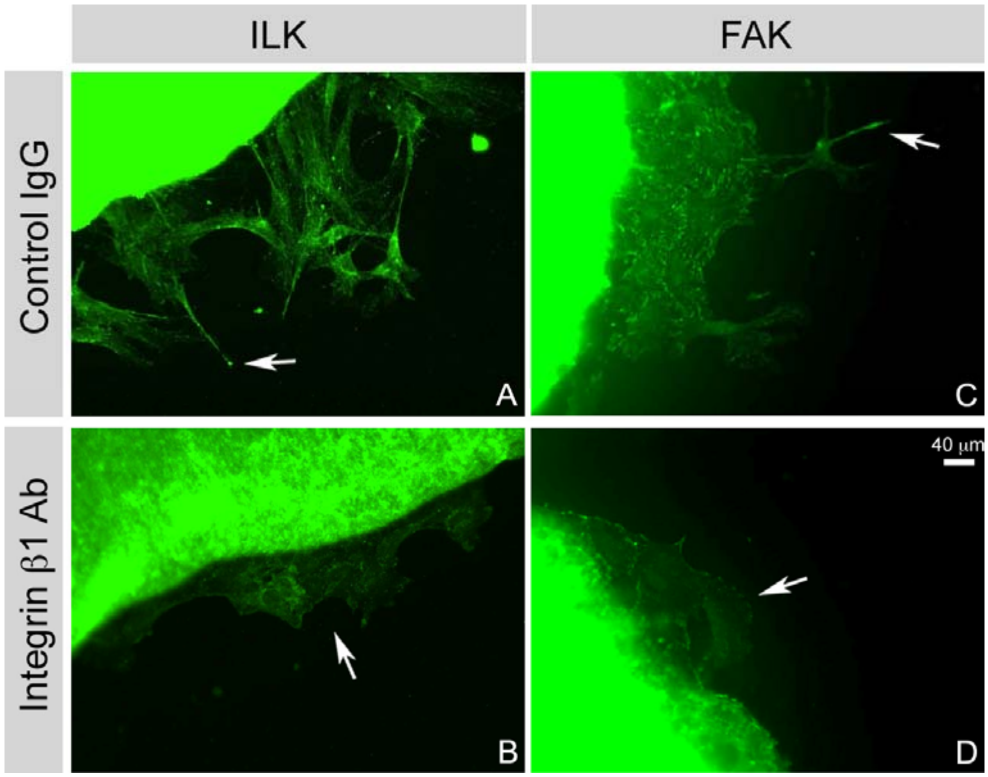

Fig.5. Functional blocking of laminin-integrin β1 receptor interaction affects ILK and FAK localization in astrocytes.

(A) WT P2 ONH culture was treated with control IgG (25μg/ml) containing media for 48hr and analyzed for ILK Expression. Arrow in (A) indicates long filopodial extension with ILK localization.
(B) WT P2 ONH culture was treated with integrin β1 antibody (25μg/ml) containing media for 48hr and analyzed for ILK Expression. Arrow in (B) indicates loss of filopodial extension and ILK localization.
(C) WT P2 ONH culture was treated with control IgG (10μg/ml) containing media for 48hr and analyzed for ILK Expression. Arrow in (A) indicates long filopodial extension with FAK localization.
(D) WT P2 ONH culture was treated with integrin β1 antibody (25μg/ml) containing media for 48hr and analyzed for ILK Expression. Arrow in (B) indicates loss of filopodial extension and focal localization of FAK.

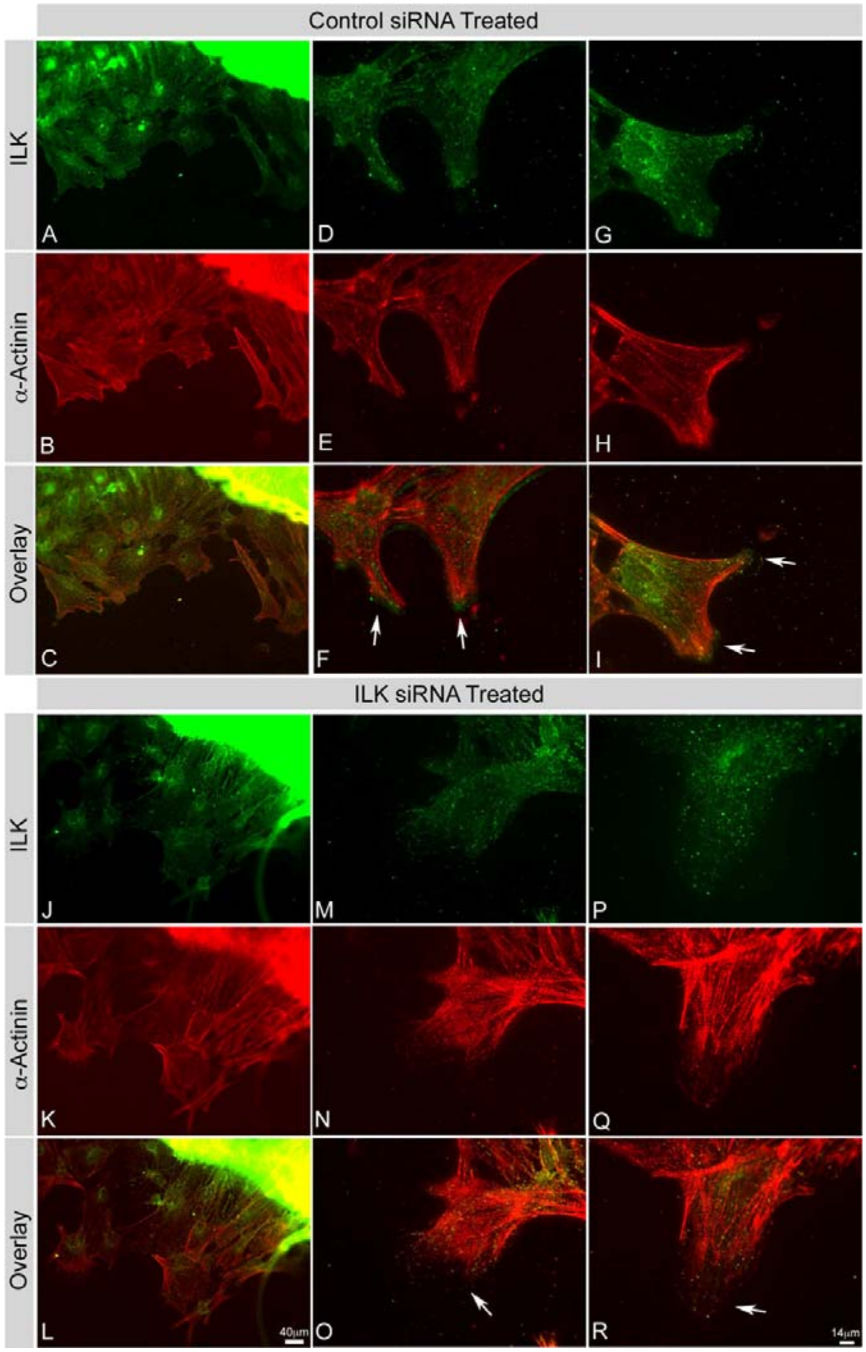

Fig 6. ILK siRNA treatment downregulates ILK levels at the filopodial structures.

(A & B) WT P0 ONH cultures were treated for 48hr with control siRNAs and analyzed for ILK expression and α-actinin organization by performing immunostaining for anti-ILK and anti- α-actinin.

(C) is the overlay of (A) and (B).

(D & I) Higher magnified images of control siRNA treated samples immunostained for anti-ILK and α-actinin to see ILK recruitment at the filopodial structure and α-actinin organization.

(J & K) WT P0 ONH cultures were treated for 48hr with ILK siRNAs and analyzed for ILK expression and α-actinin organization by performing immunostaining for anti-ILK and anti- α-actinin.

(L) is the overlay of (J) and (K).

(M-R)) Higher magnified images of ILK siRNA treated samples to see ILK recruitment at the filopodial structure and α-actinin organization. Arrow in (O) and (R) are showing the loss of ILK at the tip of the filopodial structures.

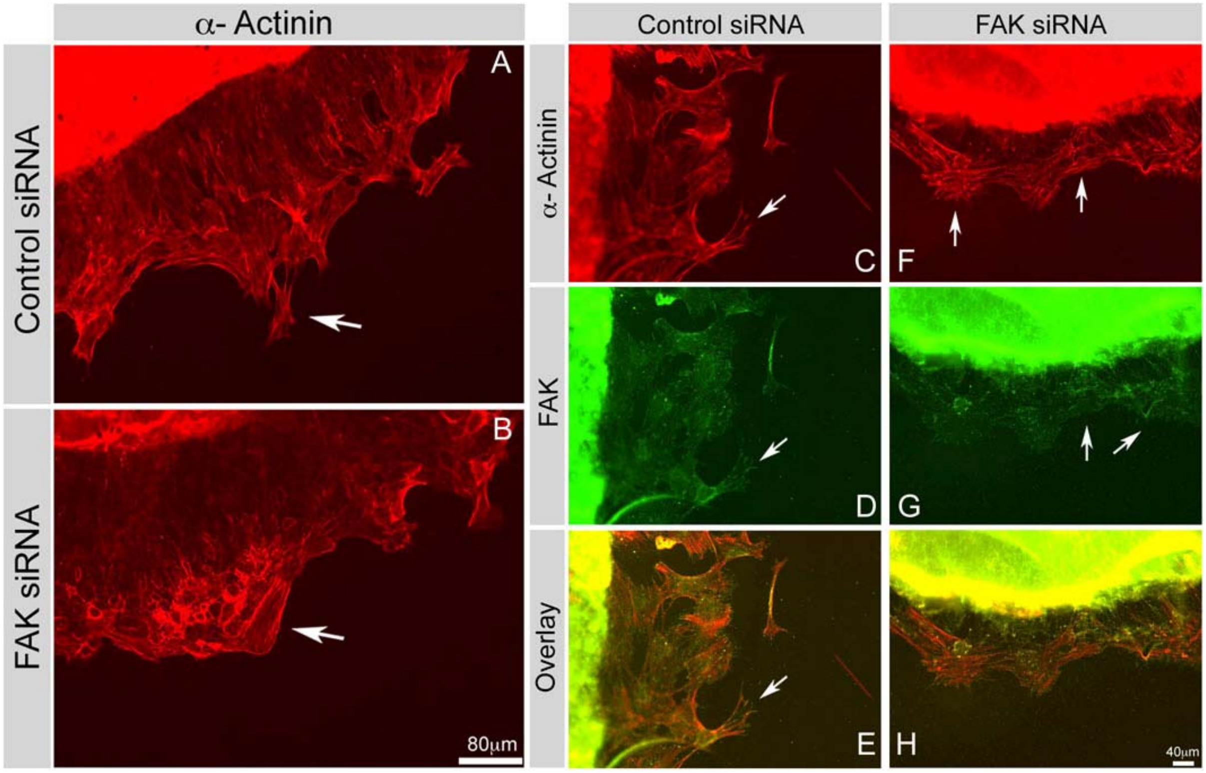

Fig.7. Laminin mediates astrocyte migration through FAK.

(A & B) WT P0 ONH cultures were treated for 48hr with control and FAK siRNAs respectively and analyzed for α-actinin organization. Control siRNA treatment did not affect astrocyte filopodial extension and α-actinin organization, arrow (A). On the other hand, FAK siRNA treatment shows abnormal astrocyte filopodial extension and α-actinin organization, arrow in (B).

(C & D) WT P0 ONH cultures were treated for 48hr with control siRNA and analyzed for α-actinin and FAK.

(D) Control siRNA treatment did not affect FAK expression at the process extension and α-actinin organization (C).

(E) is overlay of (C) and (D).

(F & G) WT P0 ONH cultures were treated for 48hr with FAK siRNA and analyzed for α-actinin and FAK.

(F) FAK siRNA treatment downregulates expression of FAK, which affects both filopodial formation and α-actinin organization (G).

(H) is overlay of (F) and (G).

Discussion

Retinal blood vessel related pathologies are among the major causes of blindness around the world. A thorough understanding of the cell intrinsic and extrinsic regulation of vascular development in the retina is required to address such vasculature related pathologies. Much attention has been given to cell intrinsic regulation of retinal angiogenesis. The goal of this study is to understand how the extrinsic cues derived from ECM molecules (laminin in particular) regulate angiogenesis in the retina. Unlike the other organs, angiogenesis in the retina is orchestrated by specialized glial cells called astrocytes. Astrocytes provide a template for endothelial cells to migrate (Dorrell et al., 2002; Uemura et al., 2006b) Disruption of astrocyte template formation affects subsequent vascular growth (Uemura et al., 2006b). So far the mechanism by which astrocytes invade the retina is unclear. In this study, using both in vivo and in vitro studies, for the first time we have shown the mechanism by which laminins at the ILM regulate angiogenesis in the retina.

The mechanism of astrocyte migration into the retina is poor understood. It has been suggested that astrocyte proliferation and migration in the retina are regulated by PDGF-A (Fruttiger et al., 1996), but the over-expression of PDGF-A causes astrocyte hyperplasia (Fruttiger et al., 1996), suggesting that PDGF-A is likely regulating astrocyte proliferation not migration. ECM molecules are well known to regulate cell migration, we hypothesize that laminin present at the ILM regulate astrocyte migration. Our hypothesis is supported by an in vitro study, where a preferential migration of brain derived astrocytes towards laminin was observed (Armstrong et al., 1990).

In the retina, laminins have been shown to be critical for ILM formation (Pinzon-Duarte et al., 2010; Hirrlinger et al., 2011; Halfter et al., 2005; Edwards et al., 2010). Similarly, mutations in α-dystroglycan (a laminin receptor) and in an enzyme that participates in glycosylation of α-dystroglycan (POMGnT1) also show defective laminin deposition at the ILM, resulting in defective ILM formation (Lee et al., 2005; Takahashi et al., 2011). In addition to ILM disruption, laminin, dystroglycan, and POMGnT1 mutants also display abnormal astrocyte distribution and blood vessel formation (Edwards et al., 2010; Lee et al., 2005; Takahashi et al., 2011). These studies demonstrate that an intact ILM is necessary for normal astrocyte distribution in the retina.

From the previous studies it is unclear whether laminins in the ILM guides astrocytes. Our in-vivo and in-vitro studies clearly suggest that laminins at the ILM is likely acting as a haptotactic factor, regulating astrocyte migration. At P0, PDGFR-a expressing astrocytes that are migrating into the retina interact with laminins containing β2 chain at the ILM. This in-vivo observation suggests that laminins containing the β2 chain provide a substrate for astrocytes to enter into the retina. Moreover, astrocytes seemed to prefer to migrate and stay within the β2 chain rich ILM (Chapter1, Figure 2). This data further confirms that β2 chain rich stripped ILM guides astrocyte migration.

Our analysis of the Lamb2-null further supports our hypothesis, where the deletion of the laminin β2 chain disrupted astrocyte migration and spatial patterning. Interestingly, although the ILM in the Lamc3 null was relatively intact, the deletion of the laminin γ3 chain reduced the rate of migration at early postnatal days. The astrocyte migration and spatial distribution appeared normal at later stages. This suggests that the laminin γ3 chain may regulate astrocyte-ILM interactions during migration. The C-terminus of the laminin γ3 chain has shown to lack a critical Glu residue, which prevents it from binding to integrins (Ido et al., 2008). It is possible that deletion of the laminin γ3 chain increases integrin-BM interaction and causes a delay in migration.

The combined deletion of β2 and γ3 chains had a severe effect on astrocyte migration and patterning, which could be due to the severe loss of the substrate (Chapter1, Figure 2). In addition to their defective distribution, astrocytes in both Lamb2 and Lamb2:c3 nulls clumped together in a net-like pattern. These data suggest that laminins containing β2 chain seem to provide cues for both migration and spatial organization of astrocytes.

The clustering of astrocytes in Lamb2— and Lamb2:c3—nulls could also be due to the loss of integrin β1 receptors. It has been reported that differentiated integrin β1-null neurons exhibit clustering properties in culture similar to that in Lamb2 and Lamb2:c3—nulls (Andressen et al., 1998).

These in vivo and in vitro data clearly suggest that ILM is regulating astrocyte migration and spatial organization. The next question we tried to address was whether astrocyte migration in the retina is directly dependent on laminin, as ILM contains other BM molecules. Our in vitro and ONH explant migration assays clearly demonstrate that astrocytes prefer to migrate on EHS-laminin coated substrates compared to laminin-332 coated or uncoated substrates. The preferential migration of astrocytes on an EHS-laminin substrate (which contains largely laminin-111) shows that astrocyte migration is laminin-isoform dependent. In addition, treatment of Lamb2:c3—null retinal explants with EHS-laminin rescued astrocyte migration and their spatial patterning (Figure 3). These data demonstrate that, besides providing substrate for migration, laminin also helps the astrocytes to attain their spatial positioning. Having said this, we cannot eliminate a possibility that the addition of laminin may have restored the expression or stabilization of other unknown molecules, which has rescued astrocyte migration.

Laminins-Integrin β1 Interaction Regulate Astrocyte Migration Through FAK

Cell migration is a critical event that occurs during development. During this process, cells polarize to form of protrusions or filopodia at the leading edge and form focal adhesions, which results in actin rearrangement leading to cell migration (Ridley et al., 2003). These events are mediated, in part, by the interaction of extracellular matrix molecules including laminins with integrins. One of the known receptors for laminin, integrin β1 has shown to interact with ILK and FAK at the cytoplasmic tail (Hannigan et al., 1996; Hannigan et al., 2005; Wennerberg et al., 2000; Mitra et al 2005). This interaction leads to the activation of Rac/Cdc 42 (Filipenko et al 2005; Suh and Han, 2010), resulting in actin rearrangement and cell migration.

Integrin β1 have shown to regulate astrocyte migration. Deletion of this receptor affects astrocytes polarization, process extension, and causes abnormal migration in culture (Peng et al., 2008). Here, we show that WT astrocytes in the retina express integrin β1 and the deletion of Lamb2 and Lamb2:c3 genes specifically affected integrin β1 expression in astrocytes, suggesting the necessity of laminins for integrin receptor localization. Previous studies also suggest that cells deposit laminins and use laminins as substrates on which to polarize and migrate (Frank and Carter, 2004). Astrocytes isolated from *Lamb2* and *Lamb2:c3*—null retinae demonstrate a defective laminin secretion in culture. Laminin immunostaining was observed mostly intracellular rather than extracellular (Chapter 1 S2). When *Lamb2:c3*—null astrocytes were grown on glass coverslips for 24 hr, most cells failed to extend processes and were unable to localize integrin β1, which could be due to the lack of laminin secretion. On the other hand, when *Lamb2:c3*—null astrocytes were grown on EHS-laminin, they rapidly induced numerous process extensions, with focal localization of integrins β1. These results clearly demonstrate that laminin is critical for focal localization of integrins, which is necessary for cell adhesion and migration. Moreover, integrin β1 receptor blocking assay further supports this hypothesis.

The addition of integrin β1 functional blocking antibodies to ONH culture stalled astrocyte migration and process extension. In addition, it also affected the recruitment of localization of both ILK and FAK. As mentioned earlier, ILK and FAK are immediate downstream signaling molecules, which are known to activate further signaling pathways for cell migration (Filipenko et al 2005; Suh and Han, 2010). This data clearly suggest that laminin is regulating astrocyte migration through integrin β1 receptor mediated signaling pathway.

The knockdown of ILK in the presence of laminin did not significantly affect astrocyte filopodial extension and α-actinin organization (Chapter 3 Fig.6). This suggests a few possibilities: one, that ILK is not important for astrocyte filopodial formation and may be involved in other functions, such as cell maturation; two, the knockdown of ILK is likely upregulating other molecules such as FAK to rescue astrocyte filopodial extension during migration; three, the filopodial extension and α-actinin organization are not affected due to insufficient knockdown of ILK.

Interestingly, knockdown of FAK in the presence of laminin showed affects astrocyte filopodial extension and α-actinin organization. The hallmark of cell migration is the formation of adhesion complexes (Hu et al., 2007), which are stabilized by interaction with the ECM molecules. FAK is known to interact with some of the adhesion complex molecules such as paxillin and talin (Scheswohl et al., 2008; Chen et al., 1995). The knockdown of FAK likely affected the adhesion complex formation, which affects filopodial extension during astrocyte migration. The analysis of α-actinin supports this hypothesis, because cells during migration orient α-actinin towards the direction of migration (Hu et al., 2007). Knocking down of FAK has affected α-actinin orientation in astrocytes. Although the FAK knockdown experiments is suggesting that FAK is likely regulating astrocyte filopodial extension, further experiments are required to clearly define whether FAK is one of the key molecules for astrocyte filopodial formation. Together, these data suggests that laminin is likely regulating astrocyte migration through integrin β1-FAK mediated signaling mechanism. It will be interesting to further investigate to see whether functional blocking of integrin b1 receptor and knock down of FAK affects the recruitment of adhesion complex molecules such as paxillin and tailn during astrocyte migration.

Laminins Are Necessary For Retinal Vascular Development

Astrocyte derived template have shown to guide endothelial tip cells in the retina (Gerhardt et al., 2003; Dorrell et al., 2002). Previous studies have suggested that astrocyte-derived vascular endothelial growth factor (VEGF) and fibronectin regulate endothelial growth (Gerhardt et al., 2003; Uemura et al., 2006). On the contrary, astrocyte-specific deletion of VEGF or fibronectin did not drastically affect endothelial growth (Scott et al., 2010; Stenzel et al., 2011). These results suggest that there may be other ECM molecules involved in regulating endothelial migration on astrocyte-derived matrix. It is highly likely that astrocyte derived laminin-rich matrix may be guiding endothelial cells. A recent study supports this statement, that laminin-integrin $\alpha6\beta1$ interaction have shown to regulate endothelial tip cell selection (Estrach et al., 2011), which leads the growing vasculature.

Our study shows that the deletion of laminin chains has differential effect on endothelial migration over an astrocyte template. Analysis of *Lamc3* null retinas showed slower vascular growth progression at early postnatal days. The effect could be due to the delay in astrocyte patterning. Interestingly, in these *Lamc3* nulls, an increase in vascular branching at the leading edges was observed. The analysis of the expression of laminin $\gamma3$ chains shows a unique deposition at vascular branch points. It is likely that the unique laminin γ3 depositions negatively regulate angiogenesis by preventing excessive branching during retinal vascular development.

In both *Lamb2*— and *Lamb2:c3*—nulls, vascular growth was severely affected in both early and late postnatal days. This effect could be due to a lack of proper astrocyte template formation. Astrocytes isolated from *Lamb2* and *Lamb2:c3*—null retinae fail to secrete laminin (Chapter 1 S3). The lack of astrocyte derived laminin in *Lamb2* and *Lamb2:c3*—null retinae possibly affected endothelial migration over the astrocyte template. Similarly, defect in vascular growth was observed in *Lama1* mutant retinas as well (Edwards et al 2010). It is possible that astrocyte derived α1 and β2 containing laminin trimers are regulating this endothelial migration.

In addition to defective vascular growth, we observed persistent hyaloid vessels (HV) in both *Lamb2* and *Lamb2:c3*—null retinae. The regression of HV has shown to be mediated by macrophage lineage cells (Lee et al., 2009). Macrophages have shown to interact with laminin through integrin α6β1 upon activation (Milner and Campbell, 2002). We detected a reduction in myeloid lineage cells in *Lamb2:c3*—null HV (data not shown), suggesting that the loss of laminin likely affected myeloid cell recruitment during HV regression. The other possibility for the reduced vascular regression could be the presence of astrocytes over the HV, inhibiting HV regression.

In summary, our analyses clearly show that laminins containing the β2 and γ3 chains play important and distinctive roles during retinal angiogenesis. In this regard, a multigenerational mennonite family carrying a mutation of LAMB2 was reported to have chorioretinal pigmentary changes (Mohney et al., 2011). Although the authors did not comment on it, the presented data also suggest the possiblility of abnormal retinal vascular development. It requires further investigation to understand how the requirement of the LAMB2 gene regulates normal retinal vascular growth.

Laminins Maintain Vascular Stability

Studies have shown that astrocytes play a crucial role in inducing properties of the blood-neural barrier in endothelial cells (Wolburg et al., 1994; Gardner et al., 1997). The astrocytes regulate barrier properties by closely interacting with the endothelial cells through vascular BM (Abbott et al., 2006). During ischemia, loss of laminin-binding integrin and dystroglycan receptors at the astrocyte-vascular interface occurs (Wagner et al., 1997; Tagaya et al., 2001; Milner et al., 2008). This suggests that laminins mediate astrocyte and endothelial interactions, thereby maintaining vascular stability.

In the *Lamb2* and *Lamb2:c3*—null retinas we observed abnormal astrocyte interaction with the vascular BM. In these nulls astrocytes abnormally clumped over the vascular wall instead of properly wrapping the vascular BM. This abnormal interaction between astrocytes and endothelial cells may have resulted in vascular leakage demonstrated by fluorescent angiography. It will be interesting to further analyze whether the expression of laminin receptors such as integrins containing $\alpha6$, $\alpha1$, and $\beta4$ subunits, and dystroglycan receptors are affected at the astrocyte-vascular BM interface in these laminin nulls.

In conclusion, our study clearly shows that laminins are indispensable for astrocyte migration and subsequent blood vessel formation in the retina. Deletion of the laminin $\beta2$ and $\gamma3$ chains disrupts astrocyte migration, spatial patterning, and subsequent blood vessel formation in the retina.

As mentioned earlier, the ultimate goal is to restore astrocyte patterning in ROP and DPR, which will prevent neovascularization events, because animal models for ROP and DPR show degeneration of astrocytes. It will be interesting to analyze if the laminin chains are affected in experimental models for ROP and DPR.

Studies have shown that alterations of BM occur during ischemic and hyperglycemic conditions (Roy et al., 1994; Roy et al., 2011; Tagaya et al., 2001). In addition, elevated glucose levels have shown to decrease laminin $\beta2$ chain synthesis in both experimental animal models and in-vitro studies in the kidney (Abrass et al., 1997; Schaeffer et al., 2010). If the BM undergoes remodeling it will affect astrocyte interaction with the ILM as well as the blood vessels. Therefore, by using experimental mice models for ROP and DPR and analyzing for the changes in BM composition would reveal novel therapeutic targets. This can be achieved by analyzing experimental ROP and DPR mice retinas and performing immunostaining for various ILM components together with proteomic analysis of stripped ILM.

Next, it will be interesting to test whether remodeling of ILM affects astrocyte interaction with the ILM as well as with the blood vessels by analyzing for cell surface receptors such as integrins, dystroglycan. Developing an *in vitro* culture conditions that replicates ROP and DPR would further help to understand the molecular changes that occurs under such conditions. This can be achieved by culturing astrocytes in cyclic oxic and elevated glucose levels in the presence or absence of laminin and analyze for changes in the protein as well as messenger RNA levels of integrin, dystroglycan and its downstream interacting partner.

Finally, treat ROP and DPR animal models with exogenous laminin by intravitreal injection and analyze for astrocyte and blood vessel patterning. These experiments will better help us to better understand the pathogenesis as well help us to find a novel therapeutic target to cure retinal vasculature related pathologies.

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