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The astrocyte-expressed integrin \(\alpha\beta8\) governs blood vessel sprouting in the developing retina

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SUMMARY

The mouse retina is vascularized after birth when angiogenic blood vessels grow and sprout along a pre-formed latticework of astrocytes. How astrocyte-derived cues control patterns of blood vessel growth and sprouting, however, remains enigmatic. Here, we have used molecular genetic strategies in mice to demonstrate that \(\alpha\beta8\) integrin expressed in astrocytes is essential for neovascularization of the developing retina. Selective ablation of \(\alpha\) or \(\beta8\) integrin gene expression in astrocytes leads to impaired blood vessel sprouting and intraocular hemorrhage, particularly during formation of the secondary vascular plexus. These pathologies correlate, in part, with diminished \(\alpha\beta8\) integrin-mediated activation of extracellular matrix-bound latent transforming growth factor \(\beta\)s (TGF\(\beta\))s and defective TGF\(\beta\) signaling in vascular endothelial cells, but not astrocytes. Collectively, our data demonstrate that \(\alpha\beta8\) integrin is a component of a paracrine signaling axis that links astrocytes to blood vessels and is essential for proper regulation of retinal angiogenesis.

KEY WORDS: Extracellular matrix, Itgb8, Cell adhesion, Blood-retinal barrier, Angiogenesis, Mouse

INTRODUCTION

Neovascularization of the mouse retina initiates in the early neonatal period when endothelial cells and pericytes proliferate and migrate along astrocytes to form a primary vascular plexus. Blood vessels subsequently sprout into deeper retinal layers to form a secondary vascular network (Dorrell and Friedlander, 2006; Fruttiger, 2007). Various growth factors and cell-cell adhesion molecules such as platelet-derived growth factor (PDGF)-BB (Benjamin et al., 1998), vascular endothelial growth factor (VEGF)-A (Ruhrberg et al., 2002; Gerhardt et al., 2003), Wnts (Phng et al., 2009) and Notch1-Dll4 (Hellstrom et al., 2007; Benedito et al., 2009) have been reported to play endothelial cell-intrinsic roles in retinal angiogenesis. Roles for astrocyte-derived factors in the regulation of retinal angiogenesis, and particularly those involving extracellular matrix (ECM) proteins and their cell adhesion receptors, remain largely uncharacterized.

Integrins are receptors for many ECM protein ligands (Silva et al., 2008), and integrin-mediated adhesion and signal transduction events are essential for blood vessel development and physiology in all mammalian organs, including the retina (Ramjaun and Hodivala-Dilke, 2009). For example, the laminin receptor \(\alpha\beta1\) integrin is expressed in vascular endothelial cells and acts to suppress pathological angiogenesis in the retina and other organs (Watson et al., 2010). Similarly, \(\alpha\beta3\) and \(\alpha\beta5\) integrins expressed in vascular endothelial cells negatively regulate hypoxia-induced retinal angiogenesis (Hodivala-Dilke et al., 1999; Reynolds et al., 2002). Fibronectin, which is expressed primarily by astrocytes in the retina, has been reported to signal via \(\alpha\beta1\) integrin to promote development of the primary vascular plexus (Stenzel et al., 2011). Lastly, TGF\(\beta\)s, which in their ECM-bound latent forms are protein ligands for some \(\alpha\)-containing integrins (Worthington et al., 2010), have also been reported to regulate retinal blood vessel physiology and homeostasis of the blood-retinal barrier (Walsh et al., 2009).

During embryonic development, the neuroepithelial cell-expressed integrin \(\alpha\beta8\) is essential for cerebral angiogenesis and blood-brain barrier development. Genetic ablation of \(\alpha\) or \(\beta8\) integrin expression in the embryonic neuroepithelium, but not the vascular endothelium, leads to abnormal cerebral blood vessel patterning and severe intracerebral hemorrhage (McCarty et al., 2002; Zhu et al., 2002; McCarty et al., 2005; Proctor et al., 2005). \(\alpha\beta8\) integrin binds to RGD peptide sequences within latent TGF\(\beta1\) and TGF\(\beta3\), mediating TGF\(\beta\) release from the ECM and subsequent receptor engagement and signaling (Cambier et al., 2005; Mu et al., 2008). Indeed, endothelial cell-specific ablation of TGF\(\beta\) receptors results in brain vascular pathologies during embryogenesis that are strikingly similar to those that develop in \(\alpha\beta8\) integrin and TGF\(\beta\) mutant embryos (Nguyen et al., 2011).

Here, functions for \(\alpha\beta8\) integrin during physiological angiogenesis in the retina have been investigated. We show that genetic ablation of \(\alpha\beta8\) integrin in astrocytes, and particularly during formation of the secondary vascular plexus, leads to defective retinal blood vessel sprouting and severe intraretinal hemorrhage. These data highlight that \(\alpha\beta8\) integrin is an essential paracrine regulator of angiogenesis in the developing retina.

MATERIALS AND METHODS

Experimental mice

\(\beta8^+/−\) and \(\beta8^{flox/flox}\) mice were obtained from the Mutant Mouse Regional Resource Center. Genotypes were determined using PCR-based methodologies as previously described (Zhu et al., 2002; Proctor et al., 2005). Nestin-Cre transgenic mice (Tronche et al., 1999) were purchased.

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from Jackson Laboratories. To generate control and β8 integrin conditional knockout mice, females harboring a floxed β8 integrin gene (β8floxB/flox) were bred with hemizygous Nestin-Cre (N-Cre+/+) transgenic males. Genotypes of F1 progeny were determined by PCR-based amplification of genomic DNA isolated from tail snips (Proctor et al., 2005). N-Cre−/−;β8floxB/flox males were then crossbred with β8 floxB/flox females. Controls (N-Cre+/−;β8floxB/flox) were heterozygous null for β8 integrin gene expression in cells that express Cre, whereas mutant littermates (N-Cre−/−;β8floxB/flox) were homozygous null for β8 integrin gene expression in Cre-expressing cells. Generation of N-Cre;αv/β3/flox/flox mice has been described previously (McCarty et al., 2005). Mouse harboring the Tgbr2floxB/flox gene were kindly provided by Dr Jonathan Currie (University of Texas M. D. Anderson Cancer Center, TX, USA) and have been described elsewhere (Chytíl et al., 2002). To generate Tgbr2 conditional knockout mice, N-Cre+/− males were bred with Tgbr2floxB/flox females. N-Cre−/−;Tgbr2floxB/flox were then bred with Tgbr2floxB/flox females to generate control and mutant littermates. Mice were genotyped using the following primers: 5′-TAAACAAGGGCAGGAGCCA-3′ and 5′-ACTTCTGCAAGGCTCCCT-3′. Genotyping of GFAP-CreERT2 transgenic mice has been described elsewhere (Hirrlinger et al., 2006).

GFAP-CreERT2+/+;β8−/− males were bred with β8+/f females to generate control and mutant F1 progeny. Alternatively, GFAP-CreERT2−/+ males were bred with Rosa26-lox-STOP-lox-YFP females. P1 neonates were injected intragastrically with 1 mg/ml DNAase (Sigma) and were injected intraperitoneally with 50 µl tamoxifen (Sigma) prepared in sterile mouse 5% deoxycholate, 0.1% SDS, 150 mM NaCl and 1 mM EDTA. Protein concentrations in detergent-soluble fractions were determined using a BCA media was pre-treated with anti-TGFβ antibodies (R&D Systems) or control isotype control antibody (Sigma). P9 neonatal mice were deeply anaesthetized by intraperitoneal injection with tribromoethanol (Sigma), and then injected intragastrically using a 10 µl Hamilton syringe. Pups were injected with 2 µl PBS, 2 µl anti-TGFβ blocking antibody (1 mg/ml, R&D Systems) or 2 µl isotype control antibody (Sigma, n=5 mice injected per condition). Six hours later, mice were sacrificed and eyes were fixed for 12 hours in 4% paraformaldehyde (PFA) in PBS. Whole retinas were isolated and then analyzed by immunofluorescence staining using anti–CD31 and anti–GFAP monoclonal antibodies or anti–GFAP rabbit polyclonal antibody, which cross-reacts with YFP (Abcam).

Isolation of primary retinal astrocytes

Astrocytes were cultured from P5 neonatal retinas and grown on laminin-coated dishes. Briefly, eyes were removed and placed in ice-cold Hank’s Balanced Salt Solution (HBSS). Retinal cups were dissected and digested for 30 minutes at 37°C in DMEM containing 100 U/ml collagenase (Worthington) and 40 µg/ml DNAase (Sigma). The tissue was then triturated and the single-cell suspension was plated onto T-25 tissue culture flasks pre-coated with mouse 3 µg/ml laminin (Sigma). After 7 days, confluent cultures were placed on a rotary shaker overnight (250 rpm) to remove contaminating cells. Astrocyte genotypes were confirmed by genomic PCR using primers specific for the β8 integrin gene (Jung et al., 2006). Retinal astrocytes were serum-starved for 18 hours and conditioned media was pre-treated with anti-TGFβ antibodies (R&D Systems) or control IgGs (both at 5 µg/ml) for one hour. Conditioned media was then added to semi-confluent monolayers of bovine brain microvascular endothelial cells (Lonza). Cells were lysed in RIPA buffer and then immunoblotted with anti-β-actin (Sigma) or anti-β-Smad3 rabbit antibodies (Epitomics).

Immunoblotting and immunofluorescence

Cells and tissues were 1% SDS RIPA buffer (10 mM Tris, pH 7.4, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl and 1 mM EDTA). Protein concentrations in detergent-soluble fractions were determined using a BCA assay kit (Thermo Scientific) prior to incubation with primary and secondary antibodies. Secondary antibodies used for fluorescence were Alexa-conjugated goat anti-rabbit and goat anti-mouse (Molecular Probes). The following commercially antibodies were used for immunofluorescence: anti–GFAP rabbit polyclonal (DAKO); anti–GFAP mouse monoclonal (Millipore); anti–CD31 rat monoclonal (PharMingen); anti–NG2 (Millipore); anti–pH3 (Cell Signaling), anti–GFAP (Abcam) and anti–laminin (Sigma). The anti–αv and anti–β8 integrin rabbit polyclonal antibodies have been described elsewhere (Tchaicha et al., 2010; Tchaicha et al., 2011).

Whole-mount retinal analysis

Neonatal mice were sacrificed and eyes were removed and fixed in 4% PFA in PBS at 4°C for 12 hours. Whole retinal cups were then microdissected, blocked in 1% bovine serum albumin (BSA) with 0.1% Triton X-100 in PBS and incubated in primary antibodies for 16 hours at 4°C. Tissues were then washed five times in PBS at room temperature and then incubated overnight in secondary antibodies. Washed retinal cups were then flat-mounted on microscope slides.

Quantitation of retinal defects

To quantify numbers of filopodia per tip cell, randomly selected micrograph images (n=3 images per retina, n=3 mice per genotype) were analyzed at 400× magnification. Individual tip cells (≥25 cells per genotype) were identified at the leading edge of the P5 vascular front and CD31-expressing filopodia were counted. Alternatively, to quantify filopodia sprouts per vessel length NIH ImageJ software was used to count projects per 100 µm at the leading edge of the migrating vessel front (n=3 images per retina, n=3 mice per genotype). Student’s t-test was performed to determine statistically significant differences between groups.

Intraocular injections of anti-TGFβ antibodies

P9 neonatal mice were deeply anaesthetized by intraperitoneal injection with tribromoethanol (Sigma), and then injected intraocularly using a 10 µl Hamilton syringe. Pups were injected with 2 µl PBS, 2 µl anti-TGFβ blocking antibody (1 mg/ml, R&D Systems) or 2 µl isotype control antibody (Sigma, n=5 mice injected per condition). Six hours later, mice were sacrificed and eyes were fixed for 12 hours in 4% PFA in PBS. Whole retinal cups were then microdissected and incubated in IsoB4-Alexa488 (Sigma) for 16 hours at 4°C. Retinas were washed five times in PBS at room temperature and flat-mounted on microscope slides for analysis.

RESULTS

αvβ8 integrin is expressed in the neonatal mouse retina and in cultured retinal astrocytes

Angiogenic blood vessels invade the retina along a pre-formed network of glial fibrillary associated protein (GFAP)-expressing astrocytes (Kubota and Suda, 2009). We interrogated αvβ8 integrin expression in cultured retinal astrocytes from wild-type and β8−/− mice. Primary cells were cultured from P5 wild-type and β8−/− littermates (n=3 mice per genotype) and grown on laminin-coated dishes. Nearly 100% of cells from both control and mutant littermates expressed GFAP and showed similar in vitro morphologies (Fig. 1A,B). However, although β8−/− cells showed impaired proliferation and survival in vitro (data not shown), Detergent-soluble lysates prepared from control and β8−/− astrocytes were immunoblotted with anti-αv and anti-β8 integrin antibodies. Robust levels of αv and β8 integrin proteins were detected in lysates from wild-type retinal astrocytes, whereas β8 integrin protein was absent in β8−/− lysates owing to gene ablation (Fig. 1C). Continued expression of αv integrin protein was detected in β8−/− lysates owing to expression of other αv-associated integrin subunits, such as β1 and β5 integrins (data not shown). Retinal lysates were also prepared from P3, P7 and P14 wild-type mice and immunoblotted with anti-integrin antibodies to reveal robust αv and β8 integrin protein expression at all developmental ages analyzed (Fig. 1D).

Angiogenesis defects and intraretinal hemorrhage upon formation of the secondary vascular plexus in β8−/− mice

Retinas from P5 wild-type and β8−/− littermates (n=7 mice per genotype) were isolated and labeled with anti–CD31 and anti–NG2 antibodies to visualize vascular endothelial cells and pericytes, respectively. Endothelial tip cells within the wild-type primary vascular plexus extended elaborate filopodial projections (Fig. 2A), whereas in β8−/− retinas endothelial tip cells displayed less elaborate and blunted morphologies (Fig. 2B). In both wild-type and β8−/− retinas, endothelial cells were closely associated with...
NG2-expressing pericytes, and no apparent differences in pericyte coverage were detected. Wild-type and β8−/− retinas from P5 littermates (n=5 mice per genotype) were also labeled with anti-CD31 and anti-GFAP antibodies to visualize vascular endothelial cells and astrocytes, respectively (Fig. 2C,D). Endothelial tip cell defects were detected in β8−/− retinas; however, no differences in the network of GFAP-expressing astrocytes were detected. We further analyzed tip cell defects in P5 wild-type and β8−/− retinas (n=3 retinas per genotype) by quantifying numbers of CD31-expressing filopodia per tip cell (n≥25 tip cells from three mice per genotype). As shown in Fig. 2E, a reduction of nearly 50% in numbers of filopodia per tip cell was detected in β8−/− retinas. We also detected reduced numbers of endothelial filopodia per defined vessel length at the leading edge of the migrating blood vessel network in β8−/− samples (Fig. 2F).

Beginning at ~P8, blood vessels in the primary vascular plexus migrate along Müller glial cells into deeper retinal layers to form a secondary vascular network (Fruttiger, 2007). Analysis of retinas from P12-14 wild-type or β8−/− mice (n≥10 per genotype) revealed grossly obvious intraretinal hemorrhage in 100% of mutants that was not present in any wild-type retinas analyzed (Fig. 3A). Anti-CD31 and anti-NG2 double immunofluorescence staining of wild-type P14 retinas (n=5 per genotype) revealed normal endothelial cell and pericyte interactions in vessels sprouting from the primary plexus into the underlying neuronal layers (Fig. 3B). Consistent with a marked defect in formation of a secondary vascular network, many capillaries within the primary vascular plexus of β8−/− mice failed to sprout into the deeper retinal layers and instead displayed glomeruloid-like tufts comprising both endothelial cells and pericytes (Fig. 3C). Double immunofluorescence staining with anti-CD31 and anti-GFAP was also performed to visualize interactions between endothelial cells and astrocytes. An elaborate astrocyte latticework was present in both wild-type and β8−/− samples (Fig. 3D,E), although increased GFAP expression was detected in mutant retinas probably owing to reactive gliosis in response to hemorrhage. Next, coronal sections from P12 wild-type and β8−/− retinas were stained with anti-CD31 and anti-NG2 antibodies to visualize endothelial cells and pericytes, respectively (Fig. 3F).
Retinal blood vessel sprouting defects in mice lacking αvβ8 integrin in astrocytes

αvβ8 integrin is reported to heterodimerize exclusively with the αv subunit (Nishimura et al., 1998); therefore, we analyzed functions for αv integrin in retinal astrocytes by selectively deleting a conditional αv integrin gene (αvflox/flox) using Cre/lox strategies. Mice harboring a homozygous αvflox/flox gene (McCarty et al., 2005) were crossed to a Nestin-Cre transgenic strain that expresses Cre recombinase in neuronal progenitors and their astroglial and neuronal progeny in the brain and retina (Tronche et al., 1999; Graus-Porta et al., 2001; Haigh et al., 2003). Nestin-Cre/+;αvflox/+ male progeny were then mated with αvflox/flox females to generate mutant progeny that were hemizygous for the Nestin-Cre transgene and hemizygous for the αv integrin conditional gene (Nestin-Cre/+;αvflox/flox). Littermate controls were hemizygous for the Nestin-Cre transgene and carried one αv integrin flox allele and one wild-type allele (Nestin-Cre/+;αvflox/+).

As shown in Fig. 4A, PCR analysis of genomic DNA isolated from retinas of P14 Nestin-Cre/+;αvflox/+ mice confirmed Cre-mediated recombination of the αvflox allele. Immunoblotting of detergent-soluble retinal lysates prepared from Nestin-Cre/+;αvflox/+ control and Nestin-Cre/+;αvflox/flox mutant littermates also revealed an obvious reduction in αv integrin protein levels in mutant retinas (Fig. 4B). Blood vessels in P5 control mice contained NG2-expressing pericytes and endothelial tip cells that extended multiple filopodia at the leading edge of the sprouting vascular network (supplementary material Fig. S2A). P5 mutant blood vessels contained NG2-expressing pericytes and endothelial tip cells that extended multiple filopodia at the leading edge of the sprouting vascular network (supplementary material Fig. S2A). P5 mutant blood vessels contained NG2-expressing pericytes and endothelial tip cells that extended multiple filopodia at the leading edge of the sprouting vascular network (supplementary material Fig. S2A). P5 mutant blood vessels contained NG2-expressing pericytes and endothelial tip cells that extended multiple filopodia at the leading edge of the sprouting vascular network (supplementary material Fig. S2A). P5 mutant blood vessels contained NG2-expressing pericytes and endothelial tip cells that extended multiple filopodia at the leading edge of the sprouting vascular network (supplementary material Fig. S2A). P5 mutant blood vessels contained NG2-expressing pericytes and endothelial tip cells that extended multiple filopodia at the leading edge of the sprouting vascular network (supplementary material Fig. S2A).
apparent in mutant astrocytes, probably owing to reactive gliosis (Fig. 4F,G). These data, coupled with a previous report showing that deletion of αv integrin in vascular endothelial cells does not result in retinal angiogenesis defects (Lacy-Hulbert et al., 2007), further support essential functions for astrocyte-expressed αv integrins in retinal angiogenesis.

Next, mice harboring a homozygous β8flox/flox gene (Proctor et al., 2005) were crossed to a Nestin-Cre transgenic strain (Tronche et al., 1999). Nestin-Cre/+;β8flox/+ male progeny were then mated with β8flox/flox females to generate Nestin-Cre/+;β8flox/+ and Nestin-Cre/+;β8flox/flox mutant progeny. PCR-based analysis using genomic DNA isolated from retinas of P14 Nestin-Cre/+;β8flox/+ control and Nestin-Cre;β8flox/flox mutant mice revealed Cre-mediated ablation of the β8flox allele (Fig. 5A). Retinal lysates from control and mutant littermates were immunoblotted with anti-β8 antibodies to reveal a reduction in β8 integrin protein in mutant retinas (Fig. 5B). Blood vessels in P5 control retinas contained pericytes with endothelial tip cells extending multiple filopodia at the leading edge of the primary vascular plexus (supplementary material Fig. S3A). Differences in vessel-associated pericytes and GFAP-expressing astrocytes were not apparent in β8 conditional knockouts (supplementary material Fig. S3B,D). However, endothelial tip cells in P5 integrin mutants displayed blunted morphologies with reduced numbers of CD31-expressing filopodia (supplementary material Fig. S3E).

Analysis of the retinal vasculature at P14 revealed grossly obvious intraretinal hemorrhage in β8 integrin conditional mutants, with many blood vessels in the primary plexus failing to sprout into the underlying retinal tissue (Fig. 5C; data not shown). Unlike controls (Fig. 5D), Nestin-Cre/+;β8flox/flox retinas contained vessels with abnormal branching patterns and glomeruloid-like tufts comprising CD31-expressing cells and NG2-expressing pericytes (Fig. 5E). Analysis of the retinal astrocyte network at P14 revealed reactive gliosis in mutants, probably owing to intraretinal hemorrhage (Fig. 5F,G). Collectively, these conditional knockout data further support essential roles for astrocyte-expressed αvβ8 integrin in the control of neonatal retinal angiogenesis.

Inducible deletion of β8 integrin in astrocytes leads to retinal angiogenesis pathologies

The Nestin-Cre transgene is expressed in embryonic neural progenitor cells that give rise to retinal astrocytes and neurons (Haigh et al., 2003) raising the possibility that loss of αvβ8 integrin functions in astrocytes as well as neurons might contribute to...
retinal angiogenesis defects in integrin mutant mice. To study more selectively functions for αvβ8 integrin in astrocytes, we employed an inducible gene knockout strategy using a tamoxifen-responsive GFAP-CreERT2 transgene (Hirrlinger et al., 2006). First, to confirm tamoxifen-inducible activation of Cre we analyzed a Rosa26-lox-STOP-lox-YFP reporter strain. As shown in supplementary material Fig. S4A,B, mosaic patterns of Cre activity were detected in GFAP-expressing retinal astrocytes, but not in CD31-expressing endothelial cells.

GFAP-CreERT2/+;β8flox/+ male progeny were mated with β8flox/flox females to generate GFAP-CreERT2/+;β8flox/+ control and GFAP-CreERT2/+;β8flox/flox conditional mutants. To induce Cre activation, P1 pups were injected intragastrically with tamoxifen once per day for three consecutive days as previously described (Pitulescu et al., 2010). Ten days later, mice were sacrificed and retinas were analyzed for vascular pathologies and by immunofluorescent labeling with anti-CD31 antibodies. Analysis of P14 control and mutant animals (n=4 mice per genotype) revealed a lack of grossly obvious intraretinal hemorrhage. However, abnormal blood vessel morphologies within the primary vascular retinal plexus of GFAP-CreERT2/+;β8flox/flox inducible mutants were detected. In comparison with controls (supplementary material Fig. S4C), many blood vessels in mutants formed tufted structures and failed to sprout into the underlying retinal cell layers (supplementary material Fig. S4D). In addition, blood vessels with tortuous morphologies were detected within cerebral cortices of mutant animals (supplementary material Fig. S5). The less severe vascular pathologies in the retinas of tamoxifen-inducible GFAP-CreERT2 mutants (as opposed to Nestin-Cre conditional mutants) might be due to more robust gene deletion patterns in nestin-expressing neural progenitor cells during embryonic development. Mosaic patterns of Cre activation in GFAP-CreERT2 transgenics probably results in heterogeneous loss β8 integrin expression with many astrocytes expressing integrin protein. It also remains possible that β8 integrin in retinal neurons as well as astrocytes determines patterns of neovascularization. Nonetheless, vascular pathologies in inducible knockout mice confirm that αvβ8 integrin in retinal astrocytes is essential for regulation of normal blood vessel sprouting in the developing retina.

Genetic ablation of TGFβ signaling in astrocytes does not cause retinal angiogenesis pathologies αvβ8 integrin is a receptor for latent TGFβ and loss of integrin expression leads to diminished TGFβ activation and signaling in the adult brain (Mobley et al., 2009; Su et al., 2010). Various
genetic studies have also linked αvβ8 integrin to TGFβs during embryonic brain development (Mu et al., 2008). To investigate a role for integrin-activated TGFβ signaling in retinal neural cells, we selectively ablated expression of TGFβR2, which dimerizes with various type 1 TGFβ receptors and is essential for the activation of intracellular signaling cascades (Massague and Gomis, 2006; Wu and Hill, 2009). Mice harboring a homozygous Tgfbr2flx/flx gene (Chytil et al., 2002) were interbred with Nestin-Cre transgenic mice (Tronche et al., 1999). Nestin-Cre/+;Tgfbr2flx/+ male progeny were then mated with Tgfbr2flx/flx females to generate mutant progeny that were hemizygous for the Nestin-Cre transgene and homozygous for the Tgfbr2 conditional allele.

Nestin-Cre/+;Tgfbr2flx/flx conditional knockout mice were born in the expected Mendelian ratios and displayed no obvious developmental phenotypes. Of 231 animals analyzed at P17, 55 mice (23%) were Nestin-Cre/+;Tgfbr2flx/flx. Analysis of postnatal life span in control (n=15) and mutant (n=13) littermates reveal that Tgfbr2 mutants survive for up to two years without developing obvious phenotypes (Nguyen et al., 2011). For example, conditional knockout mice do not display neurological deficits resulting from blood-brain barrier breakdown (data not shown). These results are in contrast to adult αv and β8 integrin mutant mice generated with Nestin-Cre, which develop progressive neurological deficits and die prematurely (McCarty et al., 2005; Proctor et al., 2005).

Retinas were isolated from P14 control and mutant mice, and Cre-mediated recombination of the Tgfbr2flx/flx gene was confirmed by genomic PCR analysis (Fig. 6A). Unlike β8−/− mice (Fig. 1) or αvβ8 integrin conditional knockout mice generated with Nestin-Cre (Figs 4 and 5), intraretinal hemorrhage was not evident in Nestin-Cre;Tgfbr2 conditional mutants (Fig. 6B). Normal associations between endothelial cells, pericytes and retinal astrocytes were detected (Figs 6C-F) and the endothelial tip cell defects were not obvious (supplementary material Fig. S6). These data reveal that ablation of TGFβ receptor signaling in neural progenitor cells via Nestin-Cre does not negatively impact angiogenesis in the post-natal retina. Hence, αvβ8 integrin-dependent angiogenesis defects are not due to loss of autocrine TGFβ signaling in retinal neural cells.

**Retinal astrocytes signal to endothelial cells via TGFβs and intraocular injection of anti-TGFβ blocking antibodies induces retinal angiogenesis pathologies**

Recently, we reported the use of a novel Alk1-Cre knock-in mouse strain to investigate roles for TGFβR2 and Alk5 receptors in endothelial cells during embryonic brain angiogenesis (Nguyen et al., 2011). Mutant animals develop abnormalities in blood vessel patterning and intracerebral hemorrhage that phenocopies defects in mouse embryos lacking αvβ8 integrin in neuroepithelial cells (McCarty et al., 2005; Proctor et al., 2005). However, TGFβ receptor mutant mice die during late stages of embryogenesis, which precludes analysis of TGFβ signaling in retinal angiogenesis. Nonetheless, we hypothesized that the retinal angiogenesis pathologies in αv and β8 integrin mutant mice were due to defective integrin-mediated TGFβ activation and signaling to vascular endothelial cells.

To investigate paracrine TGFβ signaling events between retinal astrocytes and endothelial cells, we performed in vitro cell culture experiments in which primary brain microvascular endothelial cells were treated with conditioned media taken from primary retinal astrocytes. As shown in Fig. 7A, a time-dependent increase in Smad3 phosphorylation was detected. Increased phosphorylation of Smad2 or Smad1/5/8 was not detected (data not shown). Smad3 phosphorylation was inhibited by pre-incubating conditioned medium with anti-TGFβ neutralizing antibodies, but not control IgGs. These data reveal that retinal astrocytes produce active TGFβs that can signal to endothelial cells and activate intracellular pathways.
Next, we used anti-TGFβ neutralizing antibodies to analyze roles for TGFβ signaling in retinal angiogenesis in vivo. P9 pups were anesthetized and injected intraocularly with control IgGs (n=5) or anti-TGFβ antibodies (n=5) that we have shown previously block activation and signaling functions for all three TGFβ family members (Mobley et al., 2009). Mice were sacrificed 6 hours after injection and blood vessel patterning was analyzed by staining whole retinas with IsoB4-Alexa488. Anti-TGFβ blocking antibodies (Fig. 7B), but not control IgGs (Fig. 7C), induced acute defects in angiogenesis. Unlike intraocularly injected control IgGs, anti-TGFβ antibodies generated abnormal endothelial cell morphologies and the appearance of blood vessels with glomeruloid-like tufts. Quantification of retinal blood vessel pathologies revealed statistically significant more vascular tufts in mice injected intraocularly with anti-TGFβ antibodies versus control IgGs (Fig. 7D).

**DISCUSSION**

In this report, we demonstrate functions for αvβ8 integrin-mediated TGFβ activation and signaling in neonatal retinal angiogenesis using a combination of cell type-specific gene knockout and signaling blockade strategies. Our experimental data reveal the following novel findings: (1) αvβ8 integrin is expressed in retinal astrocytes (Fig. 1) and β8−/− neonatal mice display blood vessel sprouting defects, particularly during formation of the secondary retinal vascular plexus (Figs 2 and 3); (2) selective ablation of αv or β8 integrin gene expression in astrocytes via Nestin-Cre (Figs 4 and 5) or GFAP-CreERT2 (supplementary material Figs S4 and S5) leads to retinal vascular pathologies similar to those that develop in whole body β8−/− mice; (3) genetic deletion of TGFβ signaling in astrocytes does not lead to retinal vascular pathologies (Fig. 6); and (4) antibody-mediated inhibition of TGFβ leads to acute angiogenesis defects and impaired signaling events in endothelial cells (Fig. 7). Collectively, these data are the first to identify αvβ8 integrin as an essential astrocyte-specific regulator of retinal angiogenesis.

Although we detect abnormalities in blood vessel spouting during formation of the primarily vascular network, the most severe integrin-dependent pathologies occur upon development of the secondary retinal vascular plexus. Interestingly, the secondary plexus first forms when blood vessels migrate radially along GFAP-expressing Müller glia, which are morphologically similar to brain neuroepithelial cells (Zhang and Barres, 2010). The glomeruloid-like vascular tufts that develop in integrin mutants indicate that αvβ8 integrin plays a crucial instructive role in promoting retinal blood vessel spouting along Müller glia. The vascular tufts comprise both endothelial cells and pericytes, suggesting that integrin-expressing astrocytes might communicate with both cell types to induce proper patterns of radial sprouting.

How αvβ8 integrin spatially controls endothelial cell and pericyte sprouting behaviors remains unclear, although local TGFβ activation and signaling events are likely to be necessary. One possibility is that tip cells are enriched with TGFβ receptors, which can bind to TGFβs activated locally by αvβ8 integrin in perivascular astrocytes. In support of this model, Eichmann and colleagues have shown enrichment of TGFβ receptors in endothelial tip cells of the primary retinal vascular plexus (del Toro et al., 2010). TGFβs might also bind to receptors in endothelial stalk cells or pericytes and regulate tip cell sprouting at the invading vascular front. Lastly, the retinal vascular phenotypes in integrin mutant mice might be due, in part, to defective astrocyte adhesion to ECM protein ligands other than latent TGFβ. αvβ8 integrin has been identified as a receptor for vitronectin (Milner et al., 1997) as well as collagen IV and fibronectin (Venstrom and Reichardt, 1995); therefore, impaired signaling pathways regulated by these ECM proteins might contribute to the retinal vascular phenotypes.

It is enticing to speculate that integrin-activated TGFβs cooperatively signal with other growth factors to regulate blood vessel spouting. For example, perturbation of VEGF-A interactions with the ECM leads to impaired blood vessel morphogenesis in the retina (Ruhberg et al., 2002), and these defects are similar to those
we report for integrin knockouts. However, inducible deletion of VEGF-A in astrocytes leads to only minor impairments in development of the primary and secondary vascular networks (Scott et al., 2010), revealing that other astrocyte-derived VEGF family members or other growth factors drive retinal neovascularization. One such factor is pigment epithelial-derived factor, which is expressed by Müller glia and is essential for blood vessel sprouting during formation of the secondary vascular plexus (Yafai et al., 2007). Astrocyte-expressed Norrin, a protein with structural similarities to TGFβs (Clevers, 2004) that signals via Frz4 and β-catenin, is also essential for formation of the secondary retinal vascular plexus (Xu et al., 2004; Ye et al., 2009). Norrin- and Frz4-deficient mice develop pathologies that phenocopy many of the retinal vascular defects that we report herein for αβ8 integrin mutant mice. Hence, it is possible that integrin-activated TGFβ, Wnts and other adhesion pathways cooperatively control blood vessel sprouting in the developing retina.

Lastly, abnormal regulation of angiogenesis during retinal development is associated with retinopathy of prematurity, a vascular pathology that often leads to retinal detachment and blindness (Chen and Smith, 2007). It is likely that pathways involved in physiological retinal angiogenesis, including αβ8 integrin activation of TGFβs, are altered in retinopathy of prematurity. Defining the signaling pathways that are differentially involved in neural versus vascular cells will be essential to understand better, and potentially treat, such diseases of development, as well as provide insight into how other CNS vascular pathologies might be prevented.

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