

# Neural regulation of CNS angiogenesis during development

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**Abstract** Vertebrates have evolved a powerful vascular system that involves close interactions between blood vessels and target tissues. Vascular biology had been mostly focused on the study of blood vessels for decades, which has generated large bodies of knowledge on vascular cell development, function and pathology. We argue that the prime time has arrived for vascular research on vessel-tissue interactions, especially target tissue regulation of vessel development. The central nervous system (CNS) requires a highly efficient vascular system for oxygen and nutrient transport as well as waste disposal. Therefore, neurovascular interaction is an excellent entry point to understanding target tissue regulation of blood vessel development. In this review, we summarize signaling pathways that transmit information from neural cells to blood vessels during development and the mechanisms by which they regulate each step of CNS angiogenesis. We also review important mechanisms of neural regulation of blood-brain barrier establishment and maturation, highlighting different functions of neural progenitor cells and pericytes. Finally, we evaluate potential contribution of malfunctioning neurovascular signaling to the development of brain vascular diseases and discuss how neurovascular interactions could be involved in brain tumor angiogenesis.

## Introduction

Evolution of advanced vascular systems is driven by the needs to efficiently deliver nutrients and oxygen to target tissues throughout the body of multicellular organisms. Given that blood vessels are designed to provide logistics to all tissues, it is not surprising that they are under tight regulation by target tissues that they serve. Target tissues therefore typically have special molecular machinery that modulates blood vessel function in response to different physiological states. Target tissues are also intimately involved in blood vessel network formation during development. Much of vascular biology has been focused on the study of blood vessels themselves (such as endothelial cells and mural cells), and as a result has accumulated large bodies of knowledge on vascular cell development, function and pathology. However, we argue that it is impossible to gain a comprehensive understanding of vascular systems without insight into vessel-tissue interactions, especially target tissue regulation of blood vessel development. Supported by the literature, we reason that animals are equipped with signaling pathways

dedicated to establishing close vessel-tissue interactions during development and their dys-regulation underlies a significant number of vascular diseases. Genetically accessible organisms as well as new molecular tools are beginning to allow us to explore these interactions, providing novel perspectives on vascular biology. Of note, the central nervous system (CNS) consumes much more energy per unit volume of tissue than the rest of the body, and requires a highly efficient vascular system for oxygen and nutrient transport as well as waste disposal. Therefore, neurovascular interaction is an excellent entry point to understanding target tissue regulation of blood vessel development.

Vascular and nervous systems share a variety of features at the molecular and cellular levels. Molecular approaches have identified common cues that guide both vessels and nerves during development. For example, axon guidance cues semaphorins and netrins have been found to restrict vessels to intersomitic regions during embryonic development (Lu et al., 2004; Gu et al., 2005). At the cellular level, growth cones of axons and vascular tip cells share common morphological features, with filopodial and lamellopodial projections believed to generate the force needed for extending axons and vessels (Tam and Watts, 2010). Vascular and neural cells form a neurovascular unit that maintains brain homeostasis and its dysfunction contributes to progression of brain diseases. Given such a close relationship at the cellular

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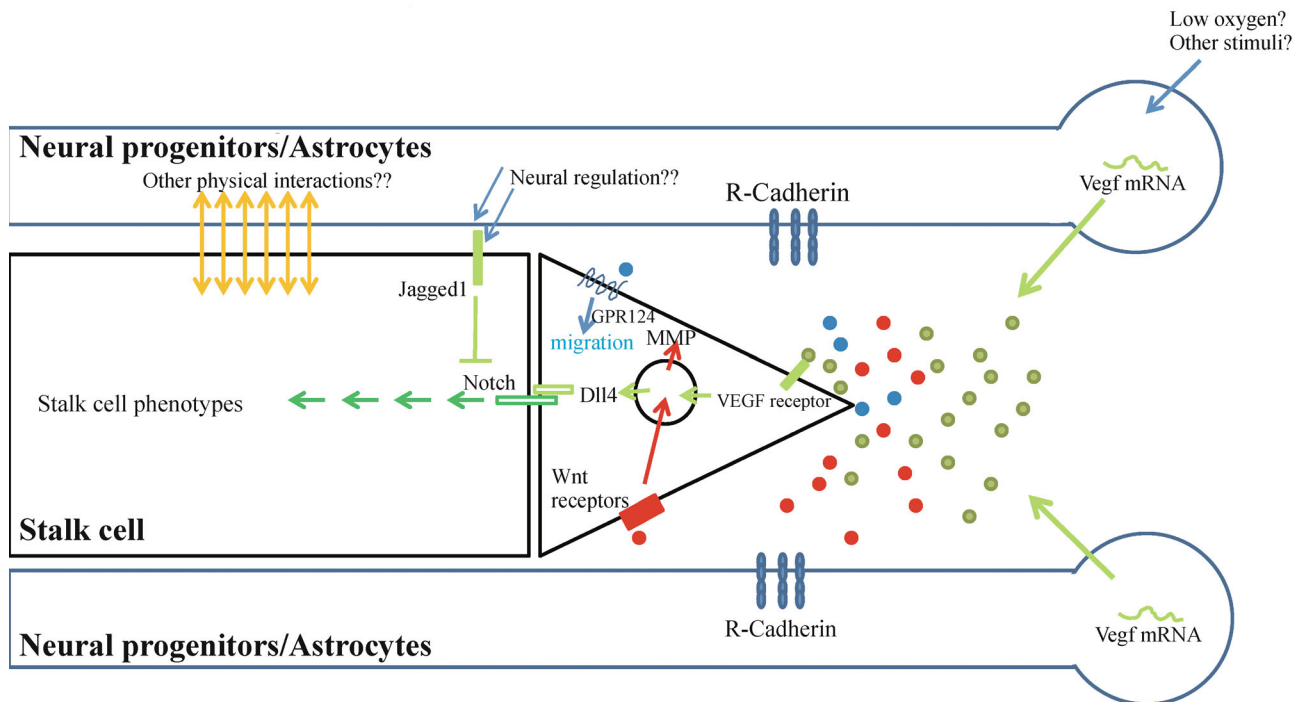
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level, vascular and nervous systems must have bidirectional communication to coordinate their functions. In fact, vascular cells play important roles in regulating neurogenesis by forming “vascular niche,” a unique anatomical structure within which both embryonic and adult neural progenitor cells divide and self-renew (Palmer et al., 2000; Shen et al., 2004). However, it still remains elusive how neural cells signal to vascular components during angiogenesis and, in general, how the neurovascular unit functions. We argue that the nature of neural-to-vascular signaling has fundamental implications for understanding tissue-specific regulation of blood vessels, which presumably show distinct properties to meet the special needs of different target tissues. In addition, we reason that many brain vascular disorders, both at the developmental and adult stage, can likely be attributed (at least partly) to neural cell dysfunction that disrupts such neural-to-vascular signaling.

## Vessel ingression and initial vascular patterning

During embryonic brain development, vascularization begins

with the invasion of blood vessels from the perineural vascular plexus (PVP) on the pial surface (Risau, 1993) (Fig. 1). The invasion of vessels is probably independent of neuronal migration, since vascular patterning is unaffected in reeler mice and SRK rats in which laminar organization is severely disrupted (Stubbs et al., 2009). Shortly after sprouting from the PVP, capillaries elongate radially into brain parenchyma, in parallel to neuroepithelial cells. Such close interactions between vessels and neural cells resemble that of the retinal vasculature, where blood vessels can be perfectly overlaid on astrocyte processes, suggesting that astrocytes are a major template for retinal angiogenesis (Dorrell et al., 2002; Gerhardt et al., 2003). This intimate neural cell association with blood vessels also raises a possible role of cell-cell contact and/or localized signaling at the neurovascular interface in mediating blood vessel development. For example, retinal astrocytes express R-cadherin and functional blockage of this adhesion molecule prevents normal vascular network formation (Dorrell et al., 2002). This suggests that astrocytes may guide vessel migration through specific cell adhesion molecules (such as R-cadherin) together with expression of VEGF isoforms. Binding to R-cadherin on the astrocytes may stabilize



**Figure 1** Neural regulation of initial vessel ingression. The blue rectangles on the top and bottom represent radial glia fibers or astrocytic processes, while the circles to the right represent cell bodies of neural progenitor cells or astrocytes. The black rectangle and triangle in the middle represent endothelial stalk and tip cells, respectively. R-Cadherin from neural cells potentially stabilizes filopodia from tip cells. Low oxygen levels or other stimuli may up-regulate Vegf gene expression within neural cells. Secreted VEGF proteins (green dots) then induce endothelial Dil4 expression, which is critical for tip cell selection and stalk cell development. Other Notch ligand such as Jagged1 has opposite functions to Dil4, suggesting that a balance between Dil4 and Jagged1 may determine tip cell number. Neural progenitor cells also secrete Wnt ligands (red dots), which stimulate endothelial cell migration probably by up-regulating MMP levels. Unidentified diffusible factors (blue dots) from neural cells may bind to GPR124 on endothelial cells to regulate Cdc42-dependent cell migration (in the forebrain).

filopodia of endothelial cells and the resulting adhesion complexes may direct their migration, in a process similar to axonal growth (Doherty et al., 2000). Some groups including our laboratory also observed radial glia directly contacting endothelial cells during brain development and our experiments suggest that this contact is required for modulating certain molecular pathways in cortical endothelial cells (discussed below) (Gerhardt et al., 2004; Ma et al., 2013). It will be interesting to further investigate the molecular underpinning of such close interactions and its function in shaping the CNS vasculature. In addition to vessels penetrating from the pial surface, vessels can populate the brain from the periventricular zone (Vasudevan et al., 2008; Stubbs et al., 2009). Vasculatures from these two sources connect poorly, leaving the cortical intermediate zone the lowest vascular density and rendering this region susceptible to ischemia risks (Volpe, 2005). Interestingly, there is some evidence that endothelial cells from these two origins differ in transcription factor expression (Vasudevan et al., 2008), suggesting that angiogenesis can follow different genetic/developmental programs in different parts of the brain. It also suggests that neurovascular interaction is region specific during development.

At the cellular level, vessel migration is achieved by specialized cell types called tip cells. Determining molecular mechanisms that control tip cell behavior is thus the first step toward understanding how neural cells may guide migration of new endothelial cells under diverse physiologic conditions. Genetic evidence shows that VEGF plays important roles in filopodial extension from tip cells as well as subsequent angiogenic sprouting in the retina and the brain (Ruhrberg et al., 2002; Gerhardt et al., 2003). VEGF expression is believed to be upregulated by hypoxia due to neuronal activities or when neural tissues attempt to meet high metabolic demands. In cultured astrocytes, this is supported by experiments showing that hypoxia increases VEGF transcript levels (Stone et al., 1995). However, the proof for hypoxia-induced VEGF expression in embryonic CNS is still lacking, partly due to difficulties in measuring oxygen levels in embryos. As the first identified angiogenic factor, VEGF has multiple isoforms produced by alternative splicing from a single gene. These isoforms differ in domains conferring heparin binding ability and therefore VEGF isoforms can bind to extracellular matrix with different affinity, forming a gradient *in vivo*. Indeed, each VEGF isoform has a distinct function in mediating CNS angiogenesis. Elegant genetic engineering that produced mice each with a single isoform showed that heparin bound VEGF is necessary for normal vessel branching morphogenesis (Ruhrberg et al., 2002), and a proper VEGF protein gradient is likely responsible for this function. Similarly in the retina, the extracellular matrix bound VEGF164 isoform provides a gradient to which tip cells respond for regulating their migration, while diffusible VEGF120 controls stalk cell proliferation (Gerhardt et al., 2003). Intriguingly, systematic examination of VEGF isoform expression pattern within the

brain showed that relative levels of VEGF isoforms are not significantly changed at different developmental stages (Ng et al., 2001), suggesting the importance of maintaining a proper distribution of VEGF proteins in the brain. It remains unclear whether the constant VEGF isoform ratio is retained in different parts of the brain and how this relates to vascular patterning. Conditional deletion of the VEGF gene provides the most direct evidence for neural cell secretion of VEGF in controlling initial vessel ingression. Conditional knockout of the VEGF gene using Nestin-cre causes reduced vessel density and neural apoptosis, probably resulting from insufficient blood supply (Raab et al., 2004). In the mutants, blood vessels are unable to ingress into the forebrain from the PVP and are not directed toward the ventricular zone in the hindbrain. This supports the hypothesis that neural progenitor cells produce high levels of VEGF (Breier et al., 1992; Breier et al., 1995), which guide blood vessels toward the ventricular zone. However, it is unclear how neural progenitor cells upregulate VEGF during development. One widely believed model is that hypoxia drives VEGF expression via HIF-1 $\alpha$ , which is a major oxygen detector in animals. But conditional removal of HIF-1 $\alpha$  from neural cells showed normal vessel density at early developmental stages, although vessel density becomes reduced in later embryos (Tomita et al., 2003), concomitant with a decrease in VEGF expression shortly before birth. Since Tomita et al. used a Nestin-cre expressed at a similar stage as Raab et al., this suggests that not all VEGF expression may be dependent on HIF-1 $\alpha$ . Therefore, more experiments are needed to definitively implicate the molecular pathway that regulates VEGF expression during development.

The Wnt signaling pathways play a diversity of roles in development and physiology. Wnt ligands are highly expressed in the developing neural tube and downstream molecules of Wnt signaling are enriched in CNS but not non-CNS endothelial cells during development (Daneman et al., 2009). Genetic removal of both Wnt7a and Wnt7b ligands results in a poorly vascularized spinal cord, and similar defects are observed in mutants that lack  $\beta$ -catenin or other Wnt signaling components (Stenman et al., 2008; Daneman et al., 2009). This suggests that canonical Wnt signaling is required for initiating CNS angiogenesis. Neural progenitor cells are the cell type secreting Wnt ligands, as it was shown that Wnt ligand expression in the ventricular zone correlates with  $\beta$ -catenin activation in CNS endothelial cells. As both Wnt and VEGF are secreted by neural progenitor cells to control brain angiogenesis, how then does Wnt coordinate with VEGF? Since vessels fail to invade neural tissue after Wnt signaling disruption, it is possible that Wnt ligands function as a factor for endothelial cell migration. *In vitro* assay suggests that Wnt7a promotes brain endothelial cell migration (Daneman et al., 2009). This is consistent with our observation that MMP2 is upregulated when Wnt signaling is ectopically elevated within endothelial cells. It is known that Wnt signaling can induce MMP expression and regulate T

cell transmigration (Wu et al., 2007). Thus, we propose that neural progenitor cells produce a gradient of VEGF protein by expressing a fixed ratio of VEGF isoforms. The vessels, in response, extend filopodia from tip cells and increase stalk cell proliferation. Canonical Wnt ligand from progenitor cells, on the other hand, induces endothelial expression of MMPs, likely among other genes, to promote extracellular matrix degradation and facilitate migration. This is consistent with findings that precise regulation of MMP activity is crucial for the balance between vessel basement membrane breakdown and stabilization (Saunders et al., 2006).

Given the paramount importance of tip cells in shaping the vascular pattern, it is necessary to first understand the molecular mechanisms that select tip cells among endothelial cell population at the leading front of the vasculature. It should be noted that most experiments on tip cell selection have been performed with the retinal vasculature as a model, for molecular markers can label retinal tip cells unambiguously. Notch and Dll4 are highly expressed in the front of the retinal vasculature in response to VEGF gradients, suggesting their functions in tip cell fate determination (Hellström et al., 2007). Both genetic and pharmacological experiments showed that endothelial cells low in Notch activity are more likely to become tip cells while those high in Notch activity are prevented from becoming tip cells and become stalk cell instead (Hellström et al., 2007; Lobov et al., 2007; Suchting et al., 2007). A model has been proposed that neural tissue-secreted VEGF stimulates a subpopulation of endothelial cells to upregulate Dll4 and these cells enriched in Dll4 become tip cells. On the other hand, Dll4 in prospective tip cells activates Notch signaling in neighboring cells that become stalk cells. Although this conclusion is based on data from the retina, it could also be generalized to brain vessels because similar vascular phenotypes were observed in the hindbrain in genetic loss-of-function experiments (Suchting et al., 2007). Surprisingly, another ligand for Notch, Jagged1 has a positive role in regulating retinal angiogenesis, by promoting tip cell formation, endothelial cell proliferation and mural cell coverage (Benedito et al., 2009). It was shown that endothelial cell specific deletion of Jagged1 results in elevated level of Hey1, which is a downstream target of Notch signaling. This suggests that normally Jagged1 inhibits Notch signaling activity within endothelial cells. Therefore, the balance between Jagged1 and Dll4 levels can determine the number of tip cells. Since Dll4 is highly expressed in tip cells where Jagged1 is absent, one possibility is that Dll4 and Jagged1 expression may be differentially regulated by neural cells. If so, it would be interesting to speculate that neural cells may regulate tip cell number by producing specific factors that change Dll4 and Jagged1 ratio under different physiological conditions. Similarly, other neural tissue secreted factors like TGF- $\beta$  superfamily members can activate the endothelial TGF- $\beta$ /BMP/Smad signaling pathway that is known to interact with Notch/Dll4 signaling to orchestrate stalk cell phenotypes (Moya et al., 2012). Neural tissues may

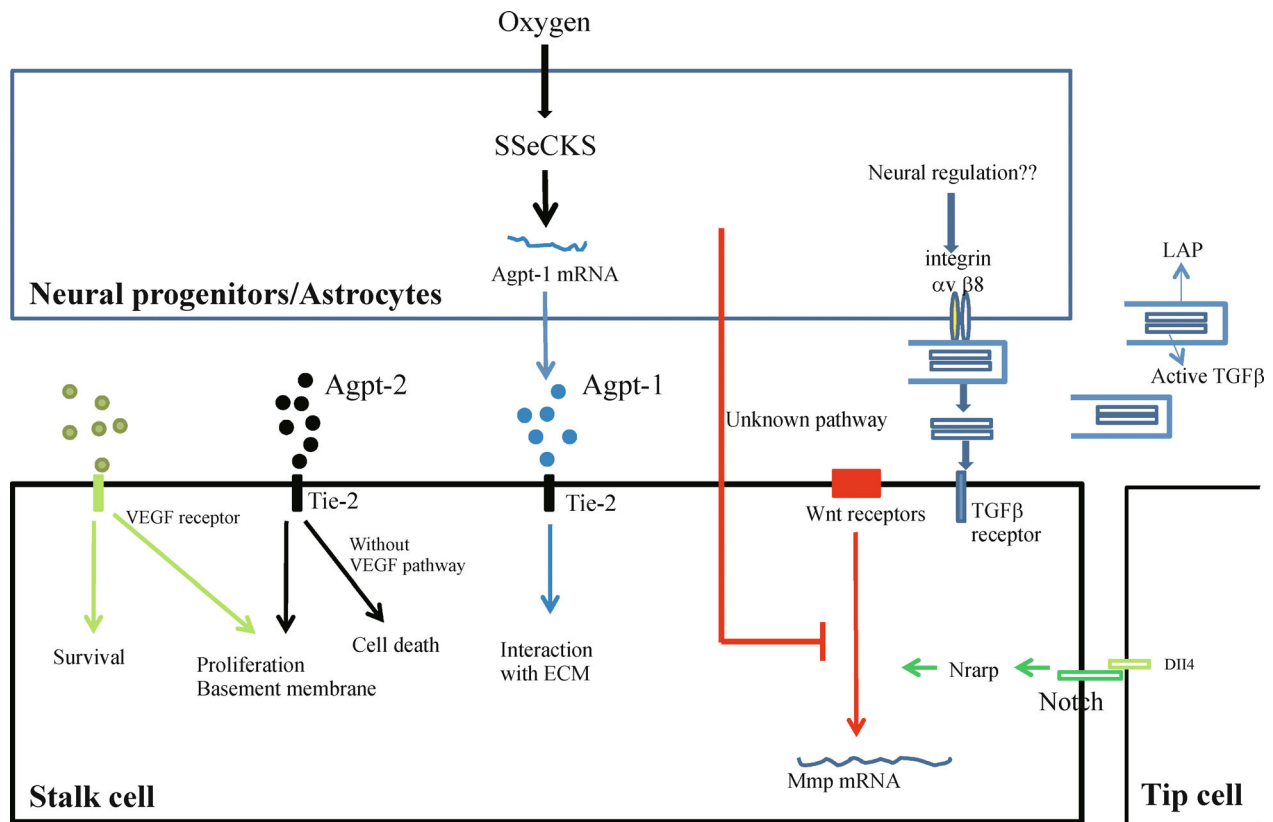
thus also regulate tip cell number through TGF $\beta$ /BMP signaling.

One way of expanding our understanding in neural regulation of vascular development is to identify and study novel receptors on CNS-specific vascular cells, followed by determining intercellular signaling that mediates the function of such receptors in controlling vessel development. Recently, G protein-coupled receptor (GPCR) GPR124, a gene initially identified in the tumor vasculature, was found expressed in CNS endothelial cells and pericytes with critical functions in CNS-specific angiogenesis (Kuhnert et al., 2010; Anderson et al., 2011; Cullen et al., 2011). Gpr124 null mutants are embryonically lethal and show forebrain and neural tube specific vascular defects. Blood vessels at early embryonic stage fail to invade neural tissues. *In vitro* experiments demonstrated that GPR124 is required to mediate Cdc42-dependent migration in response to unidentified forebrain-derived diffusible molecules (Kuhnert et al., 2010). This finding suggests that CNS angiogenesis may involve novel molecules for mediating intercellular signaling from brain to endothelial cells. Further experiments including identifying the ligand(s) for GPR124 may shed new light on the mechanisms of CNS-specific angiogenesis and of neural regulation of this process.

## Vessel stabilization and remodeling

In principle, vascular patterning may arise not only by guided migration but also by remodeling of existing vessels (Fig. 2). During the remodeling process, the vasculature eliminates some branches as well as stabilizes others. Regression can happen if blood vessels are not well stabilized during this process. At the molecular level, vessel regression occurs most likely due to endothelial cell death. So survival factors provided by neural cells may explain how neural tissues regulate vessel regression/stabilization. VEGF has been implicated in the regulation of endothelial cell survival in addition to its functions in tip cell formation and migration. In an experimental model where neonatal retina is exposed to high oxygen level condition, VEGF expression from neuroglia is down-regulated by the hyperoxia. This is followed by capillary regression via endothelial cell apoptosis. Exogenous supply of VEGF can prevent apoptosis and rescue the regression phenotype (Alon et al., 1995). Later studies using conditional knockout alleles provide direct evidence for VEGF function as a survival factor *in vivo*. Removal of VEGF from only the retinal astrocytes has minor effects on vessel development but profound effects on vessel stabilization under hyperoxic condition, suggesting that VEGF from astrocytes mainly function to stabilize vessels (Scott et al., 2010). However, we cannot exclude the possibility that astrocyte deletion of VEGF may be compensated for by VEGF upregulation of other cell types so that initial angiogenesis is not severely damaged.





**Figure 2** Regulation of vessel stabilization and remodeling by neural cells. The blue rectangle on the top represents neural cells while the black rectangle on the bottom represents endothelial cells. VEGF and Angiopoietin-2 (Agpt-2) from neural cells control endothelial cell survival either separately or by synergistic actions. SSeCKS in astrocytes stimulates Angiopoietin-1 (Agpt-1) production under hyperoxia, and Agpt-1 enhances interactions between endothelial cells and the extracellular matrix (ECM). Our data suggest that unknown pathways from radial glia stabilize nascent blood vessels by suppressing endothelial Wnt signaling during later embryonic cortical development. In contrast, evidence from the retina suggests that Notch signaling pathway positively regulates Wnt signaling by Nrarp in endothelial cells and that Wnt signaling is essential for vessel stability. Latent TGF $\beta$  is widely expressed in the brain, and neural progenitor cells can localize latent TGF $\beta$  activation via integrin  $\alpha v \beta 8$  so that active TGF $\beta$  is released locally (within the vicinity of progenitor cells) to regulate blood vessel stabilization.

Alternatively, other factors may compensate for the loss of VEGF and guide initial vessel migration in the retina. Nevertheless, this suggests that neural tissues do produce VEGF to serve as a guidance cue for initial vascular migration, which can in addition function as a survival factor for endothelial cells. Furthermore, VEGF can influence vessel stability by co-operating with other molecules. For example, it was found that VEGF switches function of angiopoietin-2 from anti-angiogenic to pro-angiogenic (Lobov et al., 2002). In the presence of VEGF, angiopoietin-2 stimulates endothelial cell proliferation, basement membrane remodeling, and new vessel sprouts in the pupillary membrane. However, when VEGF signaling is inhibited, angiopoietin-2 promotes cell death and vessel regression. Angiopoietin-1 is another blood vessel growth factor that has been studied for long time, and its receptor Tie-2 is highly expressed in endothelial cells. Genetic inactivation of either angiopoietin-1 or Tie-2 severely disrupts vascular remodeling after normal vessel sprouting is finished (Suri et al., 1998; Sato et al., 1995). Overexpression of angiopoietin-1 in mice leads to increased vascularization,

possibly due to decreased pruning and reduced regression (Thurston et al., 1999). On the other hand, angiopoietin-1 mutants show poor association between endothelial cells and surrounding extracellular matrix. Therefore, angiopoietin-1 is probably involved in vessel stabilization by promoting a strong vessel-matrix interaction. Interestingly, it was found that astrocytes can secrete angiopoietin-1 *in vitro*, suggesting one possible mechanism that neural cells may utilize for regulating vessel remodeling *in vivo* (Lee et al., 2003).

Another well understood example of vascular remodeling is hyaloid vessel regression. It is long known that hyaloid vascular networks are removed shortly after birth by vessel regression that requires the participation of macrophages (Lang and Bishop, 1993). Later studies showed that macrophages induce capillary regression by activating apoptosis of vascular cells in a cell cycle dependent manner. Genetic ablation of lymphomyeloid transcription factor PU.1 in mice showed that absence of macrophages in the mutants results in persistent hyaloid vessel networks in the postnatal eye, confirming that regression is dependent on macrophages.

Furthermore, deletion of canonical Wnt signaling molecules within hyaloid endothelial cells also causes a persistent postnatal vessel network, suggesting that the canonical Wnt pathway is required for hyaloid vessel regression. Additional experiments showed that Wnt7b ligand is expressed only in macrophages and is required for the regression. Put together, this shows that macrophages secrete the Wnt7b ligand, which then promotes vessel regression probably by activating apoptotic pathways within endothelial cells (Lobov et al., 2007). This suggests that signals from tissue-specific cell types play important roles in vascular network stabilization and remodeling. Wnt signaling also interacts with other pathways to control angiogenesis and vessel stability. In the mouse retina as well as in the zebrafish intersegmental vasculature, Dll4-induced Nrarp expression down-regulates Notch signaling and promotes Wnt signaling in endothelial stalk cells (Phng et al., 2009). Genetic deletion of Nrarp in the mouse retina results in decreased stalk cell proliferation and vessel regression marked by the presence of collagen empty sleeves. Lef1 and Ctnnb1 knockouts also have the similar phenotypes. Therefore, Nrarp functions as a molecular link between Notch and Wnt signaling in orchestrating angiogenesis including vessel stability. Altogether, it seems that canonical Wnt signaling may play multiple and sometimes opposite roles in angiogenesis, depending on specific tissues and interactions with other pathways. This raises the question of how Wnt pathways regulate brain angiogenesis. Wnt reporter lines show that endothelial cells are high in canonical Wnt signaling in both the hindbrain and cerebral cortex at early stages of brain development but the level decreases later in development (Liebner et al., 2008; Ma et al., 2013). This suggests neural mechanisms that shut down Wnt signaling to avoid potential vessel destabilization/regression observed in hyaloid vessels. Consistent with this idea, our group found that genetic ablation of radial glial cells leads to vessel regression, concomitant with ectopic activation of Wnt signaling in endothelial cells. This suggests that radial glial progenitors in the late embryonic cortex signal to nascent blood vessels and regulate vessel stabilization via suppression of canonical Wnt signaling. Interestingly, our acute primary coculture experiments suggested that neural progenitors suppress endothelial Wnt signaling only when the two cell types are in very close proximity (Ma et al., 2013). We are now pursuing the mechanisms that underlie these intercellular interactions. In short, we have provided an explanation for the dual role of Wnt signaling in brain angiogenesis, from a perspective of neural-to-vascular signaling. Wnt ligands likely facilitate endothelial cell migration by upregulating MMPs, among other genes, to degrade extracellular matrix components. Later during development, when blood vessels are being stabilized, radial glial cells may down-regulate endothelial Wnt pathways to suppress MMP activities.

TGF $\beta$  pathways also control a variety of developmental and physiological processes and genetic studies suggest that it plays important roles in angiogenesis. Unlike many other

common ligands, TGF $\beta$  is secreted in a latent form that is generated by binding of TGF $\beta$  to a latency-associated peptide (LAP). Regulated processing is necessary for the release of bioactive TGF $\beta$  molecules from LAP. TGF $\beta$  activation involves molecules such as thrombospondin-1, MMPs, and integrins  $\alpha$ v $\beta$ 6 and  $\alpha$ v $\beta$ 8. Integrins  $\alpha$ v and  $\beta$ 8 are particularly relevant to brain vascular research because null mutants for each have extensive cerebral hemorrhage and alteration of extracellular matrix, which are very similar to phenotypes from mutations in TGF $\beta$  signaling (Zhu et al., 2002; McCarty et al., 2002). *In vitro* data showed that  $\alpha$ v and  $\beta$ 8 are paired to bind the RGD domain of LAP, followed by MMP14 cleavage of LAP to release active TGF $\beta$  (Mu et al., 2002). Genetic mutation of RGD domain in mice has a similar vascular phenotype as TGF $\beta$ 1/3 null mutant, suggesting that *in vivo* TGF $\beta$  activation probably functions via this mechanism to regulate angiogenesis (Yang et al., 2007). The same mechanism also acts in the retina to regulate angiogenesis as genetic data demonstrate that  $\alpha$ v $\beta$ 8 integrin in neural cells is essential for retinal vessel development. Deletion of the integrins specifically from neural cells results in intraretinal hemorrhage and reduced vessel sprout formation in the secondary plexus. The phenotypes, at least partly, correlate with diminished  $\alpha$ v $\beta$ 8 mediated activation of TGF $\beta$  (Hirota et al., 2011; Arnold et al., 2012). Earlier work in the postnatal brain also found that astrocytic  $\alpha$ v $\beta$ 8 localizes latent TGF $\beta$  and subsequently activates it to regulate the function of endothelial cells in close proximity (Cambier et al., 2005). These studies suggest a regulatory link between neural cells and blood vessels: integrins expressed in glial cells controls activation of TGF $\beta$  ligands that then bind to endothelial cells to stabilize vessels. We reason that one purpose of using integrin-dependent TGF $\beta$  activation as a mechanism to regulate vessel stability may be to gain the power of localized control over endothelial cells. Since TGF $\beta$  ligands may be expressed widely in CNS, only certain neural cell types or cells in certain locales would localize TGF $\beta$  using  $\alpha$ v $\beta$ 8 followed by its activation to stabilize surrounding vessels. Consistent with this reasoning,  $\beta$ 8 was found to be required only in neural progenitor cells but not in neurons or endothelial cells for regulating CNS angiogenesis (Proctor et al., 2005). However, it remains unclear how neural progenitor cells control integrin dependent TGF $\beta$  activation at the molecular level.

## Pericytes in CNS angiogenesis

A neurovascular unit consists of endothelial cells, astrocytes, neurons and pericytes. Known as a major supporting cell type, pericytes are in direct contact with endothelial cells and ensheath the capillary wall. Endothelial cell-secreted PDGF-B binds to PDGFR-B expressed on pericytes, controlling pericytes proliferation, migration and recruitment to vessels. In the developing CNS, endothelial cells of sprouting vessels

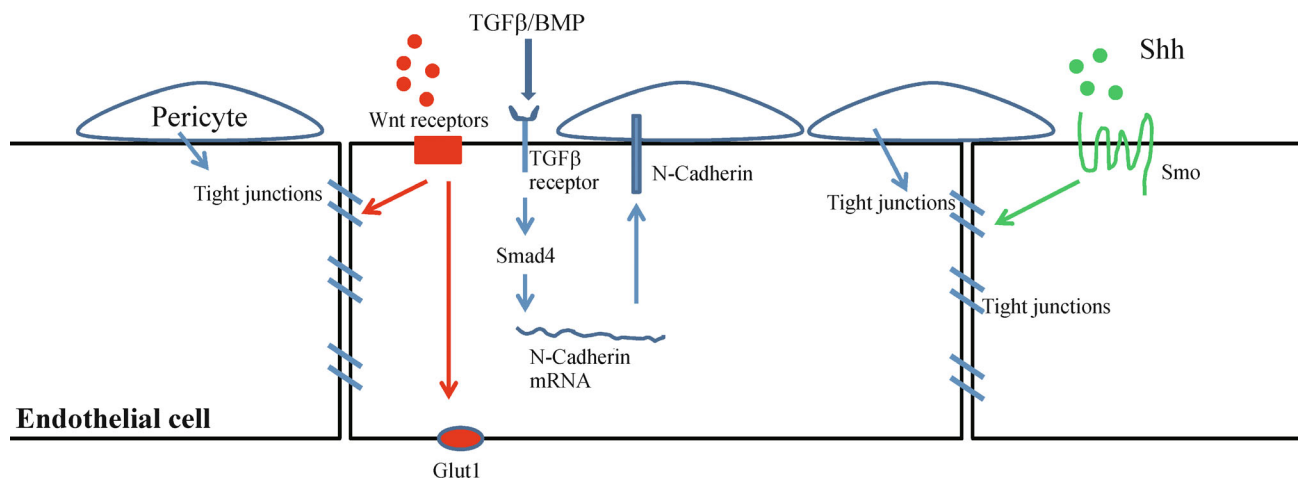
release PDGF-B that attracts PDGFR-B positive pericyte progenitors. Mouse genetics show that both PDGF-B and PDGFR-B null mutants appear to be defective in this process, causing loss of pericytes (Hellström et al., 1999). This results in microaneurysm in the cerebral cortex and later data also showed endothelial cell hyperplasia, suggesting that pericytes control endothelial cell proliferation (Lindahl et al., 1997). This is consistent with previous *in vitro* results showing inhibition of endothelial cell proliferation by pericytes (Armulik et al., 2005). Loss of pericytes also causes redistribution of adherens and tight junction proteins, implying that pericytes may contribute to vascular permeability (see further discussion in next section). In the adult brain, loss of pericytes causes reductions in microcirculation and age-dependent vascular damage even before neuronal degenerative changes (Bell et al., 2010). Given the importance of pericytes in neurovascular function, it is not surprising that complex signaling pathways from neural cells control pericyte recruitment, differentiation and function. It seems that endothelial cells are the primary cell types responsible for pericyte recruitment by secreting PDGF-B, but during later development and adulthood neural cells start to regulate crucial aspects of pericyte differentiation and interaction with endothelial cells. An elegant study using a brain endothelial cell specific Cre driver show that Smad4 deletion from EC causes impaired physical association between pericytes and brain endothelial cells, suggesting that TGF $\beta$ /BMP ligands (presumably secreted and processed by neural cells) tightly control such interactions (Li et al., 2011). More recently, astrocyte specific deletion of laminin was found to lead to breakdown of the blood-brain barrier (BBB) (Yao et al., 2014). This could be due to defective astrocyte regulation of the BBB or secondary effects due to pericyte problems. Indeed, further studies found that, in the

absence of laminin, pericyte marker is downregulated, suggesting that normally astrocytic signals are required for pericyte differentiation. Thus, pericytes are a key player in brain angiogenesis and are also under direct or indirect regulation by neural cells.

## Blood-brain barrier development

BBB is a specialized structure present in the brain vasculature. Outside the nervous system, blood vessels are generally leaky to allow substance exchange between blood and tissues. BBB restricts such flow into brain tissues to maintain CNS homeostasis, by preventing toxins and pathogens from entering so that normal neural communication is not disrupted. BBB accomplishes this by acquiring tight cell junctions, special transporters and different exocytosis rates along endothelial cells that line brain vessels. Compromise in this barrier is associated with and contributes to a variety of diseases including stroke and brain trauma. There are several excellent reviews on this topic (e.g. Zlokovic, 2008). Here we focus on neural signals that contribute to BBB establishment and maintenance (Fig. 3).

Several decades ago, transplant experiments showed that BBB is not an intrinsic endothelial cell property but is rather induced by astrocytes when endothelial cells invade neural tissues (Stewart and Wiley, 1981). Later coculture experiments also suggest that astrocytes induce barrier properties of non-neural endothelial cells (Hayashi et al., 1997). However, barrier property *in vivo* is established during embryogenesis and astrocytes are largely absent in the developing brain so that they are unlikely to induce BBB *in vivo*. Mouse genetics offers new approaches to study BBB at the molecular level.



**Figure 3** Regulation of blood-brain barrier (BBB) establishment and maturation by neural signals. Neural progenitor cells secrete Wnt and Sonic hedgehog (Shh) ligands that activate formation of tight junctions and expression of BBB specific transporters (such as Glut1) within endothelial cells. Pericytes are important for maintaining endothelial tight junction and other BBB properties, but unlikely to be responsible for inducing BBB. Pericyte-endothelial cell interaction is mediated by N-Cadherin, which is positively regulated by TGF $\beta$ /BMP/Smad4 signaling within endothelial cells.

Since BBB is a CNS-specific feature, CNS endothelial cell differentiation must involve unique processes that confer barrier properties. So it will be important to understand the molecular pathways that control CNS endothelial cell differentiation. Besides regulating vessel ingression into neural cells, canonical Wnt signaling is necessary for CNS-specific vasculature differentiation. Wnt7a/b double knockout as well as endothelial specific removal of  $\beta$ -catenin result in hemorrhage throughout the neural tube as well as reduced expression of Glut-1, a hallmark of adult BBB (Stenman et al., 2008; Daneman et al., 2009). *In vitro* experiments showed that Wnt ligands can induce expression of tight endothelial cell junction markers, one of the characteristics of mature BBB, and that inhibition of endothelial  $\beta$ -catenin signaling abolishes these effects (Liebner et al., 2008). Since Wnt signaling plays essential roles in brain development, Wnt function in BBB development suggests that other pathways of brain development may have similar roles in endothelial cell differentiation. Recently, hedgehog pathway was also found to be important for BBB integrity (Alvarez et al., 2011). In the mouse developing brain, neural cells secrete hedgehog ligands while brain endothelial cells express hedgehog receptors. *Shh*<sup>-/-</sup> mutants show reduced expression of junctional proteins essential for BBB. In accordance, endothelial cell specific deletion of smoothened, a receptor for Shh, has a similar phenotype at embryogenesis. Therefore, it seems that brain morphogens such as Wnt and Shh control CNS endothelial cell differentiation and BBB establishment during development and may also be required for BBB maintenance later in adulthood.

Since astrocytes are unlikely the primary cell types that induce BBB properties, what could be the cell types that perform this task? It has been found that pericyte recruitment to blood vessels in the brain is temporally correlated with the onset of barrier properties (Daneman et al., 2010). Mice defective in pericyte generation show increased vascular permeability and impaired tight junction as well as vesicle trafficking. Expression of extracellular matrix components together with that of MMP9 is also altered and these changes likely contribute vascular permeability. Interestingly, gene expression levels of many BBB markers are not changed in the absence of pericytes, suggesting that pericytes may not be responsible for inducing BBB but rather contribute to BBB maturation. Therefore, it is likely that neural progenitor cells, which outnumber postmitotic neurons during early development, induce most BBB molecular markers, which is then followed by pericyte coverage. Indeed, coculture experiments using neural progenitor cells and brain microvascular endothelial cells have shown that neural progenitor cells induce barrier properties in endothelial cells (Weidenfeller et al., 2007). Such conclusions, however, need to be validated *in vivo* using animal models. In addition, the brain has the most numerous pericytes compared to other tissues, suggesting that neural progenitor signals may also be involved in pericyte generation. Multiple TGF $\beta$  ligands have been found

expressed in the brain during development and mutations in TGF $\beta$  pathways such as Endog1, ALK1 and Smad4 have been linked to human vascular diseases (McAllister et al., 1994; Johnson et al., 1996; Berg et al., 1997; Gallione et al., 2004). To evaluate specific role of TGF $\beta$  signaling, Li et al. (2011) took advantage of a brain endothelial cell specific Cre driver to delete Smad4 protein, an indispensable component for TGF $\beta$  signaling. The deletion causes hemorrhage in many parts of the brain and also disrupts pericyte-endothelial cell interaction. Although Evan blue extravasation shows that the mutant brain is very leaky, this could result from secondary effects of impairment in endothelial cell differentiation and/or pericyte-endothelial cell interaction. At the molecular level, it was shown that N-Cadherin is a target for Smad4 signaling. Interestingly, the upregulation of N-Cadherin by Smad4 requires Notch signaling, which suggests that cooperation of these two pathways control proper pericyte-endothelial cell interaction. It is known that Notch signaling in stalk cells is activated by tip cell Dll4 expression, which is in turn induced by VEGF from neural cells. Thus, it is possible that, besides VEGF, neural cells also secrete TGF $\beta$  that, in cooperation with Notch signaling, facilitates pericyte-endothelial cell interaction in the brain.

Although astrocytes are unlikely to induce BBB properties, they are likely to contribute to BBB maturation, given the intimate association between astrocytes and vascular cells (Abbott et al., 2006). For example, it was suggested that oxygen tension of astrocytes regulates BBB maturation (Lee et al., 2003). This elegant study used *in vitro* approaches and revealed that Src-suppressed C-kinase substrate (SSECKS) function decreases VEGF and activates angiopoietin-1 expression within astrocytes. Furthermore, conditioned medium from SSECKS-overexpressing astrocytes stimulate expression of BBB marker genes such as ZO-1, ZO-2 and Claudin-1. In addition, oxygen levels of astrocytes were found to affect SSECKS levels as hypoxia down-regulates while reoxygenation upregulates SSECKS expression. This study thus potentially connects neural cell physiology to blood vessel differentiation. Despite of such a powerful *in vitro* system, however, the conclusion stills need to be confirmed *in vivo* using genetically tractable models. It also remains an intriguing question whether similar intercellular interactions exist between other neural cells and blood vessels and at other developmental stages. Such questions could be addressed by using inducible cell type-specific Cre drivers to delete candidate genes from specific neural cells, followed by analysis of vascular phenotypes. Lastly, cultured endothelial cells also express receptors for small molecules that function as neurotransmitters and modulators in the brain, suggesting rapid and complex signaling within the neurovascular unit in response to neural activity. From the viewpoint of brain physiology, it makes sense that BBB is modulated by neural activity so that transient opening or tightening of the barrier can control substance passage between blood and brain tissues to meet metabolic needs at short time intervals (Abbott



et al., 2006). Recently, a breakthrough study identified Mfsd2a (major facilitator super family domain containing 2a), which is specifically expressed in endothelial cells, as a key regulator of BBB establishment and function (Ben-Zvi et al., 2014). Further investigation of how Mfsd2a expression or activity is regulated may suggest novel intercellular signaling in BBB development and function.

## Disease

As we have discussed above, neural signaling is critical to initiate and maintain a functional vascular system in the brain throughout life. Thus, any disruption of such communication may lead to brain vascular disorders. In addition, neurological disorders could also change blood vessel functions and subsequently confound the symptoms.

### Germinal matrix hemorrhage/intraventricular hemorrhage (GMH/IVH)

GMH/IVH is a major neonatal disease that retains a high incidence rate of 20% in premature newborns (Heuchan et al., 2002). Germinal matrix is a transient brain structure present during embryonic development and is a very neurogenic region, so to meet metabolic demands, it may have a more rigorous angiogenesis than other parts of the brain. Indeed, high expression of VEGF and angiopoietin-2 are found in the germinal matrix, and they are triggered by hypoxia (Mu et al., 2003). This is consistent with chemical detection of hypoxia in this region (Ballabh, 2010). Pericytes are important vascular support cells required for BBB maturation and vessel integrity. Quantification of immunostainings showed that the germinal matrix has the least number of pericytes in comparison to the cortex and the white matter in human fetuses, premature infants, and rabbit pups (Braun et al., 2007). Therefore, paucity of pericytes, active angiogenesis, and remodeling may contribute to the instability and fragility of germinal matrix vessels, making vasculature prone to hemorrhage. As a result, anti-angiogenesis treatment has been devised to prevent or alleviate GMH/IVH. For example, prenatal suppression of VEGF with cercecoxib or VEGFR-2 inhibitor ZD6474 can reduce the incidence of experimentally induced GMH/IVH in animal models (Ballabh et al., 2007). However, such strategy only emphasizes the contribution of active angiogenesis to the disease and neglects the fact that vessel instability is also a major factor. In addition, the long-term effects of such angiogenic suppression are unclear and subject to further evaluation. Therefore, we propose that it is necessary to develop new intervention strategies by stabilizing pre-existent vessels instead of preventing new vascular sprouts. Mouse molecular genetics will likely be helpful in generating useful models for further basic and therapeutic research on GMH/IVH.

### Cerebral cavernous malformation (CCM)

CCM mainly affects the brain and is characterized by enlarged vessels associated with brain hemorrhage. It is caused by genetic mutations in one of the three genes, CCM1, CCM2 and CCM3 (Bergametti et al., 2005). Mice with mutations in any of the three genes have vascular malfunctions similar to human CCM symptoms (Boulday et al., 2011). Interestingly, CCM3 mutation in glia cells was found to contribute to the disease, suggesting that normal neural signaling/neurovascular interaction is critical for vascular functions (Louvi et al., 2011). Neural specific removal of CCM3 using *gfap-* and *emx1-cre* shows vascular phenotypes characterized by disorganized and dilated vessels and other lesions similar to CCM. This highlights the importance of non-cell-autonomous effects in the progression of vascular diseases. Despite RNA sequencing data revealing changes in genes for cytoskeleton remodeling, detailed molecular mechanisms, however, still remain unknown. Recently, deregulated endothelial-to-mesenchymal transition (EndMT) was also found to contribute to the progression of CCM pathogenesis (Maddaluno et al., 2013). Endothelial cell specific deletion of CCM1 gene causes CCM-like vascular abnormalities in mice, concomitant with a switch from VE-Cadherin to N-Cadherin, a hallmark for EndMT. Further experiments demonstrated that CCM1-deficient endothelial cells isolated from the brain, but not from the lung, show upregulation of EndMT markers. This suggests that CCM1 is required to suppress EndMT within brain tissues, which raises the possibility that brain tissues may intrinsically promote EndMT.

### Angiogenesis in glioblastoma

Glioblastoma (GBM), the most common brain tumor, remains one of the most deadly human cancers. Current therapy only ameliorates GBM modestly and thus much more needs to be understood for developing new drugs. The emerging concept of tumor stem cells offers a new avenue both for understanding and controlling cancers including GBM (Clarke and Fuller, 2006). Brain tumor stem cells (BTSCs) are a subtype of tumor cells with higher propagation capacity, partly due to strong angiogenic ability (Bao et al., 2006). Indeed, BTSCs reside in a perivascular niche primarily consists of endothelial cells (Calabrese et al., 2007). *In vitro*, BTSCs can physically interact with endothelial cells, suggesting that cross talks between these two cells types are important for the tumor microenvironment. Endothelial cells maintain BTSC self-renewal and promote tumor propagation capacity *in vivo*. Given the importance of vascular contribution to BTSC biology, it is an attractive strategy to target blood vessels for brain tumor intervention. Although BTSCs are different from neural stem or progenitor cells, they share fundamental

properties so that knowledge in the stem cell field may shed lights on BTSCs. Since BTSCs are characterized by a strong drive for inducing angiogenesis, they are reminiscent of neural progenitor cells that produce different angiogenic factors for recruiting blood vessels during embryogenesis (as discussed in the first part of this review). Like neural progenitor cells, BTSCs secrete VEGF to stimulate angiogenesis (Bao et al., 2006). It was found that stem cell-like glioma cells have elevated VEGF levels that are further increased by hypoxia. On the other hand, VEGF-neutralizing or VEGFR-2 blocking antibodies added to BTSC conditioned medium inhibit endothelial cell proliferation and vascular tube formation, strongly suggesting a role of BTSC-produced VEGF in tumor blood vessel regulation. Stromal-derived factor-1 (SDF-1) or CXCL12 is another angiogenic factor produced by both BTSCs and endothelial cells. It was shown that CXCL12 can activate its receptor CXCR4 in glioma and increase the expression of VEGF (Yang et al., 2005). This autocrine/paracrine loop has not been observed between neural progenitor cells and blood vessels, suggesting that BTSCs have distinct properties that drive angiogenesis. TGF $\beta$ , as an important regulator of normal angiogenesis, is also expressed in glioma cells including those with BTSC features (Qiu et al., 2011). Glioma cell conditioned medium has been found to promote endothelial cell tube formation in a TGF $\beta$  dependent manner (Pen et al., 2008), suggesting that tumor-produced TGF $\beta$  may play a role in angiogenesis. Interestingly,  $\beta$ 8 integrin has also been found to control GBM angiogenesis and invasiveness (Tchaicha et al., 2011). Manipulation of  $\beta$ 8 integrin protein levels within glioma changed their angiogenic and invasive properties, concomitant with changes in latent TGF $\beta$  activation. For example, overexpression of  $\beta$ 8 in gliomas that normally have low levels of  $\beta$ 8 reduced hemorrhage around the tumor *in vivo*, suggesting a similar role of this integrin in regulating blood vessel stabilization as during normal brain development. Further studies of how neural cells, especially neural progenitor cells, signal to vascular cells may reveal more similarities between normal brain and BTSC-induced angiogenesis and may facilitate efforts at controlling brain tumor.

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## Compliance with ethics guidelines

Shang Ma and Zhen Huang declare that they have no conflict of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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