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Defective vascular signaling & prospective therapeutic targets in brain arteriovenous malformations

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ABSTRACT

The neurovascular unit is composed of endothelial cells, vascular smooth muscle cells, pericytes, astrocytes and neurons. Through tightly regulated multi-directional cell signaling, the neurovascular unit is responsible for the numerous functionalities of the cerebrovasculature – including the regulation of molecular and cellular transport across the blood-brain barrier, angiogenesis, blood flow responses to brain activation and neuroinflammation. Historically, the study of the brain vasculature focused on endothelial cells; however, recent work has demonstrated that pericytes and vascular smooth muscle cells – collectively known as mural cells – play critical roles in many of these functions. Given this emerging data, a more complete mechanistic understanding of the cellular basis of brain vascular malformations is needed. In this review, we examine the integrated functions and signaling within the neurovascular unit necessary for normal cerebrovascular structure and function. We then describe the role of aberrant cell signaling within the neurovascular unit in brain arteriovenous malformations and identify how these pathways may be targeted therapeutically to eradicate or stabilize these lesions.

1. Introduction

Neuronal function requires a constant supply of oxygen, glucose and other nutrients to meet metabolic needs, while ensuring clearance of toxic metabolic waste products. Neuronal metabolic demands are not static, but rather change as a result of functional activation and synaptic transmission on a cellular level. Complex neurologic tasks – such as speech, movement or sensation – requires activation and coordination of millions or possibly billions of cells in vast integrative networks (Herculano-Houzel, 2009). As a result, the brain utilizes roughly 20% of the body's oxygen and glucose, and disruption of blood flow may have profound effects on neuronal function and viability within minutes (Iadecola, 2017; Kisler et al., 2017a; Sweeney et al., 2018a, 2018b).

To meet this need, a vast integrated networking of tapering and branching blood vessels has evolved. The large muscular arteries which give rise to the cerebrovasculature arise off the aortic arch and form an anastomotic circle in the subarachnoid space along the base of the brain – known as the Circle of Willis (Menshawi et al., 2015). The large muscular arteries then progressively taper and form pial arteries along the brain surface. These arteries in turn dive into the brain parenchyma as penetrating arteries which progressively arborize to give rise to arterioles and ultimately the expansive capillary network tasked with molecular exchange between blood and brain (Iadecola, 2017; Zhao et al., 2015). The brain is a densely vascular organ; estimates have suggested that the capillary-to-neuron ratio is nearly 1:1, and neurons rarely exceed a distance of 15 μm from an adjacent capillary (Tsai et al., 2009). Capillaries coalesce into postcapillary venules, which further coalesce to form veins that ultimately drain into the venous sinuses. The venous sinuses in turn allow egress of blood out of the cranium back into the systemic circulation for re-oxygenation and filtration or removal of metabolic waste products (Kilic and Akakin, 2008).

This vast vascular network, however, is not a series of passive conduits. Rather, multicellular processes, such as sprouting angiogenesis and neurovascular coupling, allow dynamic remodeling of vascular structure and the functional allocation of blood flow to meet ever changing neuronal metabolic needs (Iadecola, 2017; Kisler et al., 2017a; Sweeney et al., 2018a, 2018b). These processes rely on coordinated signaling through multiple interconnected cell types. Cerebral blood vessels are anatomically comprised of endothelial cells, vascular smooth muscle cells, and pericytes, which are embedded in an

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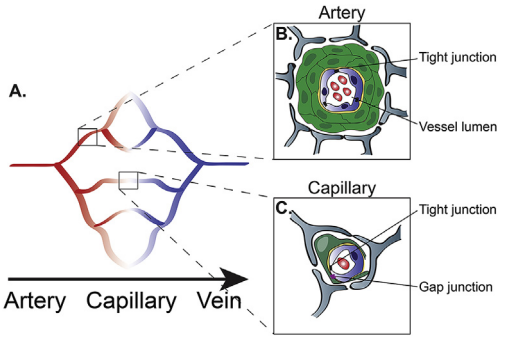

Fig. 1. Cells composition of the cerebrovasculature along the arterial-venous axis. (A) Extensive arborization of arteries and arterioles (red) gives rise to a dense capillary network (light gray) – the principal site of molecular exchange between circulating blood and brain. Capillaries coalesce to form venules which converge to form veins (blue). Large venous sinus drain into the systemic circulation for clearance of toxic metabolic waste products and re-oxygenation of circulating blood cells. Arrow, direction of blood flow. (**B**) Cartoon showing the cellular composition of a cerebral artery in cross-section. Endothelial cells (blue) are embedded within a protein rich basement membrane (yellow) and form a continuous lining along the lumen which contains circulating blood plasma and cells, such as erythrocytes (red). Endothelial cells are connected to one another by tight junctions (black rectangles). This prevents leakage of circulating cells or plasma-derived proteins into the brain. In arteries, concentric rings of vascular smooth muscle (green) and then astrocyte end-feet (gray) surround the endothelium. The perivascular space between astrocyte end-feet and vascular smooth muscle is called the Virchow-Robin space and is the site of the "glymphatic" clearance pathway. (**C**) Cartoon showing the cellular composition of a cerebral capillary in cross section. Endothelial cells (blue) form a continuous lining in and are connected by tight junctions (black rectangle). Unlike arteries, the vessel wall is comprised by pericytes (green) embedded in a common vascular basement membrane (yellow). Pericytes consist of a prominent cell body and extend finger-like processes wrapping around 70–90% of the endothelial surface area. At discrete points which are devoid of basement membrane – known as "peg-and-socket contacts" – connexin gap junctions (purple) electrically couple endothelial cells and pericytes. Pericytes are ensheathed by astrocyte endfeet (gray) which form a permeable barrier with brain perivascular spaces.

extensive protein-rich extracellular matrix known as the vascular basement membrane (Winkler et al., 2011, 2014) (Fig. 1). Functionally, vascular cells are in tight juxtaposition and in constant communication with neurons, astrocytes, and inflammatory cells (microglia, perivascular macrophages and blood-borne leukocytes) (Abbott et al., 2006; Iadecola, 2017; Ransohoff, 2016). This has led to the coining of the term "neurovascular unit" to emphasize the functional interdependence between cell types (Iadecola, 2017; Zlokovic, 2005). In the ensuing subsections, the cellular components of the vasculature and key molecular pathways are summarized to provide a mechanistic understanding of distinct cerebrovascular functionalities. Thereafter, we describe how disruptions in normal cellular function contribute to formation of brain arteriovenous malformations and identify pathways for future therapeutic drug development.

2. Cells of the neurovascular unit

2.1. Endothelial cells

Brain endothelial cells form a one cell thick lining of the vascular lumen, serving as the vital interface between blood and brain known as the blood-brain barrier (BBB) (Sweeney et al., 2018b; Zlokovic, 2008). Unlike systemic vessels, the endothelial membrane is continuous and without interruption with rare exception – such as discrete circumventricular organs (Kaur and Ling, 2017). Brain endothelial cells are connected through tight and adherens junctional protein complexes, which limit unregulated paracellular influx of blood-borne circulating molecules and cells. The endothelium also lacks fenestrae and has infrequent pinocytosis and bulk-flow vesicular transport, thus limiting unregulated transendothelial transport. As a result, entrance of large, non-lipophilic molecules (> 40 Da) and blood-borne cells is tightly regulated through specific transport systems (Sweeney et al., 2018b; Winkler et al., 2011, 2014; Zlokovic, 2008). The endothelium also senses sheer stress from circulating blood and molecular cues from adjacent neurons and glia which stimulates secretion of vasoactive molecules such as nitrous oxide, prostanoids and endothelin-1 to help initiate and/or propagate vasomotor responses in adjacent mural cells (Iadecola, 2017). Studies utilizing single cell sequencing have demonstrated a subspecialized continuum within the endothelium along the arteriovenous axis, which allows endothelial cells to accomplish these diverse functions (Vanlandewijck et al., 2018).

2.2. Pericytes

Single cell transcriptomics has also confirmed the presence of two distinct mural populations – pericytes and vascular smooth muscle cells (Vanlandewijck et al., 2018) (Fig. 1). Subpopulations within each cell population have also been described (He et al., 2016). Pericytes are confined to capillaries, venules and rarely terminal arterioles along the arterial-venous axis depending on the species (Armulik et al., 2011; Sweeney et al., 2016; Winkler et al., 2011). Pericytes are embedded within a vascular basement membrane that is shared with the adjacent endothelium. The vascular basement membrane serves as an important structural scaffolding facilitating endothelial, pericyte and astrocyte interactions and is predominately comprised of laminin, collagen IV, nidogen and heparin proteoglycans (Thomsen et al., 2017). Fibronectin-rich adhesion plaques anchor pericytes with the vascular basement membrane (Diaz-Flores et al., 2009).

Pericytes are comprised of a cell body with prominent nucleus and minimal cytoplasm from which stellate finger-like cell processes extend and cover roughly 70%–90% of the endothelial cell membrane (Armulik et al., 2010; Bell et al., 2010, 2012; Daneman et al., 2010; Sagare et al., 2013; Winkler et al., 2012, 2018). In discrete areas, pericytes directly contact the brain-facing endothelial plasma membrane and form "peg-and-socket" cell-to-cell contacts (Winkler et al., 2011, 2014). These points of contact contain gap junctions comprised of connexin-43 (Ivanova et al., 2017). Pericytes may therefore communicate through both direct electrical coupling or local paracrine signaling cascades with the endothelium. Astrocyte end feet also directly contact pericytes, though the release of secreted factors appears to be the primary form of astrocyte-pericyte signaling (Sweeney et al., 2016; Yao et al., 2014).

Unlike peripheral organs, pericytes are more abundant in the central nervous system, a fact which likely reflects their functional importance – including regulatory roles in the blood-brain barrier, angiogenesis, cerebral blood flow, and neuroinflammation (Sweeney et al., 2016; Winkler et al., 2014). In pathologic conditions, such as hypoxia or ischemia, a growing body of evidence suggests that pericytes may also serve as multipotent stem cells (Dore-Duffy et al., 2006; Esen et al., 2016; Nakagomi et al., 2015; Tatebayashi et al., 2017). Histochemically, pericytes may be identified through a number of cell surface or other antigens including chondroitin sulfate proteoglycan (NG2), platelet-derived growth factor receptor-β (PDGFRβ), N aminopeptidase (CD13), regulator of G-protein signaling-5 (RGS5), cell surface glycoprotein MUC18 (CD146)), vitronectin and interferen-induced transmembrane protein 1 (He et al., 2016; Sweeney et al., 2016).

2.3. Vascular smooth muscle cells

Vascular smooth muscle cells are the predominant cellular constituent of the vessel wall in arteries and veins (Fig. 1). In larger arteries traveling within the subarachnoid space or along the pia, vascular smooth muscle cells form concentric rings that are several cells thick

and are separated from the endothelium by the elastic lamina (Iadecola, 2017). As the arteries penetrate and course distally through the brain parenchyma, vascular smooth muscle cells gradually become less numerous, no longer forming concentric rings. At this level, connexin-based gap junctions directly couple vascular smooth muscle cells with adjacent endothelial cells (Figueroa and Duling, 2009). Vascular smooth muscle cells express receptors to numerous vasoactive molecules including adenosine, prostaglandins or catecholamines, and myogenic or flow-related stimuli, such as stretch activated cation channels (TRPC6, TRPM4) (Kisler et al., 2017a; Koller and Toth, 2012). Activation of many of these receptors leads to influx of extracellular calcium through voltage-gated ion channels in the plasma membrane and/or release of intracellular stores resulting in actin-myosin mediated contraction (Iadecola, 2017; Kisler et al., 2017a; Koller and Toth, 2012), and vascular smooth muscle cells are believed to principally serve as contractile cells regulating cerebral autoregulation and cerebral blood flow (Fernandez-Klett et al., 2010; Hill et al., 2015; Iadecola, 2017). Vascular smooth muscle cells also stabilize the vascular wall and help maintain the blood-brain barrier in larger arteries (Henshall et al., 2015). Other functions such as the phagocytosis of extracellular molecules have also been described (Bell et al., 2009). The relative contributions of vascular smooth muscle cells and pericytes to regulating blood flow responses to neuronal activation – a process known as "neurovascular coupling" - remains controversial (Hall et al., 2014; Hamilton et al., 2010; Hill et al., 2015; Kisler et al., 2017b; Winkler et al., 2017).

2.4. Astrocytes

Once within the brain parenchyma, the cells of the vascular wall are further ensheathed by astrocyte endfeet which form a permeable membrane – the glial limitans (Fig. 1). The perivascular space confined within the glial limitans – called the Virchow-Robin space – contains cerebrospinal fluid (CSF) which is circulated between the subarachnoid space and brain interstitial fluid (ISF) as a result of arterial pulsations. This represents an important clearance pathway for toxic metabolic byproducts in the brain known as the "glymphatic pathway" (Louveau et al., 2017; Rasmussen et al., 2018). Expression of transmembrane pores (e.g., aquaporin-4) or ionic pumps (e.g., the Kir4.1 potassium channel) within astrocyte end feet help to regulate glymphatic transport and the ionic composition of ISF, respectively (Abbott et al., 2006; Iliff et al., 2012). Astrocytes also express metabotropic glutamate receptors (mGluR) and purinergic receptors (P2YR) which detect glutamate or adenosine triphosphate released from activated neurons. Activation of these receptors leads to intracellular calcium currents via inositol triphosphate (IP3) signaling within astrocytes, thereby promoting secretion of vasoactive molecules including arachidonic acid, associated derivatives and prostaglandin E2, which act on adjacent vascular smooth muscle cells and pericytes in arteries, arterioles or capillaries (Abbott et al., 2006; Iadecola, 2017; Kisler et al., 2017a). Astrocytes therefore help link neuronal activation with the contractile cells of the vasculature and fulfill an important role in neurovascular coupling. Astrocytes also help contribute to endothelial blood brain barrier properties and further stabilize the vasculature through secretion of basement membrane proteins, such as laminin (Abbott et al., 2006; Yao et al., 2014).

2.5. Other cells

Although this review highlights several of the predominant cell types, additional functionally important cell types are found within or interact with the cerebrovasculature. Recent works have identified a fibroblast-like cell within the Virchow-Robin space of all vessels with the exception of capillaries (Vanlandewijck et al., 2018). The functional significance of these cells has yet to be defined, but they are thought to promote scar formation in response to injury. Perivascular macrophages and adjacent microglia play important roles in the phagocytosis of extracellular proteins, immune surveillance and neuroinflammatory responses (Park et al., 2017; Ransohoff, 2016). Adjacent neurons contribute to cerebral blood flow through initiation of signals either directly or through other cells, e.g., astrocytes, as described in ensuing subsections (Iadecola, 2017; Sweeney et al., 2018a).

3. Cerebrovascular function

3.1. Angiogenesis

Angiogenesis is the process of generating new blood vessels from pre-existing vessels and relies on coordinated paracrine cell signaling and direct contact between pericytes and endothelial cells. Two forms of angiogenesis have been proposed: endothelial sprouting and non-sprouting/intussusceptive, which is characterized by the splitting of pre-existing vessels by transcapillary pillars (Groppa et al., 2018; Risau, 1997). Here, we will focus on mechanisms of sprouting angiogenesis – the cellular cascade where endothelial cells migrate from the side of existing blood vessels by degrading local extracellular matrix to establish nascent vessels in surrounding tissue.

Trigged by local tissue hypoxia and secretion of vascular endothelial growth factor A (VEGF-A), endothelial tip cells spearhead new vessel sprouts by extending long filopodia that are responsive to environmental cues such as VEGF-A, and thus help direct the direction of sprout growth (Gerhardt et al., 2003; Jakobsson et al., 2010). Tip cells are followed closely by stalk cells; morphologically, stalk cells are characterized by a high rate of cell proliferation to establish adherens and tight junctions to stabilize existing vascular sprouts (Carmeliet and Jain, 2011; Gerhardt et al., 2003).

The crosstalk of the VEGF and Notch signaling pathways help regulate endothelial tip and stalk cell phenotypes (Carmeliet and Jain, 2011; Hellstrom et al., 2007). High levels of local VEGF-A binds to endothelial VEGF receptor 2 (VEGFR2), which differentially activates tip cells to guide migration and stalk cells to promote proliferation (Gerhardt et al., 2003). VEGF stimulation in tip cells induces nearby stalk cells to express transmembrane Delta-like ligand 4 (Dll4) (Hellstrom et al., 2007); this in turn may bind to Notch1 and Notch3 receptors to limit endothelial sprouting and promote vessel morphogenesis (Kofler et al., 2015). Notch signaling within vascular smooth muscle also regulates endothelial sensitivity to angiogenic stimulation (Yang and Proweller, 2011). By balancing the ratios of endothelial tip and stalk ratios, Notch and VEGF signaling regulate continued sprouting, branching and formation of vascular anastomoses (Blanco and Gerhardt, 2013; Carmeliet and Jain, 2011; Hellstrom et al., 2007). Genetic deletion of either VEGF-A or Dll4 results in embryonic lethality as a result of pathologic vascular development (Carmeliet et al., 1996).

Following endothelial sprout formation, secretion of platelet-derived growth factor B (PDGF-B) activates platelet-derived growth factor receptor β on pericytes and vascular smooth muscle cells promoting their proliferation and recruitment to the nascent endothelial tube (Enge et al., 2002; Hellstrom et al., 1999; Lindahl et al., 1997). Mural cells express VEGF receptor 1 (VEGFR1), which helps spatially restrict VEGF signaling and helps to promote endothelial sprouting (Eilken et al., 2017). Once activated, these pericytes project into the perivascular space, disrupting their basement membranes (Diaz-Flores et al., 1992) and appear to guide endothelial cells as they sprout into newly formed vessels (Nehls and Drenckhahn, 1993). The matrix metalloproteinases MMP-2 (Virgintino et al., 2007), MMP-3, and MMP-9 (Candelario-Jalil et al., 2009) degrade nearby extracellular matrix during this step to facilitate the mechanical migration of endothelial cells. NCK1 and NCK2, a family of adaptor proteins, have also been shown to be selectively required for PDGF-B induced pericytes migration to sprouting endothelial cells (Dubrac et al., 2018). Pericytes help promote and maintain endothelial cell survival through induction of autocrine VEGFA within endothelium (Franco et al., 2011), and a loss

of pericytes has been associated with endothelial apoptosis in some models (Bell et al., 2010; Kisler et al., 2017b).

Pericytes, however, have been shown to have context-specific effects on endothelium and angiogenesis (Winkler et al., 2011, 2014). In the adult brain and in select tumors, pericytes promote endothelial cell survival through secretion of VEGF-A (Darland et al., 2003), whereas a loss of pericytes contributes to endothelial cell death (Bell et al., 2010; Song et al., 2005). Pericyte loss at other times, such as during embryonic development, leads to endothelial hyperplasia and proliferation (Hellstrom et al., 2001).

3.2. Vascular stabilization

Extracellular proteins and cells of the vascular wall help stabilize an otherwise fragile endothelial cell tube, such as pericytes and vascular smooth muscle cells. Coordinated signaling from pericytes, vascular smooth muscle cells and astrocytes induce endothelial differentiation and helps stabilize the vascular wall through multiple signaling pathways –including transforming growth factor β (Dave et al., 2018; Diniz et al., 2018; Li et al., 2011), angiopoietin 1 (Suri et al., 1996; Uemura et al., 2002), Notch (Henshall et al., 2015; Li et al., 2011), and Wnt/β-catenin (Cho et al., 2017; Wang et al., 2018). Pericytes, vascular smooth muscle cells and astrocytes synthesize vascular basement membrane proteins which stabilize the vasculature and provide an adhesive scaffolding promoting cell adhesion (Chasseigneaux et al., 2018; Chen et al., 2013b; Gautam et al., 2016; Stratman et al., 2009; Yao et al., 2014). Abnormalities in basement membrane extracellular matrix proteins and/or adhesion proteins, e.g., integrins or N-cadherin, responsible for connecting mural cells to the extracellular matrix are associated with intracerebral hemorrhage (Arnold et al., 2014; Chen et al., 2013b). A loss of brain pericytes is frequently associated with tortuous, ectatic vasculature with associated microaneurysms in small blood vessels (Armulik et al., 2010; Bell et al., 2010; Daneman et al., 2010; Lindahl et al., 1997; Winkler et al., 2011). Degeneration of vascular smooth muscle cells is associated with ectasia and aneurysm formation in large muscular arteries of the Circle of Willis and comparatively smaller pial arteries (Chalouhi et al., 2012; Henshall et al., 2015; Wang et al., 2014).

3.3. Blood-brain barrier

Endothelial vascular barrier properties are dependent on strong, uninterrupted tight and adherens junctional protein complexes (Sweeney et al., 2018a; Zlokovic, 2008).

Junctional complexes and sparse pinocytosis limit unregulated influx of molecules and cells from circulating blood into brain via paracellular or transcellular routes, respectively. Transport proteins, adhesion molecules and transmembrane cell signaling receptors are asymmetrically distributed on the blood- or brain-facing endothelial plasma membrane, which facilitates transport across the blood-brain barrier. For example, carrier mediated transporters allow diffusion of many nutrients, including carbohydrates, amino acids, vitamins, and fatty acids, down concentration gradients into the brain (Sweeney et al., 2018b; Zlokovic, 2008). Other carrier mediated transporters remove excitatory amino acids, such as glutamate and aspartate, or metabolic by-products, such as lactate, from the brain into the circulating plasma (Cohen-Kashi-Malina et al., 2012; Knudsen et al., 1991). Larger peptides are transported into or out of the brain through a multitude of receptor-mediated transport systems (Sweeney et al., 2018a, 2018b). The brain-facing or abluminal endothelial membrane also expresses numerous ionic pumps – such as the $Na+/K$ + ATPase – which contribute to regulating the ionic composition of the brain ISF (Mokgokong et al., 2014).

Through expression of chemokines or adhesion molecules, such as p-selectin, vascular cell adhesion molecule 1 or intercellular adhesion molecule 1, the endothelium also modulates entrance of circulating inflammatory cells into the brain following injury (Takeshita and Ransohoff, 2012). Subspecialization of the endothelium at a single cell level facilitates the molecular and cellular trafficking across the blood-brain barrier. That expression of transporters is most prominent in smaller capillaries and venules which serve as the principal site of molecular exchange given their immense surface area and comparatively thin vascular wall. Endothelial transcription factors and/or adhesion molecules alternatively demonstrate enrichment in arteries or veins, respectively (Vanlandewijck et al., 2018).

The blood-brain barrier is functionally organized in endothelial cells. However, both induction and maintenance of the blood-brain barrier require cell signaling from adjacent pericytes, vascular smooth muscle cells and astrocytes. In smaller vessels, pericytes suppress a pro-inflammatory, leaky phenotype of brain endothelial cells during embryonic development prior to appearance of astrocytes. This is accomplished through down-regulation of pro-permeability factors which promote non-specific transcytotic pathways, such as caveolin-1 or plasmalemma vesicle-associated protein, and inflammatory adhesion molecules, such as intercellular adhesion molecule 1 or activated leukocyte adhesion molecule (Daneman et al., 2010; Hellstrom et al., 2001). Throughout the postnatal period and adulthood, pericytes further maintain the endothelial blood-brain barrier (Armulik et al., 2010; Bell et al., 2010; Winkler et al., 2012). In pericyte deficient rodents, a loss of brain pericytes results in disruption of endothelial tight junctional proteins zonula occludens-1 and claudin 5 and the adherens junctional protein vascular endothelial cadherin, resulting in non-specific paracellular influx of circulating solutes and plasma-proteins into the brain (Bell et al., 2010; Winkler et al., 2012). In other pericyte deficient rodents, a loss of pericytes leads to increased pinocytosis and activation of transcytotic cascades (Armulik et al., 2010).

Comparatively less is known about the role of the vascular smooth muscle cells in the induction and maintenance of the blood-brain barrier in larger arteries. Degeneration of arterial vascular smooth muscle cells through deletion of *Notch3* are associated with breakdown of the blood-brain barrier and leakage of either exogenous vascular tracers or circulating plasma-derived proteins (Henshall et al., 2015; Wang et al., 2014). Despite widespread vascular smooth muscle degeneration, the blood-brain barrier breakdown is focal and much less frequent. Tight junctions between adjacent endothelial cells appear intact, and in the context of frequent aneurysms it is hypothesized that the breakdown occurs from local rupture of the arterial wall (Henshall et al., 2015; Wang et al., 2014). However, the underlying mechanisms have yet to be fully characterized and the generation of pericyte- or vascular smooth muscle specific animal models are needed to better delineate relative contributions of mural cell populations along the arterial-venous axis in the central nervous system.

3.4. Cerebral blood flow regulation

Cerebral blood flow is tightly coupled to brain activation to meet constantly changing neuronal metabolic needs. This is achieved through tightly regulated communication between neurons, astrocytes, pericytes and vascular smooth muscle cells. Secretion of neuronal glutamate was believed to activate the metabotropic glutamate receptor 5 (mGluR5) on astrocytes activating IP3 (Mulligan and MacVicar, 2004; Zonta et al., 2003). This in turn releases intracellular $Ca2+$ stores to activate phospholipase A2 and generate downstream arachidonic acid derivatives to evoke vasodilation (Stephenson et al., 1994). However, it was noted that mGluR5 expression is down-regulated in astrocytes of adult animal models (Sun et al., 2013). Recent experiments in adolescent rat cortical slices show that ATP-gated channels increase astrocytic calcium ultimately generating arachidonic acid derivatives through phospholipase D2 and diacylglycerol lipase instead of phospholipase A2. Astrocytic secretion of arachidonic acid derivatives then acts on adjacent pericytes, but not vascular smooth muscle cells, to evoke vasomotor responses (Mishra et al., 2016). Vasomotor responses of

vascular smooth muscle in brain arterioles is controlled by an astrocyte-independent pathway. NMDA receptor activation and calcium influx triggers interneurons to directly release nitrous oxide, which diffuses to adjacent vascular smooth muscle cells promoting relaxation (Mishra et al., 2016). Differential molecular regulation may therefore occur along the arterial-venous axis with respect to alterations in blood flow to neuronal activity.

The relative contributions of brain contractile cells to neurovascular coupling, and whether this occurs within arteries, arterioles or at a capillary level remains controversial. Both pericytes and vascular smooth muscle cells express contractile proteins and receptors for vasoactive molecules – such as endothelin-1, vasopressin and catecholamines (Diaz-Flores et al., 2009; Hamilton et al., 2010; Kisler et al., 2017b; Koller and Toth, 2012; Winkler et al., 2017). Pericytes have been demonstrated to contribute to resting cerebral blood flow (Bell et al., 2010; Winkler et al., 2017), and to alter capillary diameter in response to endogenous neuron- or astrocyte-derived cues or in pathological states, such as brain ischemia (Hall et al., 2014; Peppiatt et al., 2006). Pericyte-mediated vasodilation has been shown to precede arteriolar relaxation in live rodents in response to brain activation, and that a loss of brain pericytes leads to neurovascular uncoupling, hypoxia and neuronal metabolic stress (Kisler et al., 2017b). Others, however, have failed to show pericyte contraction *in vivo* or casted doubt about the functional significance of pericyte contractions (Fernandez-Klett et al., 2010; Hill et al., 2015), and controversy persists.

3.5. Brain arteriovenous malformations

Brain arteriovenous malformations (bAVMs) are a collection of dysplastic, ectatic blood vessels – also known as the "nidus" – which is formed by a direct connection between feeding arteries and draining veins without an intervening capillary bed (Lawton et al., 2015). Whether this is the result of abnormal sprouting angiogenesis and direct arterial-venous connection or progressive dilation of the capillary bed remains controversial. Functionally this forms a fragile, high flow shunt between the arterial and venous systems in the brain which is prone to rupture and may result in intracerebral hemorrhage. bAVMs are estimated to have a prevalence of 10–18 per 100,000 people, and roughly 2–4% annual risk of hemorrhage (Al-Shahi and Warlow, 2001; Rangel-Castilla et al., 2014; Stapf et al., 2003). With rupture, rates of significant neurologic disability and mortality are 20–30% and 10%, respectively (Rangel-Castilla et al., 2014), and in select populations, such as pediatric patients (ages $<$ 18 years old), AVMs are the leading cause of intracerebral hemorrhage (Lo et al., 2008).

Insights into the cellular and molecular mechanisms of bAVM pathogenesis have come both from models of known genetic syndromes associated with bAVMs – such as hereditary hemorrhagic telangiectasia (HHT) and capillary malformation-arteriovenous malformation syndrome (CAMS) (Walcott et al., 2016). In the ensuing subsections, we will describe cellular and functional abnormalities of the neurovascular unit in AVMs. We will then describe the genetic and molecular pathways thought to contribute to bAVM formation in available rodent models, and how these pathways may represent novel therapeutic targets. Finally, we will conclude with avenues of ongoing future research designed to better understand the biology and guide treatment of these potentially debilitating vascular malformations.

4. Aberrant cytoarchitecture

4.1. Endothelial cells

To date, most bAVM research has focused on the endothelium. On histologic evaluation, significant endothelial heterogeneity has been described (Fig. 2). Endothelial cells can be either single- or multi-layered, and frequently display a hyperactive, immature phenotype

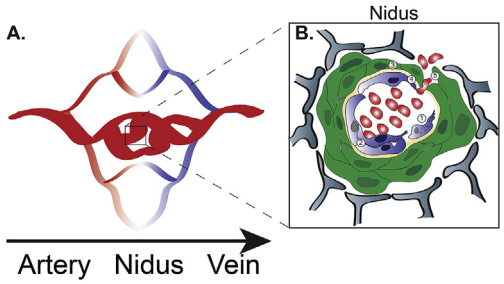

Fig. 2. Alterations in cerebrovascular structure and cytoarchitecture in brain arteriovenous malformations. (A) A direct connection between arterial and venous circulations without intervening capillary bed forms the arteriovenous malformation. This high flow shunt manifests as a tangle of dysplastic and tortuous vessels – called the nidus (bright red). Shunting of arterial blood directly into the venous system results in higher venous pressures. Affected veins appear "arterialized" and are more red in coloration than adjacent veins (blue). Arrow, direction of blood flow. (**B**) Cartoon showing the cellular composition of a nidal blood vessel in cross section. Heterogeneity of the endothelium (blue) is observed: (1) endothelial degeneration; and (2) endothelial hyperplasia. (3) Vascular smooth muscle cells (green) are reduced. Remaining cells show alterations in both cytoskeletal and contractile elements. (4) Alterations in vascular basement membrane proteins (yellow), such as perlecan, contribute to overproduction of angiogenic factors and vessel fragility. (5) Loss or alteration of structural components of the vascular wall and disruption of endothelial tight junctions ultimately result in blood-brain barrier breakdown, microhemorrhage or larger intracerebral hemorrhage.

with filopodia, cytoplasmic vesicles and vacuolization (Tu et al., 2006b). Even within the same blood vessel, however, adjacent segments may be characterized by endothelial hypoactivity and microvascular collapse or endothelial hyperplasia (Tu et al., 2006b, 2010). Barrier properties also vary. Some regions of the nidus are lined with endothelial cells connected via intact tight junctional protein complexes (Gault et al., 2004; Wong et al., 2000). Other regions may have multiple points of discontinuity in the endothelial lining – including disruption of tight junctional complexes or the presence of fenestrae (Tu et al., 2010). Disruption of the blood-brain barrier is well documented (Chen et al., 2013a; Winkler et al., 2018), and microhemorrhages are frequently observed in unruptured AVMs and may predict future rupture (Abla et al., 2015; Guo et al., 2012).

At a molecular level, endothelial cells express higher levels of a number of pro-angiogenic factors (Ferreira et al., 2014; Hashimoto et al., 2005; Jabbour et al., 2009; Koizumi et al., 2002), and as a result frequently assume a pro-angiogenic phenotype in bAVMs. Endothelial turnover and division is increased 5-to-7 fold in human bAVM specimens – a value intermediate between normal cerebral vessels and aggressive tumors (Hashimoto et al., 2001; Hatva et al., 1996; Sure et al., 2001). bAVM endothelial cells proliferate and migrate more rapidly and form aberrant vascular tubules *in vitro* (Ferreira et al., 2014; Jabbour et al., 2009; Stapleton et al., 2011; Wautier et al., 1999). Endothelial cells also lose their capacity to terminate proliferation when exposed to pro-inflammatory cytokines, such as interleukin 1β, tumor necrosis factor α, transforming growth factor-β and interferon- γ (Wautier et al., 1999).

The endothelium within a bAVM also assumes a pro-inflammatory phenotype. More specifically, endothelial cells upregulate the adhesion molecules intercellular adhesion-molecule-1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1) and E-selectin (Sammons et al., 2011; Storer et al., 2008). Endothelial cells have also demonstrated enhanced *in vitro* secretion of pro-inflammatory cytokines and chemokines, such as interleukin-8 (Jabbour et al., 2009). Together, upregulation of endothelial adhesion molecules, cytokines and blood-brain barrier breakdown facilitate the infiltration of circulating blood-derived inflammatory cells observed in bAVMs (Chen et al., 2008; Guo et al., 2014). Endothelial expression of adhesion molecules for platelets, such

as p-selectin or platelet adhesion molecule 1 (PECAM-1), or the pro-thrombotic factors thrombomodulin or tissue factor, are unchanged in bAVMs (Sammons et al., 2011; Storer et al., 2008). Therefore, endothelial cells do not appear to assume a pro-thrombotic phenotype in bAVMs.

4.2. Pericytes & vascular smooth muscle cells

Reductions in pericytes have now been described in both human bAVMs and rodent models (Chen et al., 2013a; Tu et al., 2006b; Winkler et al., 2018). Pericyte reductions are greatest in ruptured human AVMs (Winkler et al., 2018). In unruptured AVMs, the magnitude of pericyte loss correlates with the severity of blood-brain barrier disruption and microhemorrhage (Winkler et al., 2018). In bAVM rodents, a similar relationship has been described (Chen et al., 2013a), and therapeutic restoration of pericyte and vascular smooth muscle cell populations via lentiviral PDGF-B overexpression or treatment with thalidomide or related analogs decreases level of vessel dysplasia and hemorrhage suggesting a more direct causal role (Zhu et al., 2018a). Residual pericytes also have a number of histologic abnormalities – including greater abundance of pinocytotic vesicles, vacuoles and cytoskeletal filaments (Tu et al., 2006b).

Reductions in vascular smooth muscle cells in bAVMs have also been described (Fig. 2)(Chen et al., 2013a; Zhu et al., 2018a). Overexpression of micro RNA (miRNA) signatures which are up-regulated in bAVMs – such as miRNA-137 and miRNA-195 – has been shown to impair vascular smooth muscle cell migration and survival *in vitro* (Huang et al., 2017). Even so, the rate of vascular smooth muscle turnover in human and rodent models has yet to be fully characterized. Alterations in cytoskeleton proteins, such as actin and collagen, and contractile proteins, such as smoothelin, have also been described (Kim et al., 2018; Uranishi et al., 2001a; Wong et al., 2000). In rodent models, microhemorrhage coincides with blood vessels lacking β-actin positive smooth muscle cells (Chen et al., 2013a).

5. Molecular pathways implicated in brain arteriovenous malformations

Numerous changes in gene expression have been described within bAVMs (Hashimoto et al., 2004; Shenkar et al., 2003). Less than ~5% of arteriovenous malformations are associated with autosomal dominant disorders – such as such as hereditary hemorrhagic telangiectasia (HHT) and capillary malformation-arteriovenous malformation syndrome (CAMS) (Walcott et al., 2016). Generation of rodent models of these genetic syndromes or alterations in other molecular pathways implicated in bAVMs have begun to shed light on mechanisms which contribute to bAVM pathogenesis. Several of these studies have suggested that a secondary insult which activates brain angiogenesis, such as overexpression of VEGF or wound healing, is required in addition to a genetic deficit in endothelial cells, pericytes or vascular smooth muscle, to promote bAVM formation *in vivo* (Chen et al., 2013a; Choi et al., 2014; Milton et al., 2012; Walker et al., 2011). However, whether similar molecular pathways contribute to more prevalent "sporadic" bAVMs which account for > 95% of bAVMs remains to be seen.

Two hypothesized mechanisms for bAVM formation have been postulated: 1) abnormal sprouting angiogenesis leading to an anomalous direct arterial-venous connection; and (2) the progressive dilation of existing capillary beds resulting in high flow shunting from arterial to venous circulations. Both mechanisms have been described and appear specific to the rodent model being studied (Murphy et al., 2014; Park et al., 2009; Walker et al., 2011). Below, we summarize the transforming growth factor-β (TGF-β), Notch, and VEGF pathways. We also summarize emerging research geared towards a postulated genetic source for lesions once thought to be "sporadic" and without underlying genetic mutation.

6. *Transforming growth factor receptor beta (TGF-β)*

Transforming growth factor beta (TGF-β) is a multifunctional cytokine which has multiple effects on brain vascular development implicated in vascular malformations – including both bAVMs and cavernous malformations (Cunha et al., 2017; Gaengel et al., 2009; Sweeney et al., 2016). Latent TGF-β is secreted by endothelial cells, pericytes, neurons and astrocytes, and is activated by thrombospondin or integrins in the extracellular space (Lebrin et al., 2005). Activated TGF-β binds to a type 2 TGF-β receptor, such as TGFβR2, which then recruits and activates up to seven possible type I TGF-β receptors, collectively referred to as activin-like kinase 1–7 (ALK1-7) (Cunha et al., 2017). Depending on the type I receptor activated, different Smad-signaling protein complexes are recruited. For example, activation of ALK1 recruits and phosphorylates Smad 1/5/8 whereas activation of ALK5 recruits and phosphorylates Smad 2/3 in endothelium. The phosphorylated Smad complex then binds Smad4 and translocates to the nucleus to alter transcription of target genes. In this example, the ALK1-Smad1/5/8 and ALK5-Smad 2/3 pathways result in endothelial proliferation and migration or quiescence, maturation and formation of barrier properties, respectively (Cunha et al., 2017; Goumans et al., 2002; Lebrin et al., 2005; Winkler et al., 2011). Signal transduction and cellular responses may also be further modulated through the recruitment of additional accessory receptors, such as endoglin (Sugden and Siekmann, 2018).

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant disorder which results from mutations in endoglin (*ENG*), activin-like kinase 1 (*ACVRL1; Alk1* in rodents) or *SMAD4* (Walcott et al., 2016). HHT results in capillary telangiectasias along mucosal surfaces which are prone to bleeding, such as nose or gastrointestinal bleeds. bAVMs are also found in a subset of HHT patients, with a reported incidence of 5–43% (Bharatha et al., 2012; Krings et al., 2015). Deletion of *Alk1* or *Eng* results in vascular malformations resembling human AVMs in rodents, but requires activation of angiogenesis through viral-mediated overexpression of VEGF in addition to genetic deficits (Chen et al., 2013a; Choi et al., 2012; Walker et al., 2011). Real-time imaging of AVM formation in other organs using *Alk1* deletion showed *de novo* formation of arterial-venous shunts through nascent vessel formation (Park et al., 2009). Additional studies have shown that endothelial deletion of *Alk1 or Eng* may result in bAVMs following induction of angiogenesis (Chen et al., 2014; Choi et al., 2014). Others have shown that deletion of *Eng* or *Alk1* from mural cells may also induce bAVMs that are prone to rupture (Choi et al., 2014; Milton et al., 2012), which raises the question as to which vascular cell is responsible for bAVM formation in these models (Chen et al., 2014). More recently, deletion of *Smad4* was shown to also result in spontaneous AVMs in brain and other organs with higher blood flow, such as the retina, without need for additional angiogenic activation (Kim et al., 2018; Ola et al., 2018).

In humans, other studies have suggested that polymorphisms in *ACVRL1* or *ENG* may be associated with heightened risk of sporadic AVMs (Pawlikowska et al., 2005; Simon et al., 2006). Significant up-regulation of TGF-β and receptors, such as endoglin, or alterations in extracellular matrix proteins, such as perlecan, which increase TGF-β levels have been demonstrated in bAVMs (Hashimoto et al., 2004; Kahle et al., 2012). More recently, whole exome sequencing has identified a novel *SMAD9* mutation associated with a recurrent, sporadic AVM. Oligonucleotide-mediated knockdown in developing zebrafish confirmed formation of brain arterial-venous shunts suggesting a causal role (Walcott, 2014). However, the role of TGF-β signaling in the formation of non-HHT, sporadic bAVMs remains to be defined.

6.1. Notch

Notch signaling is important for a multitude of functions implicated in initial brain vascular development and subsequent angiogenesis –

including arterial-venous differentiation (Gridley, 2007; Hill-Felberg et al., 2015; Proweller et al., 2007; Walchli et al., 2015). Notch is a transmembrane receptor protein which is activated by cell-bound ligands in adjacent cells via direct cell-cell interactions. This leads to cleavage of the Notch receptor by the γ-secretase complex and liberation of the activated Notch Intracellular domain (NICD) which may then translocate to the nucleus and evoke transcriptional changes in additional downstream effectors, such as Hes1 or Hey 1 (Bray, 2006; Takada et al., 2017; ZhuGe et al., 2009). Four distinct Notch receptors have been described in mammals (Notch1-Notch4) (Gridley, 2010). Notch receptors and ligands are differentially expressed in arteries and veins, with Notch1 and Notch4 expressed on arterial endothelial cells and Notch 2 and Notch3 expressed on venous endothelial cells (Gridley, 2010; Villa et al., 2001). Five different ligands have also been described – including Jagged-1, Jagged-2, and delta-like ligand (DLL) 1, 3 and 4 (Gridley, 2010). Notch signaling induces the formation of ephrin-B2 – a transmembrane ligand marker of arterial identity (Bray, 2006; Carlson et al., 2005; Krebs et al., 2004; Lawson et al., 2002). Conversely, EphB4 (the ephrin-B2 receptor) is expressed in veins but not arteries.

Recent works have suggested a role for Notch signaling in the formation of non-syndromic, sporadic bAVMs. Elevated expression of Notch1 and NICD and increased activity of the ligands Jagged-1 and DLL-4 have been described in human bAVM specimens (Murphy et al., 2009; ZhuGe et al., 2009, 2013). Increased expression of the downstream effector Hes1 has also been shown to be elevated in surgically resected bAVMs (Mouchtouris et al., 2015b). Other studies have shown that Notch1, but not Notch4, Jagged 1, and or Dll4, was overexpressed in ruptured AVMs when compared to unruptured cases (Li et al., 2014). However, others have failed to reproduce findings of Notch1 overexpression in bAVMs and rather demonstrated increased expression of Notch3 and Notch4 (Hill-Felberg et al., 2015). Notch4 gene polymorphisms have also been associated with human AVM formation and hemorrhage (Delev et al., 2017). Whether differences in the Notch receptor identified suggest that there are different molecular subgroups and/or other nuances with Notch receptor signaling in humans remains to be defined.

In rodents, constitutive activation of the Notch4 intracellular domain in young mice has led to the production of brain arteriovenous shunts (Carlson et al., 2005; Murphy et al., 2009, 2014). bAVMs formed in the setting of constitutive activation of the Notch4 intracellular domain regress after repression of the transgene, suggesting that Notch signaling may play a role in both induction and maintenance of bAVMs (Murphy et al., 2009, 2014). Inducible deletion of other Notch signaling components, such as the transcription factor recombining binding protein suppressor of hairless (*Rbpj*), also recapitulated brain arteriovenous shunting in the early postnatal period, but not in adults (Nielsen et al., 2014). Collectively, these data suggest that aberrant Notch signaling plays a contributory role in the formation of AVMs in brain and may represent a potential therapeutic target.

6.2. Vascular endothelial growth factor (VEGF)

As described above (see *Angiogenesis* subsection), vascular endothelial growth factor is a potent inducer of brain angiogenesis under both physiologic and pathologic conditions. Within bAVMs, ECs express higher levels of the angiogenic factors vascular endothelial growth factor and endothelin-1 (Ferreira et al., 2014; Hashimoto et al., 2005; Jabbour et al., 2009; Koizumi et al., 2002). VEGF is expressed in four isoforms – VEGF A, VEGF B, VEGF C and VEGF D. All isoforms are overexpressed with greatest levels of VEGF A noted in the majority of AVMs (Ferreira et al., 2014; Koizumi et al., 2002). VEGF C and D are overexpressed in AVMs with larger niduses (Koizumi et al., 2002), whereas VEGF A and D are the predominant isoforms expressed by bAVMs endothelial cells *in vitro* (Ferreira et al., 2014). Upregulation of VEGF signaling is greatest in ruptured AVMs (Weinsheimer et al., 2011). Increased expression of VEGF may in part be explained by a loss of the angiogenic inhibitor thrombospondin-1 (Ferreira et al., 2014; Stapleton et al., 2011) or alterations in the extracellular matrix protein perlecan (Clarke et al., 2012; Kahle et al., 2012). Expression levels of VEGF receptors (Flt-1 and Flk-1) are less clear, and conflicting reports suggest either upregulation or downregulation (Hashimoto et al., 2000; Jabbour et al., 2009; Uranishi et al., 2001b). Whether such alterations arise due to cell autonomous or non-cell autonomous mechanisms or as the result of alterations in hemodynamics or shear stress remains unclear. As evidenced in *Alk1* or *Eng* mutant rodents, heightened VEGF stimulation is required for formation of bAVMs and has attracted attention as a potential therapeutic target (Bray, 2006; Carlson et al., 2005; Krebs et al., 2004; Lawson et al., 2002).

6.3. Additional pathways

Unbiased genome wide association studies failed to identify single nucleotide polymorphisms which confer risk for development of non-syndromic bAVMs – so called “sporadic” AVMs (Weinsheimer et al., 2016). However, more targeted approaches have identified bAVM associated polymorphisms in multiple genes – including G-Protein Coupled Receptor 124, angiopoietin-like 4, or multiple inflammatory cytokines such as interleukin-6, interleukin-1α and interleukin-1β (Kim et al., 2009; Mikhak et al., 2011; Moftakhar et al., 2009; Mouchtouris et al., 2015a; Pollock et al., 2003; Weinsheimer et al., 2012). Others have identified genetic polymorphisms associated with bAVM hemorrhage, such as erythropoietin-producing hepatocellular receptor B4 (*EPHB4*) (Weinsheimer et al., 2009). Differential gene expression analysis with microarray has also shown upregulation in a multitude of additional angiogenic, extracellular matrix and apoptotic proteins (Hashimoto et al., 2004). Similar analysis has also shown that upregulation in MAPK, VEGF, Wnt and multiple inflammatory pathways is greatest in ruptured AVMs (Weinsheimer et al., 2011). How many of these pathways confer risk for bAVM formation remains to be determined.

Newer next generation sequencing technology has identified somatic activating mutations in the *KRAS* proto-oncogene in the majority of sporadic bAVMs suggesting there may be an underlying genetic cause (Nikolaev et al., 2018). Activating *KRAS* mutations led to activation of the mitogen activated protein kinase (MAPK) – extracellular signal-regulated (ERK) pathway in endothelium *in vitro*. This in turn lead to greater expression of multiple angiogenic pathways, Notch signaling and pathways regulating endothelial-to-mesenchymal transition, and ultimately enhanced endothelial migratory capacity (Nikolaev et al., 2018). With greater depth of sequencing, *KRAS* mutations were found in 81% and 100% of brain and spinal AVMs, respectively (Hong et al., 2019). Activating *BRAF* mutations were also identified in a small subgroup of AVMs (Hong et al., 2019). This raises questions as to whether distinct molecular subgroups as is observed for brain tumors are present for brain AVMs, and whether they may be therapeutically targeted with specific blood-brain barrier permeable inhibitors. Further characterization is also needed to determine whether dysregulated signaling as the result of activating mutations in *KRAS* influence TGF-β or other convergent pathways responsible for occurrence of bAVMs in genetic syndromes, such as HHT. Therefore, whether a common molecular pathway is shared between familial and sporadic bAVMs is presently unknown.

6.4. Prospective therapeutic targets in brain arteriovenous malformations

Multiple treatment options exist for AVMs – including surgical resection, stereotactic radiosurgery, endovascular embolization, or medical management of co-morbidities and observation (Lawton et al., 2015). Selection of the most appropriate treatment modality is influenced by a multitude of factors – including the size of the nidus, location and functionality of involved brain (so-called “eloquence”), rupture status, patterns of venous drainage, presence of high risk features,

such as aneurysms, and patient age or comorbidities (Lawton et al., 2010, 2015; Spetzler and Martin, 1986). However, existing treatment modalities are not without risk. Even less invasive therapies, such as endovascular embolization or radiosurgery, are associated with risks such as stroke or radiation necrosis or delayed cyst formation (Ilyas et al., 2018; van Beijnum et al., 2011). With unruptured AVMs, recent randomized controlled trials have suggested that medical management alone may be superior for prevention of death and stroke (Mohr et al., 2014). This study was not without flaws, and concerns exist with observation as to the risk of rupture in those with untreated AVMs. Continued research has focused on identifying additional risk factors in efforts to identify patients at highest risk who may benefit most from treatment.

Existing therapies either remove the AVM (surgery) or obstruct blood flow either acutely (endovascular embolization) or over a delayed interval (radiosurgery). Without blood flow, the risk of bAVM rupture is reduced. Careful consideration of radiosurgery sheds insights into how the underlying biology may be manipulated to evoke a therapeutic response without invasive therapy. Radiation ultimately induces the endothelium to degenerate and the vascular smooth muscle to proliferate to progressively occlude or compress the vascular lumen, respectively (Friedman and Bova, 2011; Tu et al., 2006a). Radiotherapy also reduces circulating levels of multiple pro-angiogenic factors within three months, such as VEGF, TGF-β, angiopoietin-2, and basic fibroblast growth factor (Xu et al., 2018), and leads to upregulation in inflammatory adhesion molecules such as ICAM-1 and E-selectin and the pro-thrombotic molecule thrombomodulin (Liu et al., 2012; Sammons et al., 2011; Sharp et al., 2003; Storer et al., 2008; Yuan et al., 2005). However, radiosurgery takes years to occlude an AVM and induces inflammatory changes in adjacent brain (Ding et al., 2017; Ilyas et al., 2018).

An ideal therapy would be non-invasive, immediate in action, and would be specific to the abnormal cells of the AVM without effect on adjacent blood vessels or brain. Considerable efforts have been placed on using existing therapies to target molecular pathways disrupted in bAVMs – including TGF-β, Notch and VEGF. For example, losartan – an angiotensin II receptor antagonist used for treatment of hypertension – decreased vascular dysplasia and arteriovenous-shunting in a zebrafish model of bAVMs induced through knockdown of the TGF-β receptor *alk1* (Walcott, 2014). Others have explored the therapeutic potential of thalidomide or related analogs. Mutations in *Eng* or *Alk1* result in reduced expression endothelial PDGF-B – a potent recruitment factor for pericytes and vascular smooth muscle cells (Lebrin et al., 2010; Zhu et al., 2018a). As described previously, this results in reductions in bAVM associated pericytes and vascular smooth muscle cells (Chen et al., 2013a; Winkler et al., 2018). In HHT, treatment with thalidomide was shown to restore endothelial PDGF-B expression leading to recruitment of mural cells and vessel stabilization (Lebrin et al., 2010). Induced bAVMs in *Alk1* deficient rodents were shown to respond similarly to thalidomide and the less toxic analog lenalidomide. Thalidomide or lenalidomide restored endothelial PDGFB expression and brain pericyte and vascular smooth muscle populations resulting in reduced vascular dysplasia, hemorrhage and inflammation (Zhu et al., 2018a). However, these studies were limited to mouse models of HHT and whether they would exert similar effects in other rodent models or bAVMs which arise outside the context of HHT remains unknown.

Inhibitors of Notch signaling may represent another prospective therapy for future development. BAVMs in mice with constitutively active Notch4 intracellular domain regress once the transgene is turned off which also leads to improvement in neurologic sequelae, such as ataxia and seizures (Murphy et al., 2009, 2014). Notch inhibitors have been developed and have been shown to be safe in clinical trials with Alzheimer's disease and cancer (Coric et al., 2015). With extracranial vascular malformations, Notch inhibitors reduce the rate of endothelial migration and formation of vascular networks (Davis et al., 2018). Notch-inhibiting drugs have not yet been tested in bAVMs, but these results suggest they may represent a prospective promising therapeutic direction.

Direct targeting of pro-angiogenic pathways such as VEGF has also attracted attention for prospective therapeutic development in AVMs. As previously described (see “Vascular Endothelial Growth Factor” subsection), VEGF has consistently been shown to be overexpressed in human bAVMs and is required for induction of bAVMs in select animal models (Chen et al., 2013a; Choi et al., 2012, 2014; Ferreira et al., 2014; Hashimoto et al., 2005; Jabbour et al., 2009; Koizumi et al., 2002; Walker et al., 2011). VEGF may be targeted indirectly by inducing expression of endogenous inhibitors, such as thrombospondin. This approach has proved effective *in vitro* and restoration of thrombospondin levels via expression of a microRNA (miRNA-18a) or the addition of thrombospondin to the culture medium reversed the proliferative and disorganized phenotype of culture bAVM endothelial cells (Ferreira et al., 2014; Stapleton et al., 2011). However, evidence for such an approach *in vivo* is presently lacking.

Others have begun to explore a direct approach using bevacizumab – a humanized VEGF monoclonal antibody. Bevacizumab has an established safety profile and has been trialed as anti-angiogenic therapy in a number of neoplastic conditions – including the aggressive brain cancer glioblastoma (Chinot et al., 2014; Gilbert et al., 2014). In *Alk1* deficient rodents with bAVMs, treatment with bevacizumab induced vascular apoptosis reducing the number of proliferating vascular cells and dysplastic vessels (Walker et al., 2012). The efficacy for bevacizumab therapy in human bAVMs remains unclear, and a phase I trial examining the safety of bevacizumab in adult patients with high grade AVMs is currently enrolling patients (NCT02314377).

7. Future directions

Advances in next generation sequencing technologies have led to identification of unrecognized genetic mutations which contribute to the genesis of bAVMs – such as *KRAS*, *BRAF* and *SMAD9* (Hong et al., 2019; Nikolaev et al., 2018; Walcott, 2014). Development of new rodent models utilizing newer techniques, such as CRISPR, have begun to be undertaken (Zhu et al., 2018b). Genetically engineered rodents harboring newly identified mutations, such as *KRAS* or *BRAF*, may offer the first model of sporadic bAVMs and represent an important platform which better recapitulates human AVMs for testing future therapeutics. Initial experiments with MAPK-ERK pathway promoted vascular barrier properties and quiescence in patient-derived *KRAS*-mutant endothelial cells *in vitro* (Nikolaev et al., 2018). Inhibitors of KRAS- or BRAF-related pathways are clinically available with established safety profiles, e.g., vermurafenib, dabrafenib and trametinib, and are currently being trialed in a number of brain tumors including pilocytic astrocytomas, craniopharyngiomas and metastatic melanoma (Brastianos et al., 2016; Davies et al., 2017; Olow et al., 2016). Whether these agents may be repurposed to accelerate translation of a similar approach in human patients with bAVMs remains to be seen. To inform such an approach, comprehensive next generation sequencing should continue to be performed to better understand and identify divergent molecular subgroups in bAVMs – similar to what has been done for human gliomas (Cancer Genome Atlas Research et al., 2015; Eckel-Passow et al., 2015).

Only recently have contributions to other cell-types – such as pericytes, vascular smooth muscle cells and inflammatory cells – have begun to be appreciated in bAVMs (Chen et al., 2008, 2013a; Winkler et al., 2018). How molecular cross-talk between these cell types is disrupted remains poorly understood in bAVMs, and systematic characterization of other cell types, including astrocytes and resident microglia, has yet to be performed. Generation of pericyte and vascular smooth muscle cell specific rodent models may also help better delineate the relative contributions of different segments of the arterial-venous axis to initial bAVM formation. A more comprehensive understanding of the dysfunction of the neurovascular unit in its entirety will likely yield additional targets for therapeutic development. Whether a

progenitor cell population may contribute to bAVM formation also remains unknown and has yet to be identified. Continued advancements with induced pluripotent stem cells may also open opportunities for cell replacement therapies targeting the vascular wall – such as pericytes or vascular smooth muscle (Yoshida and Yamanaka, 2017). Despite the present limitations, the recent convergence of a better molecular and cellular understanding of bAVM biology with new technologies likely marks the dawn of a new promising era of therapeutic discovery for treatment of bAVMs.

Conflicts of interest

None.

Appendix A. Supplementary data

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