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Biochemical- and Biophysical-Induced Barriergenesis in the Blood–Brain Barrier: A Review of Barriergenic Factors for Use in In Vitro Models

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Central nervous system (CNS) pathologies are a prevalent problem in aging populations, creating a need to understand the underlying events in these diseases and develop efficient CNS-targeting drugs. The importance of the blood–brain barrier (BBB) is evident, acting both as a physical barrier to drug entry into the CNS and potentially as the cause or aggravator of CNS diseases. The development of a biomimetic BBB in vitro model is required for the understanding of BBB-related pathologies and in the screening of drugs targeting the CNS. There is currently great interest in understanding the influence of biochemical and biophysical factors, as these have the potential to greatly improve the barrier function of brain microvascular endothelial cells (BMECs). Recent advances in understanding how these may regulate barriergenesis in BMECs help promote the development of improved BBB in vitro models and therefore novel interventional therapies for pathologies related to its disruption. Herein, an overview of specific biochemical and biomechanical cues in the formation of the BBB, with a focus on in vitro models and how these might recapitulate the BBB function, is provided.

1. The Blood-Brain Barrier

The blood–brain barrier (BBB) is a dynamic interface that separates the central nervous system (CNS) from the circulatory system, maintaining homeostasis in the neurological niche, which is easily affected by fluctuations in the concentration of small molecules and metabolites and protecting neurons from pathogenic agents present in the bloodstream. This barrier is formed by a series of unique properties of the nonfenestrated brain microvascular endothelial cells (BMECs), namely the tight junctions between BMECs which form a diffusion barrier limiting paracellular movement, as well as the enzymatic barriers and increased expression of efflux transporters and pumps, which regulate the transcellular transport.[1,2] All these characteristics come together to form a diffusion barrier which impedes the influx of most compounds into the brain, including most drugs targeting the brain with a molecular mass over 400 Da.[3] While the major component of the BBB are the BMECs, the properties that allow BMECs to exert this barrier function are not intrinsic to the cells but are induced through their interaction with other cell types found in close association with the brain microvasculature.[4–6] This complex, well-organized multicellular anatomic structure is known as the neurovascular unit (NVU). The NVU consists of BMECs, pericytes, astrocytes, microglia, and their shared acellular basement membrane (BM), which together ensheath the brain microvasculature and are thought to establish and maintain the BBB, as shown in **Figure 1**.[4,7,8]

The components of the NVU secrete biochemical factors and provide mechanical support required to maintain barrier integrity, as shown in **Figure 2**. Astrocytes play a key role in the development and maintenance of the BBB, partially by releasing growth factors, through both their direct interaction with endothelial cells and their secretory protein communication, resulting primarily in a restricted permeability through tight junctions.[4,9–12] While defining the role of astrocytes has historically been successful, and thus their importance in the NVU inflated, finding the exact role of pericytes within the NVU is challenging. This is as there is currently no distinct pericyte-specific marker, although extensive pericyte recruitment is a hallmark of a functional BBB.[13,14] However, in early BBB development, the adhesion between BMECs and pericytes has been hypothesized to release chemotactic factors from the BMECs

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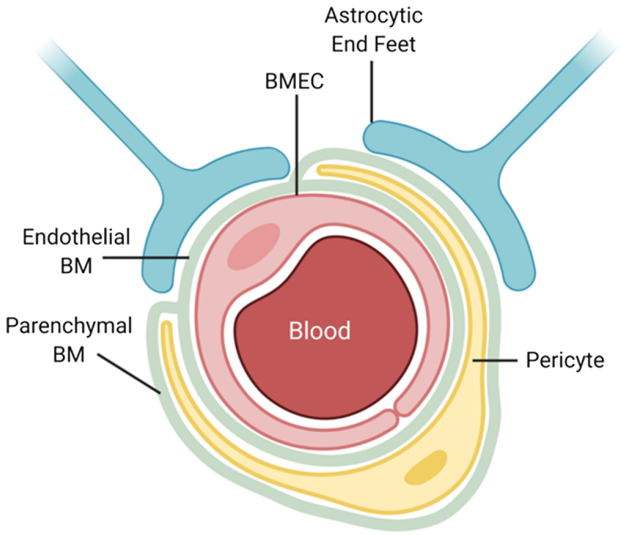

Figure 1. The cells of the NVU. BMECs form the lumen of the blood vessel by wrapping around themselves, forming TJs with the BMECs either side. Pericytes are discontinuously distributed along the CNS microvessels, partially enclosing the vessels, and have been found to contribute to the barrier properties of the BBB. BMECs and pericytes share a BM, named the endothelial or perivascular BM. This is distinct from the parenchymal BM, which splits the astrocytic end feet from the pericytes and BMECs. Astrocyte end feet additionally coat the microvessels of the NVU with a lacework of lamellae and additionally aid the BMECs in forming a barrier.

to induce the migration of pericytes to the endothelial wall and subsequent maturation of the vessels via the increased production of ECM components through the activation of growth factors such as the transforming growth factor (TGF)-β and platelet-derived growth factor (PDGF).[7,15–17] The BM is an important acellular component of the NVU that provides mechanical support to BMECs and acts as a reservoir of growth factors secreted by the surrounding cells, which strongly influences BBB integrity.[18,19]

Neurological diseases, such as neurodegenerative diseases, infection, pain, and psychiatric disorders, are the leading cause of disability, morbidity, and mortality worldwide.[20,21] As the BBB prevents most therapeutic agents from entering the brain, the lack of a priori knowledge of drug delivery kinetics to the brain remains a bottleneck in the development of CNS therapies.[3,20,22,23] A notable example is glioblastoma, a particularly deadly and very aggressive cancer which begins within the brain, whose treatment is ineffective in part due to the inability of chemotherapeutic drugs from reaching the tumor site due to the BBB.[24] This is additionally accompanied with changes in the NVU near the tumor site, such as altered junctional protein expression, loss of astrocytic end feet, and increased permeability.[25] There is further correlation between the disruption of the BBB and pathologies such as Alzheimer's, multiple sclerosis, and Parkinson's.[26–28] For example, a breakdown of the primary tight junction (TJ) seal proteins, claudins, between BMECs has been demonstrated in stroke and inflammation.[2,29]

This results in delayed and extended drug development timelines and a high failure rate of related clinical trials.[30] As a result, the need for an accurate and physiological in vitro model of the BBB has become an indispensable pursuit, to model CNS pathologies and test the BBB-penetrating potential of novel drugs.

Advances in bioengineering, stem cell technology, microfluidics, as well as general knowledge of the CNS microvasculature have led to rapid advances in the development of in vitro BBB models capable of exhibiting a wide range of in vivo-like BBB properties, although no current model is capable of reproducing the full range of physiological functions and responses or maintaining a stable barrier function.[31–34] Importantly, BMECs are capable of sensing the mechanical stimuli exerted by blood flow, resulting in cytoskeleton rearrangement and impacting BBB function.[35,36] The value of an in vitro BBB model resides in its ability to recapitulate the in vivo and ex vivo properties and its ability to facilitate CNS drug discovery and develop novel CNS therapeutics.

The current models can be categorized in four general archetypes: 1) the Transwell model, 2) microfluidic chips, 3) spheroid-based models, and 4) hydrogel-laden microfluidic chips.[33] Within these models, the BMECs are exposed to numerous biochemical signals, in the form of cell culture media composition and extracellular matrix (ECM) protein coatings, as well as mechanical cues, such as fluid flow and substrate/scaffold physical properties and architecture, all of which impact barrier formation. While shear stress and biochemical in vitro signaling have been well reported, the impact of matrix-induced mechanical signaling is somewhat less discussed. Furthermore, some of the common pitfalls of these models involve the use of substrates that poorly represent the native BMEC environment in terms of mechanical stiffness, protein composition, and potentially cell curvature. In addition, while complex co- or triculture conditions offer tighter barriers, there remain gaps in the literature on the core required biochemical signals that can entirely skip the use of cocultures with BMECs.

While there remains a long way for in vitro BBB models to go, some innovative microfluidic devices can overcome these pitfalls, both mimicking the 3D geometry and cell curvature of the BBB in combination with shear stress.[34,37] In the model described by Campisi et al., increasing complexity models are created with the addition of pericytes and pericytes plus astrocytes, which were shown to self-assemble into microvessels of increasing BBB characteristics with the addition of further cell lines.[34] This model has limited applicability as a drug permeability model, as the polydimethylsiloxane (PDMS), a widely used polymer in microfluidic applications, shows a nonspecific protein adsorption behavior. As testing the barrier permeability is limited in this model, it is additionally a shame that transendothelial electrical resistance (TEER) measurements are another metric of the BBB function, which this model does not currently support. Furthermore, the general complexity of the model, with the use of triculture conditions and iPSCs to achieve BBB characteristics, may limit its usability by the average lab or medical center, and therefore the use of BBB-inducing mechanisms aside from coculture may be helpful.

Creating relevant BBB models requires an understanding of the complex in vivo CNS microcapillaries, as well as the dynamic microenvironment that surrounds the BBB and maintains or disrupts its barrier integrity.[38] Within this Review, we will outline the biochemical and mechanical cues linked to BMEC

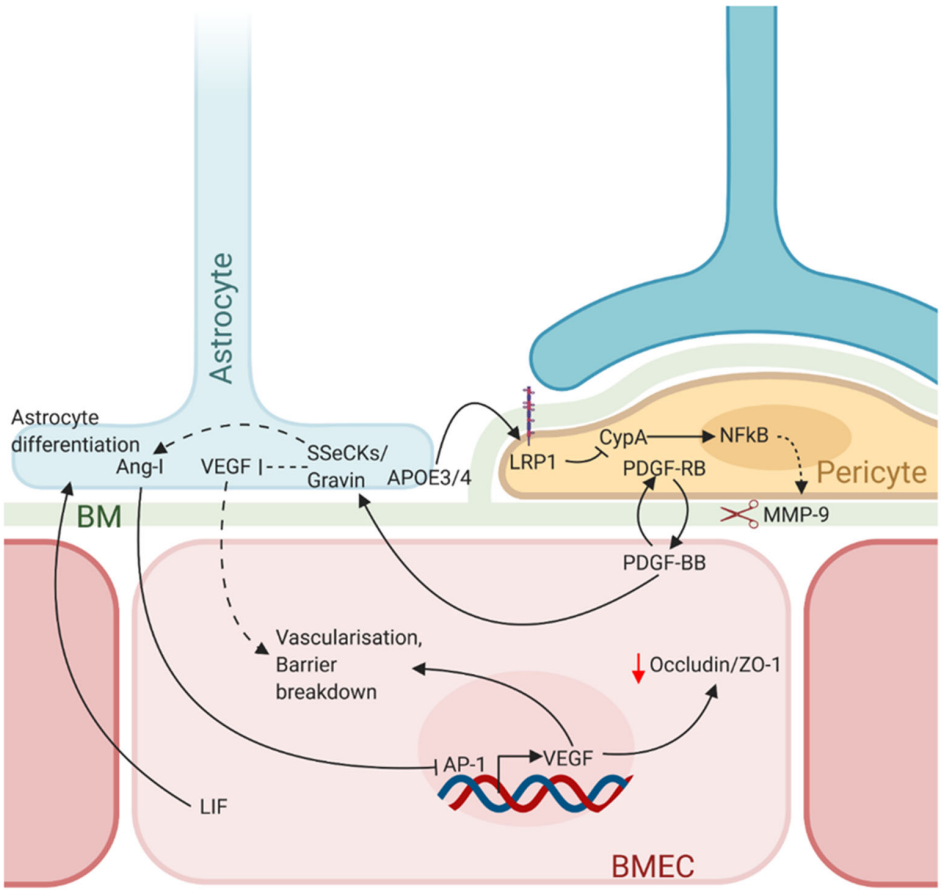

Figure 2. Mechanisms of the bidirectional interactions between astrocytes, pericytes, and BMECs, as they aid the formation and maintenance of the BBB. Of the mechanisms known to the authors, the majority depicted in this figure are concerned with overcoming proangiogenic forces, such as VEGF. Other mechanisms induce the retention of pericytes in the NVU (PDGF-BB), preservation of the BM through inhibiting matrix MMPs or through the differentiation into favorable astrocyte phenotypes (LIF). Solid line represents a direct action, dashed line indicates that intermediary steps have been omitted for clarity.

barriergenesis and how these may be used to create in vivo-like BBB in vitro models.

2. Biochemical Insights into Barriergenesis

The cells of the NVU display a clear biochemical interaction with the microvasculature, as shown in Figure 2, and by the improvement of in vitro TEER measurements through use of coculture involving both immortalized, primary, and iPSC-derived cells.[39–60] Bidirectional signaling between pericytes and BMECs, and astrocytes and BMECs, leads to the formation of tight junctions and barrier integrity into adulthood, which makes the secretomes of both astrocytes and pericytes of great interest in the search for barriergenic factors in the BBB.[4,7,10,61] The formation of the BBB can be broken down into three phases, consisting of: 1) angiogenic, 2) barriergenic, and 3) maturation phases, each of which are characterized by differing levels of angiogenic and morphogenic biochemical signals, the latter two of which will be explored in this section. In short, the angiogenic phase is driven by vascular endothelial growth factor (VEGF) and angiopoietin (Ang)-II-driven angiogenic sprouting in early embryogenesis.[62] The recruitment of pericytes to the developing microcapillaries is a critical step in the formation and maintenance of the BBB, and much of the insight into their importance has been ascertained from pericyte-deficient mice studies with PDGF-BB disruptions.[7,63,64] In this barriergenic phase, angiogenic factors are overridden by barrier-inducing signals, such as Sonic Hedgehog (Shh), Norrin, PDGF-BB, and Ang-I.[62] While biochemical signaling is key to the differentiation and formation of the barrier, the molecular crosstalk needed for BBB maturation and maintenance is still an emerging field. As cells in in vitro models maintain their barrier characteristics for a short period of time, the maintenance of the barrier may require contacts with the cells of the NVU; however, at this time this is pure speculation.[62] While astrocytes are recruited at a later stage to further assist BBB formation, and therefore are uninvolved in early BBB barriergenesis, the full extent of their role in BBB formation and maintenance remains murky waters, as regional genetic removal of astrocytes shows little effect on BBB permeability, although their role in the cross communication between cells of the NVU shows astrocytic importance for maintaining an optimal BBB phenotype[31,65–67] Below, we will discuss some of these biochemical signals in detail as they have been shown to affect the BBB in in vivo ablation studies and their ability to produce barriergenic properties in vitro, which are further summarized in **Figure 3**.

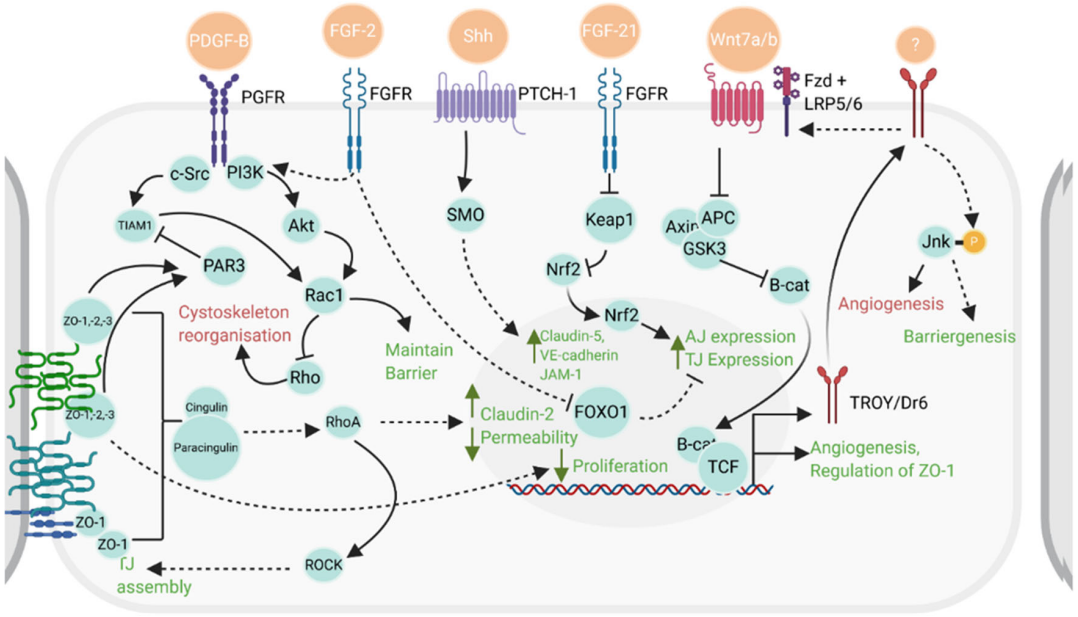

Figure 3. Selection of signaling pathways activated by barriergenic growth factors and cytokines in BMECs. Pathways function primarily through three pathways: 1) through increasing expression of junction proteins and 2) efflux transporters, and 3) prevention of angiogenesis. PDGF-BB signaling through Rac1 maintains barrier function. FGF signaling additionally signals through the PI3K-AKT pathways as well as inhibits transcription regulator forkhead box protein O1 (FOXO1) and occurs via the translocation of transcription factor Nrf2 into the nucleus, thereby increasing the expression of AJs and TJs. Wnt signaling inhibits the breakdown of B-catenin as well as allows β-catenin translocation into the nucleus, where it causes the expression of many genes, including TROY/DR6, which additionally causes barriergenesis to occur.

3. PDGF Signaling

PDGF is a potent mitogen which plays a significant role in blood vessel formation. During CNS angiogenesis, BMECs recruit pericytes to the nascent vessel by releasing PDGF-BB, a high-affinity ligand for the PDGF-Rβ receptor found on both pericytes and endothelial cells.[64,68] PDGF-BB secreted by BMECs binds to two receptors, PDGFR-α and PDGFR-β found on pericytes, although it is unclear whether PDGF-BB/PDGFR-α interaction supports BBB integrity, the binding of which on pericytes leads to the enhancement of BBB integrity.[69,70] PDGF signaling has been shown to be involved in both vascularization in the brain and pericyte recruitment and retention. The disruption of the attachment of pericytes leads to BBB disruption as well as neuroinflammation and CNS disease.[49] While it is a known barriergenic factor, and numerous mice studies show the restorative ability of PDGFR-β and -α after stroke (which involves BBB disruption), to our knowledge, no in vitro models utilize PDGF-BB signaling, except in the form of pericyte/astrocyte cocultures. It would be of interest to see whether PDGF-BB autocrine signaling from BMECs is an effective instigator of barriergenesis.

While the PDGF interactions between pericytes and BMECs play a key role in BBB formation, the BMEC–astrocyte interaction additionally leads to the formation and maintenance of the barrier. The endothelial-derived PDGF-BB caused the downregulation of VEGF expression by astrocytes, a known disruptor of the BBB.[71] This is caused by an upregulation of Gravin (the human homolog to mouse SSeCKS), which was shown to further markedly upregulate Ang-I, a potent vascular maturation factor, which overall leads to the strengthening of the interendothelial junctions, partially through the increased expression of TJ proteins occludin and claudin-5.[49,71] Pericyte-BMEC PDGF-BBB signaling therefore works not only to recruit and retain pericytes but additionally reduce and overpower proangiogenic signaling from nearby astrocytes.

In animal studies of the interaction of PDGF-BB and the BBB, a decrease in the number of pericytes was seen, as a result of reducing PDGF-Rβ signaling in mice through the deletion of tyrosine phosphorylation sites or ECM retention motif compared with wild-type littermates, which results in leaky vessels and increased vascular permeability.[7,72,73] Counter to what might be thought, the effect on vascular permeability was shown not to be caused through compromised TJ protein expression, but through defective regulation of endothelial transcytosis. The existence of large numbers of efflux transport systems in BMECs, as well as the enzymatic barriers which exist, is one of the key reinforcers of BMEC barrier properties.

4. Wnt and B-Catenin Signaling

The name Wnt is a portmanteau from the names Wingless and Int-1 and acts as the name for a group of signal transduction pathways, which all begin from the binding of a Wnt-protein ligand to a Frizzled (Fzd) family receptor.[74,75] The effectors of the Wnt signaling pathway are highly upregulated in BMECs in comparison with peripheral endothelial cells, such as those in the liver and lung, which suggests the role of

Wnts in the regulation of CNS microvasculature.[61,76] Wnt factors act in three known independent pathways, a canonical β-catenin-dependent pathway and two non-canonical pathways.[74,76] However, research has indicated that only canonical signaling is important in barriergenesis, as well as essential for the development of the brain microvessels.[61,76–81] Studies have shown that the activation of the Wnt/β-catenin pathway is both necessary and sufficient to induce BBB-type endothelial TJs in vivo on both a molecular and a structural level.[78–81] While the use of biochemical factors in lieu of co- or triculture conditions is a field still emerging, Laksitorini et al. showed that in a BBB in vitro model, utilizing immortalized BMECs, manipulating exogenous Wnt signaling through the inhibitor LiCl, or activating Wnt signaling with Wnt3a improved P-gp efflux transporter activity and decreased paracellular permeability, although manipulating the autocrine Wnt signaling of BMECs resulted in more modest and minimal effects.[82] Overall, Wnt activators to date have been shown to be an exciting avenue of exploration in the creation of BBB in vitro models.

In the canonical pathway, the binding of Wnt to Fzd and LRP5/6 results in the translocation of a stabilized β-catenin into the endothelial cell nucleus and interacts with TCF/LEF-1 complexes, which regulate the expression of specific genes. Genes controlled in this manner in BMECs include glucose transporter Glut1, a hallmark of the BMEC phenotype, as well as death receptors DR6 and TROY, and TJ proteins claudin-5 and occludin.[76,80,83,84] Tam et al. showed that the increased expression of TROY and Dr6, which are downstream elements of the VEGF pathway, drives brain angiogenesis and BBB formation through the regulation of zonula occludens-1 (ZO-1) expression in BMECs, independent of their canonical apoptotic and prodegenerative activity.[83] The ablation of β-catenin in endothelial cells results in normal vascularization in all organs except the CNS, where vessel formation fails, and BMEC accumulation of β-catenin has been proposed to regulate the formation of TJs via the increased expression of claudin-3.[61,79]

When looking at specific activators of the Wnt pathway in the BBB, Wnt7a and Wnt7b are shown to be particularly important in BMECs and have been implicated in BBB development in vivo.[61,77] Furthermore, genetic ablation in mice caused the failure of developing BMECs to invade the embryonic CNS, as well as a lack of expression of proteins characteristic of the BBB. Wnt7a b^{-1} as well as Wnt3a have been shown to induce the expression of claudin-3 and Glut-1.[61,80,85] Wnt5a was shown to regulate endothelial cell survival, proliferation, and gene expression.[78,86]

Norrin, a divergent member of the TGF-β superfamily, shows high affinity of the Fzd4 receptor while sharing no homology with Wnt family proteins and, through binding the receptor, activates the canonical Wnt pathway.[87] Norrin signaling upregulates the expression of Glut-1 and claudin-5 in BMECs, while downregulating proangiogenic markers, plvap/meca32.[87] The genetic ablation of Norrin or Frizzled4 was shown to result in defective angiogenesis in the CNS and barrier disruption.[88] In a study on mice, when Norrin was expressed, loss or inactivation of Norrin or Frzd4 led to the loss of barrier characteristics and upregulation with permeability-associated genes, plvap/meca32.[62,87]

5. Hedgehog Signaling

The hedgehog (Hh) pathway is involved in embryonic morphogenesis, neuronal guidance, and adult vascular proliferation and differentiation. The Hh pathway has been identified as playing a key role in the expression of claudin-5 and maintenance of barrier properties with the Hh family member Shh being the best described with CNS morphogenic events.[65,89–91] Astrocytes are the main Shh secretors in the CNS, which bind to the endothelial patched homolog 1 receptor (Ptch-1), a 12-pass transmembrane protein. The binding of Shh to Ptch-1 causes the release of the inhibitory protein smoothened (SMO), allowing for the activation of Gli family transcription factors and the expression of Shh-regulated genes such as claudin-5, VE-cadherin, and JAM-A.[65] The selective deletion of SMO results in decreases in TJ protein expression and is associated with the vessel leakage of plasma proteins.[92] To our knowledge, no in vitro models of the BBB utilize activators of the Hh pathway.

The maturation phase and type of astrocytes from which Shh appears to have an effect on how Shh acts upon the BBB, with Shh released by astrocytes in the adult BBB, contribute to the integrity of the barrier, particularly during inflammatory disease and Shh secreted by immature astrocytes, increasing the expression of occludin and claudin-5, as well as the regulation of these genes.[65,89] During brain angiogenesis, Shh is secreted by astrocytes, resulting in a decrease in SMO activity in ECs that leads to the decreased expression of TJ proteins such as occluding and claudin-3 and −5, but also ZO proteins. This resulted in the increased leakiness of the BBB. By regulating the expression of p120-catenin, the Shh pathway may modulate the maturation of both adherens junctions (AJs) and TJs in CNS vascular endothelium.[65] Shh was shown to preserve BBB integrity by increasing ZO1, occludin, and Ang-I expression in animal models for ischemic stroke.[88] At a cellular level, Shh was shown to induce the expression of Ang-I and repress Ang-II, which led to increased expression via Tie1 and downstream junctional protein expression and vascular maturation.[93]

In animal models for stroke, Shh is found to be transiently upregulated in the focal ischemic brain.[94] In animal models for acute ischemic stroke, loss of Shh signaling led to aggravate brain edema.[94] As this research may suggest, intracerebroventricular injections of Shh in ischemic stroke animal models reduce brain edema and preserve the BBB integrity by inducing expressions of ZO-1, occludin, and Ang-I.[89] Shh knock-out (KO) mice are not viable and express with BBB formation abnormalities, maintaining normal numbers of vessels but decreased expression of occludin and claudin-5.[5] This data suggest that Shh is not required for CNS blood vessel formation, but for maturation of the BBB once vessels are formed, as well as maintaining and repairing barrier characteristics in the BBB.

6. TGFB Signaling

The multifunctional cytokine, TGF-β, is a known disruptor of the BBB; it has also been reported to cause the induction of claudin-5 expression in BMECs but also decrease claudin-5 expression in BMECs. TGF-β promotes BBB integrity via enhanced endothelial TJ protein expression, both in vivo and in vitro.[95,96] BMECs and

pericytes adhesion are additionally mediated by the TGF-β and TGF-β receptor 2 (TGF-βR2) by both cells, after the recruitment and proliferation of pericytes at spouting vessels within the CNS occurs via PDGF-BB.[68] In addition, deletion of TGF-β was reported to result in embryonic lethality, pericyte loss, faulty vascular development, and hemorrhaging.[97–99] While TGF-β is often grouped with growth factors involved in BBB disruption and stated to exacerbate BBB permeability in disease, there exists a duality within the data that additionally show a potential for maintenance of the BBB.

TGF-β signaling in pericytes initiates production of ECM proteins and in BMECs promotes pericyte adhesion through N-cadherin. Within the TGF-β signaling cascade, Smad4 is a key protein, and mice deficient in it show pericyte detachment and increased vessel diameter, BBB permeability, and hemorrhage.[68] Garcia et al. showed experimentally that treatment of cultured endothelial cells with TGF-β increased the activity of BBB marker γ-glutamyl-transferase (GGT) in a dose-dependent manner, which occurred concurrently with a reduction in the number of endothelial cells, indicating an inverse relationship between proliferation and GGT expression, which is consistent with differentiation into BMECs forming a barrier.[100,111] A study by Shen et al. indicated that TGF-β may be a downstream effector of PDGF signaling and that PDGF's pivotal role in BBB restoration after cerebral ischemia may be in part due to PDGF regulation of TGF-β signaling.[69]

GRP124, an orphan member of the G protein-coupled receptor family, was identified as an essential endothelial receptor for CNS-specific angiogenesis. Orphan G-protein coupled receptor, Gr124, additionally acts as a specific coactivator of the Wnt/β-catenin signaling pathway inn the BBB.[102,103] GRP124 signaling induces Glut-1 expression and is required for TGF-β signaling in BMECs.[104,105] Mice with GRP124 KOs are embryonic lethal, with defects in the CNS vasculature with signs of hemorrhages, which result from impaired endothelial cell survival, outgrowth, and migration.[102,104,105] There appears to be some overlap between Wnt signaling and GRP124, such as a lack of Glut-1 expression in KO mice, which may indicate some interaction between these pathways during development.[61,80]

7. Fibroblast Growth Factor Signaling

Fibroblast growth factors (FGFs) are a family of growth factors which are known to play key roles in angiogenesis, wound healing, and embryonic development.[106] FGF protects from BBB breakdown through reduction of RhoA activity via the phosphoinositide 3-kinase (PI3K) Akt-Rac1 signaling pathway.[107] In experiments conducted by Reuss et al., the ablation of FGF-5 and FGF-2 in mice resulted in decreased levels of glial fibrillary acidic protein (GFAP), an astrocytic marker, as well as BBB breakdown.[108] Although the BBB research community has established that FGF plays a vital role in brain physiology and astrocytes, as it induces the proliferation and maturation of astroglia cells and activates astrocytes, there has been a long silence in the FGF research and how it may induce barrier formation in in vitro models.[108]

In an in vitro model which used slice cultures from mice, Bendfeldt et al. found that FGF-2 (also known as basic FGF or bFGF) helped preserve the cerebral vessels and maintained the tight junctions.[109] FGF-2 is primarily produced by astrocytes in close association with BMECs in the BBB and binds the FGF receptor 1 (FGFR1) on BMECs.[110] Sobue et al. hypothesized that the barrier-improving properties of astrocyte coculture may be due to FGF-2 secretion by astrocyte; they did not find a significant increase in the number of TJs, nor the expression of mdr and Glut-1 in immortalized BMECs, although FGF-2 addition to media did increase the tightness of the barrier and decrease L-glucose permeability (but not as much as coculture conditions did).[110,101] FGF-2 was also shown to decrease in vitro permeability by el Hafny et al.[112] It has also been hypothesized to be one of the barrier-tightening factors by C6 glioma cells in rats.[113]

There is a high level of colocalization of the FGFR1 receptor with laminin in the BM and no colocalization of the receptor with astrocytes.[109] This is in agreement that FGFR1 signaling is dependent on the ECM composition in capillary EC differentiation and that laminin plays a crucial role in this.[114]

8. Ang Signaling

Both Ang-I and II are released from astrocytes in the NVU, where Ang-I binding the Tie2 receptor on BMECs results in the upregulation and subcellular distribution of TJ proteins and Ang-II binding AT1 is involved in the posttranslational modification of occludin and its subcellular distribution.[60,115,116] Ang-I's role in barrier stability was shown by Ang-I positively regulating B-catenin in BMECs, through activation of Akt and GSK2B phosphorylation, leading to the upregulation of Notch signaling.[116]

9. Notch Signaling

Notch1, which is expressed by stalk cells within the neural niche, leads to the suppression of an angiogenic phenotype and therefore leads to vessel stabilization and potential for barrier formation.[117] Notch signaling originating from pericytes additionally binds Notch1 on the BMEC surface, which leads to the increased expression of N-cadherin, increasing pericyte retention in CNS vasculature.[118]

10. Other

Other relevant growth factors include cluster of differentiation 146 (CD146), glial cell line-derived neurotrophic factor (GDNF), as well as apolipoprotein E (APOE). CD146 is a known marker of the endothelial cell lineage, although it has also been identified as a spatiotemporal molecule which orchestrates BBB development as a critical regulator of claudin-5 expression and BBB permeability. Pericyte expression of CD146 promotes the pericyte coverage of ECs, as well as enhancing BBB integrity more directly by regulating PDGF-BB/PDGF-Rβ signaling.[119,120] GDNF is also known to increase claudin-5 expression, although the mechanism through which it does this remains unknown.[121,122] APOE secreted by astrocytes was also found to be involved in the post-translational modification of occludin

when it binds the LRP-1 receptor. APOE KO mice increased cerebral vessel permeability and the leakage of serum proteins into the CNS.[123–127] The different APOE isoforms were also found to have differing effects on the BBB, with APOE3 and APOE2 promoting physiological BBB tightness and APOE associated with disruption.[128] Adrenomedullin is a potent vasodilator and has been shown to increase claudin-5 expression and increase TEER and reduce the permeability of rat BMECs in vitro.[129] Vascular endothelial (VE) cadherin, while not only contributing to overall vascular stability, additionally plays a role in the endothelial response to both pro- and antiangiogenic stimuli.[130]

11. Mechanical Cues in Barriergenesis

Most cells require cues from a 3D environment to form a physiologically relevant tissue structure in vitro. At the most basic levels, tissues are composed of a population of cells interacting with their ECM. Cells are capable of remodeling their matrix during morphogenesis and differentiation or under normal physiological conditions, which are dictated to an extent by the matrix mechanical properties (stiffness, viscoelasticity) and the interactions of membrane cell receptors with their cognate extracellular motifs formed between the cell and its surroundings.[131,132] Compared with plastic or glass substrates, cells cultured on matrix or matrix-mimetic substrates can induce the expression of tissue-specific genes.[133]

The mechanical environment of cells is defined by complex interactions between local forces, generated by the movement of fluid/air pressure, gravitational forces; and intracellular tension, arising from the organization of cytoskeleton arrangement.[131] The process of mechanotransduction can be broken into 1) the initial force, such as shear stress produced by the blood flow, 2) the detection of this force by cellular structures in the cell membrane, 3) the transduction of these forces by signaling molecules and the propagation of the signal, and finally 4) the reception by cellular receptors and the physiological response.[130,134,135] Mechanical forces play an important role in the physiology and development of every organ, and the endothelial monolayer in the BBB is no exception. The endothelium is exposed to two physical cues: the fluid shear stress (FSS) on the apical face and ECM/BM nanoscale topography on the basal face of the endothelium.[136] While pivotal in the formation of the BBB, these forces have also been shown to play a role in vascular pathology in the BBB.[130,136]

12. The Endothelial Mechanosome

In vitro investigations have highlighted the activation of multiple mechanosensors in BMEC cell membranes in response to mechanical cues.[135,137] These include integrins, tyrosine kinase receptors such as G proteins and G protein-coupled receptors, ion channels, caveolae, membrane lipids, glycocalyx, gap junctions, focal adhesions, and proteins in the intracellular junctions, namely VE cadherin and occludin.[135,136] Of these, VE cadherin and PECAM-1 make up the mechanosensory complex situated in the endothelial AJs.[137]

12.1. VE Cadherin

VE cadherin is responsible for the assembly of AJs and the maintenance of the BMEC monolayer integrity and plays a major role in the assembly of the mechanosensory complex and as a mechanoadaptor to VEGFR-2.[138–140] Tzima et al. discovered the mechanotransductory role of VE cadherin, showing that endothelial cell alignment with the direction of the fluid flow required for VE cadherin.[141] As shown below, since the discovery of its role in endothelial cell response to flow, VE cadherin has become a well-documented vascular mechanosensor.

VE cadherin is particularly important for the formation of stable AJs, in which VE cadherin couples to the actin cytoskeleton and acts as a crucial mechanotransducer for mechanical sensing. Cytoskeleton pulling on the adherin complex is known to enhance cell–cell adhesion.[138,139,142] Actin dynamics furthermore control the assembly/disassembly of VE cadherin junctions. In mature junctions, VE cadherin is linearly organized in cells, supported by actin bundles running in parallel. Remodeling driven by actomyosin contractions results in the formation of discontinuous junctions connected to perpendicular tensile actin bundles.[138,142] The equilibrium between these two mechanisms is tightly controlled by Rac and Rho GTPases. Rac GTPase activation supports linear junctions, corresponding to a release of tension from VE cadherin, and Rho GTPase activation increases actomyosin-mediated pulling forces on VE cadherin junctions.[141,143] In flow conditions, laminar flow elevates the tension of the actin cytoskeleton, increasing BMEC cell–cell interaction forces through Rho GTPase-dependent alignment of actin fibers in the direction of the flow.[138,141] VE cadherin is additionally responsible for AJ formation, which is presumed to be essential for TJ formation and the BBB architecture.[144]

VE cadherin expression levels and phosphorylation play vital roles in the permeability of the BBB, with enhanced expression promoting BMEC barrier function and the phosphorylation of VE cadherin showing a strong correlation with impaired BBB formation.[144] The signaling pathways culminate in a change of the phosphorylation at key tyrosine, threonine, and serine residues on TJ proteins, such as tyrosine phosphorylation on occludin, which has been linked to BBB breakdown.[145] VE cadherin transmits physiological cues through occludin via the activation of the Tiam1/Rac1 signaling pathway, which promotes tyrosine dephosphorylation, inducing barrier stabilization.[145] Mechanical forces, such as laminar shear stress, have also been shown to affect the expression of VE cadherin and cause upregulation of occludin.[146]

VE cadherin in tight junctions provides both vascular stability and endothelial polarity but additionally regulates the BBB endothelial response to angiogenic stimuli.[62,140]

12.2. Integrin

Integrins have an established role in mechanotransduction, acting as intermediaries in shear stress-induced signaling cascades to activate Shc and c-Hun NH2-terminal kinases (JNKs).[141,147,148] During brain development, angiogenic BMECs express integrins $\alpha4\beta1$ and $\alpha5\beta1$ which bind to

fibronectin and induce cell proliferation via the MAPK signaling cascade.[149,150] However, in the adult mouse, endothelial cell differentiation and overall vessel stabilization are promoted through the binding of integrins α1β1 and α6β1 to laminin.[149]

12.3. PECAM-1

Platelet-derived cell adhesion molecule-1 (PECAM-1) contributes to the maintenance of the endothelial barrier and is a key mechanotransducer that translates shear stress from laminar blood flow into cell alignment with the direction of flow.[138,141,151] Mechanoresponses in the endothelial layer are likely dependent on the direct force exerted on PECAM-1, as shown through the local application of tensional force on PECAM-1 which elicits global cytoskeleton stiffening, which in turn leads to remodeling of integrin-based adhesions.[138,152]

12.4. Other

Transmembrane proteins piezo1 and piezo2 have been identified as critical components of mechanically activating ion channels and have been brought forward as mechanotransduction molecules in endothelial cells, as have YAP/TAZ, which relay mechanical signals exerted by the ECM rigidity and cell shape to the nucleus.[103]

13. Basement Membrane

The BM is a type of ECM found predominantly beneath endothelial and epithelial cells. The vascular BM consists of a 3D network of proteins, mostly consisting of laminins, collagen IV, nidogen, and perlecan.[153,154] Other proteins are differently expressed in the vascular BM depending on the physiological and developmental state.[155] These include fibronectin, fibulin-1 and -2, and collagen XVIII.[156,157] The development of the BBB BM may be of interest, particularly in the roles of different BM constituents, and a hypothesized timeline of BM formation is shown in **Figure 4**. The BM has many important functions, such as cell anchoring, structural support, and signal transduction.[158] Two types of BMs are found in the NVU, the endothelial BM and the parenchymal BM, which are physically separated by pericytes, although these are indistinguishable under nonpathological conditions.[159] The thickness of the BM has been reported to be in the range of 50–100 nm or 20–200 nm.[160,161] The importance in the ECM also lies in its ability to wrap and accumulate secreted proteins, such as Wnt proteins.[162,163] Changes in its composition can be observed in both acute and chronic neuropathies, which are thought to contribute significantly to disease pathogenesis.[160,161]

The vast majority of research on the BBB focuses on cellular and molecular constituents, leaving the BM largely unstudied. This is in part brought about by its intrinsic complexity, as well as the lack of research tools.[18,164] In this section, the composition of the cerebral microvascular BM, as well as its effect on both the formation and the maintenance of the BBB, with both genetic KO and knock-down (KD) studies, as well as results achieved in in vitro BBB models, will be discussed. Recent studies have shown that the BM contributes substantially to the vascular barrier function, particularly in the migration of leukocytes through the vessel wall.[165–167] However, there is a significant gap of knowledge on how BM proteins may differentially affect BBB qualities within the same in vitro model, which would substantially benefit the field.

The three primary cells of the NVU, BMECs, pericytes, and astrocytes, adhere to the BM via specific members of the integrin or dystroglycan family.[168,169] The expression of dystroglycan is found in BMECs and perivascular astrocytes within the adult mouse brain.[170] β1- containing integrins were found to be expressed by BMECs, pericytes, and astrocytes, and it is the interaction of β1-integrins with collagen IV in the BM that is correlated to the expression of claudin-5 and increased BBB integrity in vitro.[171]

13.1. Laminin

Laminin is a heterotrimeric protein composed of α, β, and γ chains.[172] The combinations of these generate a large number of laminin isoforms, although not all combinations have been found in mammals.[173] The biological activity of laminins is largely defined by the a-chain interaction with integrins.[174,175] The formation of the vascular BM is dependent on the initial self-assembly of laminin into a sheet, which is then linked via nidogen and perlecan to collagen IV, aiding the formation of a secondary polymer network by collagen IV.[176–178]

While BMECs, pericytes, and astrocytes all synthesize laminin, they contribute different isoforms to the vascular BM.[164] BMECs predominantly generate laminin-411 and laminin-511, whereas astrocytes generate laminin-211, and pericytes contribute laminins containing the α4, α5, and γ 1 subunits.[159,179] Due to this cell-specific expression, the parenchymal and endothelial BMs differ in the distribution of laminins such as laminin-211 in the parenchymal BM and laminins-422 and -511 mainly located in the endothelial BMs.[155] Sixt et al. showed that the recruitment of mononuclear cells within the perivascular space was correlated with the laminin composition. For example, T cell recruitment exclusively occurred when the endothelial BM contained laminin a4 chains but not the a5 chains associated with the parenchymal BM which has a restrictive or inhibitory function.[159] This is due to the high affinity of integrin α6β1 of the T cells to laminin-411 and low affinity for laminin-511.[180,181] Blocking β1 interaction in vitro increases vascular permeability through the decreased expression of claudin-5.[62]

Very few, if any, in vitro models utilize laminin coatings or laminin-based scaffolds in BBB models, which would be a very exciting possibility for its potential use in producing strong barriers.

Global KOs of most laminin subunits, including those found in the BBB, lead to embryonic lethality, therefore preventing further study on their effect on the BBB.[182,183] Conditional KO lines targeting the γ1 laminin chain, a very common subunit in laminin isoforms expressed in the BBB, have been very revealing of its role. When specifically depleted in astrocytes, deletion of the laminin γ1 chain produced mice exhibiting weakened vascular integrity, resulting in hemorrhages in small arterioles in the hypothalamus, thalamus, and ganglia.[164,184] The significance of laminin, in particular astrocyte-derived laminin, can

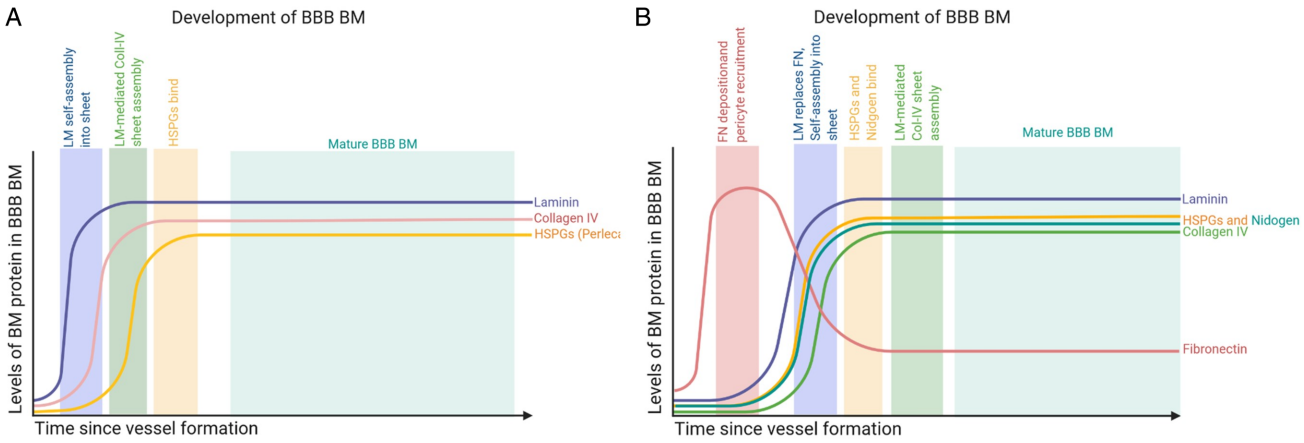

Figure 4. Two models on the temporal deposition of the BM of the BBB. A) Pastor-Pareja and Xu showed that perlecan deposition is dependent on prior collagen-IV presence in Drosophila.[231] This was supported by analysis of Col4a1 mutant mice,[277] Drosophila,[278] and C. elegans.[279] However, mechanisms for laminin-independent collagen IV recruitment to at least some BMs have been shown by Ramos-Lewis et al. and Jayadev et al.[279,280] B) Based on the review by Thomsen et al. on the formation of the vascular BM (HSPGs, nidogen, laminin and collagen IV), with addition of Wang and Milner for their theory on the role of fibronectin as the first deposited BM component in the BBB, followed by laminin and then the binding of HSPGs such as perlecan and agrin.[205,281] While fibronectin briefly aids the recruitment and attachment of pericytes, this is then replaced by laminin. Laminin-mediated collagen-IV assembly is the final step in formation of the BM.

be further shown in laminin α2 chain KO experiments. Intracerebral hemorrhage and age-dependent BBB breakdown were shown to be associated with astrocyte-derived laminin-211 loss.[9,185] Mice lacking the laminin α2 chain show defective BBBs, as shown by the presence of inflammatory cells in the brain parenchyma, as well as changes in TJ protein organization and reduced pericyte coverage.[186,187] These results suggest an indispensable role of astrocyte-derived laminin in BBB maintenance. Overall, the depletion of astrocyte-derived laminin was associated with an increase in BBB permeability and decrease in the expression of TJ proteins, changes that may be attributed to the effect of astrocyte-derived laminin on pericyte differentiation and maintenance of pericyte noncontractile state.[188]

In transgenic mice studies utilizing mice with laminin deficiencies in smooth muscle cells and mural cells, BBB breakdown and hydrocephalus were seen in the mural cell transgenic mice but not in the smooth muscle cell transgenic mice.[189] This suggests that pericyte-derived rather than smooth muscle cell-derived laminin is the cause of these changes. Hydrocephalus, a condition characterized by the build-up of fluid-containing cavities within the brain, can itself cause BBB compromise, and therefore it cannot be ascertained whether BBB disruption in the mural cell mice is due to pericyte-derived laminin or secondary to hydrocephalus.[190] Laminin α4 null mutants are viable, although they exhibit symptoms such as compromised vascular integrity as well as hemorrhage at the perinatal, but not adult, stage of development.[191] Loss of laminin α4 is thought to be compensated for by laminin α5, which has been shown to rescue hemorrhage phenotype in adulthood, when laminin α5 expression is seen.[164,192] Mice with specific KO of laminin α5 in endothelial cells were shown to have a normal phenotype without any obvious defects, supporting the laminin α4 and α5 compensatory action hypothesis.[193] While good for the mice, this compensatory action means that the role of laminin in the brain remains largely unknown. However, Russo et al. interestingly found that mice lacking laminin α5 lost the ability to respond to shear stress.[194]

13.2. Collagen IV

Collagen IV is the most abundant component of the BM. It is a trimeric glycoprotein comprising three α-chains, of which six have been identified to date (COL4A1-6).[178] While most collagen IV isoforms are spatially and temporally restricted, COL4A1 and COL4A2 can be seen in practically all BMs and are conserved across species.[195] As ablation of either is embryonically lethal, studies investigating the effect of COL4A1-2 on the BBB BM utilize more spatially restricted genetic tests.[196,197] Gould and coworkers, by introducing a splice mutation which removes exon 41 in COL4A1 in both BMECs, pericytes and astrocytes individually, showed that the relative contribution of the manor NVU cell types could be seen.[196–198] Loss of exon 41 from astrocytes caused mild intracerebral hemorrhage, whereas introducing splice mutation in pericytes or BMECs resulted in fully penetrant intracerebral hemorrhage.[196] Complementing this study, there have been numerous investigations that utilized missense mutations in COL4A1/2, resulting in varying degrees of intracerebral hemorrhage severity as well as brain malformations.[197,199,200] Taken together, these findings suggest a critical role of collagen IV in vascular integrity, which is likely why it is the most common BM protein component in BBB in vitro models.

13.3. Nidogen

There are currently two identified nidogen isoforms in mammals, nidogen-1 and nidogen-2.[177] The function of nidogen within the BM is to stabilize the collagen IV and laminin networks, particularly nidogen-1.[177,201,202] Nidgen-1 additionally

binds perlecan and fibulin.[203,204] While mice deficient in either nidogen-1 or nidogen-2 are largely normal, thinning of the vascular BM is seen compared with controls in nidogen-1 KO mice, as well as neurological defects, which presented themselves as episodes of involuntary movement, which largely resemble seizures.[201,205] Nidogen-2 is upregulated and redistributed in nidogen-1 null mice, although nidogen-1 expression does not affect nidogen-2 null mice; this still indicates the existence of a compensatory mechanism between the two isoforms.[206,207] Nidogen-2 mRNA expression is significantly downregulated at the postnatal age, implying the importance in embryogenesis but not adulthood, which may explain the differences of compensatory upregulation in null mutants.[208] The deletion of both isoforms leads to severe BM defects and perinatal lethality.[209]

13.4. Perlecan

Perlecan is a large protein present in most BMs and has various domains (*I–V*) and motifs, allowing it to interact with a large roster of molecules.[210] These include ECM proteins and heparin-binding growth factors, such as FGF-2 and VEGF, as well as perlecan core protein binding factors, such as PDGF subunit B (PDGF-B).[7,175,211,212] Perlecan plays an important role in the maintenance of BM integrity.[213]

While perlecan deficiency is embryonic lethal in mice, exhibiting complex phenotypes in many tissues, the BBB and its formation were seen to be affected. This may indicate that in embryogenesis, perlecan is dispensable in BBB formation, although how it may affect the BBB in later stages remains unknown.[214]

13.5. Agrin

Agrin is a heparin sulfate proteoglycan (HSPG) with several C-terminal sites for splicing, denoted as X, Y, and Z in rats, and is the most abundant HSPG in the vascular BM.[215] The isoform of agrin present in vascular BMs, z0, lacks an amino acid insertion a the COOH terminal of the protein.[215,216] Agrin may have a supportive role in BBB formation, as it has been shown to accumulate in the BM during the developmental period when permeability of blood vessels in the brain reduces.[215,217] This is further supported by mice studies showing the localization of AJs and associated proteins in BMECs with agrin, and that has a stabilizing effect on AJs.[216]

Agrin KOs are embryonic lethal.[218] Interestingly, z+ isoform-deficient mice were born with smaller brains and died shortly after birth due to neuromuscular defects but additionally showed lower levels of many other agrin isoforms, such as z0 reduction.[219] It is our understanding that there are no published in vitro models of the BBB which utilize agrin coatings.

13.6. Fibronectin

Fibronectin is a major adhesive glycoprotein, known to be involved in cell interaction with collagens, and is important in development and wound healing.[149] Wang and Milner proposed a model in which fibronectin promotes aspects of BMEC behavior consistent with an angiogenic phenotype, including cell survival, proliferation, and migration, unlike laminin which promoted EC differentiation and stabilization.[149] While there is an initial downregulation of Rho activity during cell spread and high cytoskeleton dynamics, in cells cultured on fibronectin-coated substrates, there is an increase in Rho GTP loading as cell spread is completed. This correlates with focal adhesion and stress fiber assembly and then eventual return to baseline.[220,221]

13.7. SPARC

SPARC, also known as osteonectin or BM-40, is a BM protein investigated as a possible modulator of TEER in BMECs, shown to have an antiadhesive effect on BMECs and reduce TEER.[222] SPARC is predominantly antiadhesive and is expressed by astrocytes both in vivo and in vitro.[223–226] It has also been suggested to be involved in cerebral endothelial cell differentiation.[227] However, when endothelial cells are grown on inserts coated with SPARC, it has a negative effect on TEER measurements compared with controls.[222]

13.8. In Vitro Results

The importance of the BM proteins in the maintenance of the tightness of the BBB has become evident, as confirmed by various in vitro studies of cell cultures grown on protein-coated Transwell inserts mimicking the BBB.[228] In a refreshing study by Katt et al., different BM protein coatings were used in the Transwell model, either on the membrane or on gels placed on the Transwell membrane.[229] They found that the coverage of human brain microvasculature endothelial cells (HBMECs) on different collagen IV (COL IV) gels greatly depended on stiffness, with their lower-stiffness gels and lower cell coverage decreasing over time, in comparison with their stiffer gels. TEER values additionally substantially decreased from Transwell membranes when cells were coated on the less-stiff collagen IV gels, although this may also be caused by the rough surface morphology of the gels, which prevents the formation of a seal along the sidewalls. They additionally compared different BM coatings such as fibronectin, laminin, collagen IV, perlecan, and agrin. BMECs grown on COL-IV gels were not fully confluent three days after seeding, and overall, the use of BM coating increased cell coverage. Cells grown on perlecan, or on combinations of perlecan with other tested proteins cells, showed poor adhesion. Agrin gels, however, showed complete monolayers, and the authors stated them as important in promoting the adhesion of BMECs, although BMECs showed weak claudin-5 staining and poor barrier function. However, the addition of fibronectin restored the barrier.[229]

Thomsen et al. set up to investigate whether the co- or triculture of murine BMECs affected the expression of different BM proteins.[45] They found there was no significant difference in the gene expressions of laminin-α5 nor COL4-α1, between mono and coculturing conditions. In proteome analysis comparing BMEC monoculture and coculture with glial cells, no statistically different compositions of BM proteins were found. BMECs expressed all the major BM proteins mentioned earlier, while also expressing laminin-421, SPARC, fibulin-1/-2, fibronectin, collagen-type XVIII, and thrombospondin 1.

Tilling et al. found that inserts coated in 1:1 type IV collagen and fibronectin (FN), and FN and Laminin, showed higher TEER measurements then the components alone, with FN/Laminin showing the highest TEER values in porcine brain capillary endothelial cell monolayers.[222]

14. Mechanical Properties of the BM and Barrier Formation

The acellular BM may hold hidden insights into the formation of the BBB. As the majority of current in vitro models utilize Transwell inserts, which mimic none of the physiological properties of the native BBB BM, there exists a lack of understanding on how different physical properties, such as topography, thickness, and stiffness, affect the formation and maintenance of BBB properties. For example, it is not yet understood as to why the BM forms hexagonal electron-dense networks of collagen IV and fibronectin.[192,223–226]

The effect of BM topography remains a topic of active research and, particularly in terms of barriergenic properties, remains incompletely understood. Both the topography, as a dense structure of interconnected protein networks, and the thickness of the BM act as additional physical barriers for immune cell migration across the BBB and permeability of large molecules. While studies of BM stiffness and topography are of particularly interest in the vasculature, this is an emerging area of understanding, although these are limited to major vessels, such as the aorta and carotid artery, to date.[230,231] The measurement of BM stiffness in vivo remains extremely challenging, although overall, it is assumed that the BM stiffness is determined by a combination of protein packaging and the hydration state of the BM.[232]

The biomechanical properties of the BM, such as thickness, stiffness, and elasticity, are of particular interest in in vitro BBB models, as vascular stiffening is associated with cardiovascular pathologies as well as stroke.[233–237] However, our understanding of the mechanisms linking barrier permeability and junctional disruption with matrix stiffness is still emerging, largely due to the lack of appropriate in vitro models where such tunable parameters exist.[138,238–242] The majority of in vitro BBB modeling is still conducted on substrates with stiffnesses in the MPa–GPa range, such as Transwell inserts, glass, and tissue culture plastic, whereas the proposed in vivo, brain ECM is $\approx$1 kPa.[232] Furthermore, our emerging knowledge of the elastic properties of the BM is constantly being updated. While it has previously been considered a purely linear elastic material, a more recent study using breast gland BM concluded that the BM acts more as a fluid-filled porous elastic solid.[138,240,243] Therefore, when designing an in vitro mode, poroviscoelastic frameworks may be considered to better represent the in vivo BM mechanical behavior.

One driver of AJ junctional disassembly and vascular leakage is increased contractility in BMECs, although actin fiber formation has also been tied to AJ presentation and barrier permeability.[233,243,244] Studies have shown a link between the edge presentation of VE cadherin and the formation and maturity of both the barrier as a whole and the junctions between BMECs. This is specifically true of linear VE cadherin structures that run parallel to the cell boundary, not with discontinuous focal adherens with serrated morphologies, which are indicative of an immature junction.[233] BMEC AJ presentation has also been shown to be modulated through the alteration of subendothelial stiffness via myosin II-mediated contractility.[240] Soft substrates and cells with low tension where shown to form stable, linear AJs, whereas cells experiencing high tension or on stiff substrates showed discontinuous junctions and increased permeability.[245] ZO-1 has been recognized as a regulator in cell–cell tension and junctional assembly through the organization of actomyosin and actin, which themselves are involved in the cell response to substrate stiffness.[130,144,246] Gray et al. found that junctional coverage, calculated using ZO-1 presentation, was the lowest on glass and increased on 1 kPa hydrogels, showing improved barrier properties on biologically relevant stiffness.[247] The cells grown on glass showed their highest contractility, which induced the poorest barrier properties of all conditions tested, which improved in response to blebbistatin, with decreasing contractility. This is supported in previous work by Onken et al., that showed increased actin stress fibers in the HBMEC cell line when seeded on polyacrylamide gels of increasing stiffness, which correlated with immature VE cadherin junctions.[240]

15. The Effect of FSS on BMEC

FSS, a tangential force caused by circulating blood, is a critical factor affecting vascular remodeling, endothelial cell polarity, and barrier properties.[144,248] The endothelial monolayer acts as a transduction interface for hemodynamic forces, which can be subsequently transmitted to the surrounding astrocytes, pericytes, and microglia of the NVU.[130,144,246] The BBB dynamically responds to FSS, with evidence that capillary-like shear stress promotes BBB functions and facilitates the differentiation of VE BMECs, with the defining characteristics of low permeability, tight junctions, and low cell proliferation, as summarized in **Figure 5**.[245,249]

At a cellular level, shear stress activates several flow-regulated transcription factors, among which are Kruppel-like factory family proteins (KLF2 ad KLF4), YAP, and NF-κB.[248,250–252] KLF2 and KLF4 are known to be expressed in response to laminar flow, causing an increase in eNOS expression and reducing endothelial permeability.[248] They further reduce the expression of known inflammatory proteins. Mechanical stimuli also regulate the cellular location of and translocation of YAP, unidirectional laminar flow, causing nuclear translocation in a transient manner, and disturbed or oscillatory flow, facilitating nuclear translocation in a more sustained manner.[248,251]

At a larger scale, VE cells outside the brain microvessels become elongated and aligned with the blood flow through a process of the redistribution of junctional proteins. BMECs reportedly do not deform from their cobblestone morphology under mechanical shear stress.[253–256] It additionally promotes cell adhesion through increase in integrin–ligand binding and induces cell alignment in the direction of flow. This is due to the increased expression of TJ and AJ proteins in BMECs, particularly ZO-1, which is advantageous to BMEC barrier properties and prevents morphological transitions, such as elongation.[257–259] While this offers to be a promising avenue in

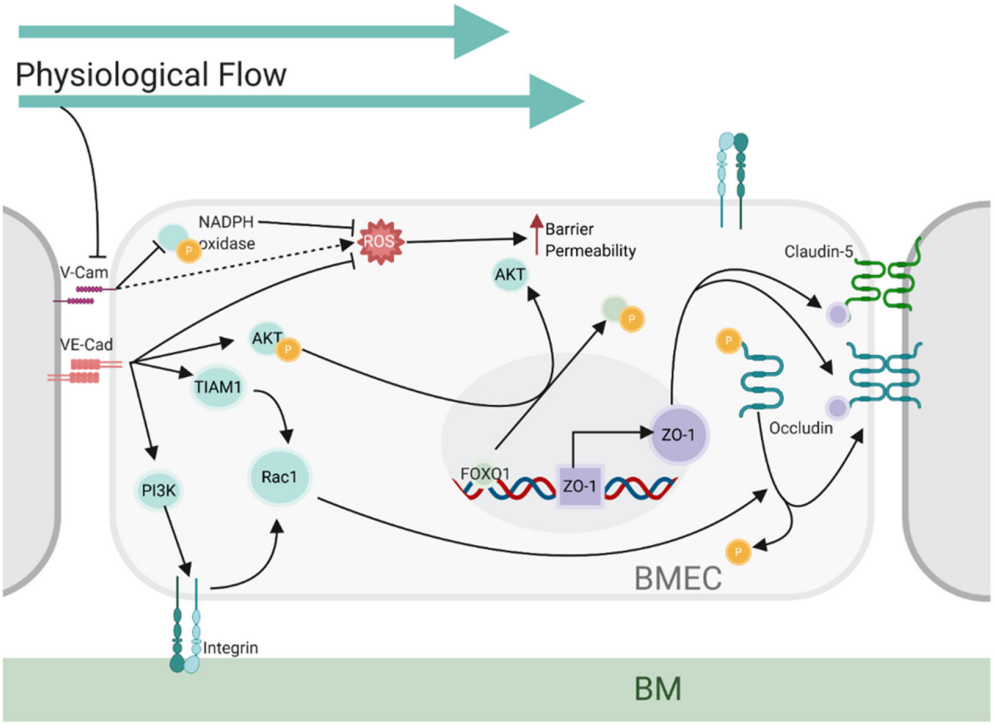

Figure 5. Effect of physiological shear stress on BMECs. Physiological shear stress acts both as an inhibitor of ROS-mediated inflammation and as barrier disruption, as well as increases expression and localization to cell–cell junctions of TJ and AJ proteins. The pathway through which the increase in AJ and TJ protein expression occurs appears to be largely unknown, although the increased expression of ZO-1 and therefore cell–cell junction localization of occludin, and potentially claudin-5, occurs through the AKT-FOXO1 pathway. It further allows for cytoskeleton reorganizing and intracellular tension.

BBB in vitro model creation, evidence suggests that cell source should be considered. Andrews et al. reported that the initiation of mechanotransduction in adult BMECs and fetal BMECs may be prevented by transformation into an immortalized D3 cell line.[260] It would be of interest to produce a series of thorough experiments by comparing and contrasting immortalized BMEC cell lines to the iPSC-counterparts, in particular in regard to the use of shear stress as a barriergenic agent so as to establish within the BBB community preferred cell line.

A large FSS range has been reported for brain capillaries, from 5–23 dyne cm^{-2} in humans to 20–40 dyne cm^{-2} in rats.[130,261] The evidence indicates that capillary-like shear stress promotes BBB functions and facilitates the differentiation of the BBB phenotype in VE cells, while additionally increasing the tightness of the endothelium and control of substance exchange at the blood–brain interface.[146,245,249] Physiological laminar shear stress is known to increase the expression of tight and adherent junctions, expression of related RNAs, drug efflux transporter genes, and causes an increase in TEER values, as well as localization of tight junction proteins to the cell–cell junctions through cytoskeleton reorganization.[145,146,245,249,262] Laminar shear stress also inhibits proliferation of endothelial cells which is early marker in cell differentiation.[245,263]

A physiological-like shear stress of 8 dyne cm^{-2} furthermore relieves the disruptive effect of interleukin (IL) on BMECs.[257] This was further evidenced by research showing the disruptive effect physiological shear stress has on tumor necrosis factor-a (TNF-a) and IL-6 and the reversibility of this under high shear stress.[249,264] Garcia-Polite et al. additionally observed the increased expression of TJ-related genes, adhesion molecules, drug transporters, and integrins related to the BBB phenotype, as well as an overall increase in cytoskeleton protein content at 6.2 dyne cm^{-2} shear stress.[262] However, low shear stress (both 4 and 12 dyne cm^{-2}) was shown to have no effect on the expression nor localization of TJ proteins claudin-5, occludin, or ZO-1 in iPSC-derived HBECS.[257] In response to 10–20 dyne cm^{-2}, BMECs were shown to upregulate TJ proteins ZO-1 and claudin5, as well as BBB formation indicator, p-GP. At 10 dyne cm^{-2}, these were shown to have increased localization to cell–cell borders.[265] There is additional increase in several cytochrome-450 enzymes, which have been suggested to regulate the entry of substances to the brain in an interdependent manner.[257,258] Rochfort et al. further showed that the suppression of nicotinamide adenine dinucleotide phosphate (NADPH) reactive oxygen species (ROS) production was stabilized in physiological shear stress conditions. This was accompanied with increased expression and decreased tyrosine and threonine phosphorylation of TJ and AJ proteins.[262,266] It has also been suggested that laminar shear stress decreases the negative effects brought on by proinflammatory cytokines through the production of a compensatory antioxidant defense. This neutralizes ROS as well as inhibits thioredoxin-interacting proteins.[262,267]

Just as there is substantial evidence on the protective effects of physiological flow, the pathogenic effect of disturbed flow and high shear stress and its ability to increase oxidative stress and inflammation, characterized by increased expression of

inflammatory cell-anchoring proteins such as VCAM1, has also been shown.[268] Steady flow inhibits VCAM1 by increasing antioxidant mechanisms and blocking inflammatory signaling through inhibiting TNF-mediated activation of ASK1 kinase1-JNK/p38 pathway.[262,268] Proinflammatory cytokines TNF-a and IL-6 induce the activation of NADPH oxidase, therefore leading to the generation of ROS in VE cells. NADPH oxidase-dependent ROS has been shown to increase paracellular permeability through the reduction of expression and the disassembly of inter-endothelial AJ and TJ proteins.[266]

High shear stress is sensed by G protein-coupled receptors, which subsequently activate Src and ERK1/2.[235,262] There has been a recent advancement in understanding the flow sensitivity of the tyrosine protein kinase (Src)/extracellular signal-related kinase (ERK1/2) pathways in VE monolayers. Under physiological conditions, these are inhibited, and there is an additional upregulation of TJ markers, but pulsatility and high shear stress lead to downregulation of TJ markers.[144] In a high shear stress model (40 dyne cm^{-2}) and pulsatile flow model, Garcia-Polite et al. showed the downregulation of TJ marker expression and ZO-1 translocation into the nucleus.[262] ZO-1 translocation to the nucleus is inversely related to the number and maturity of cell contacts, and can be a trigger for barrier loss, as ZO-1 protein downregulation is consequently followed by claudin-5 downregulation.[244] There is further decrease in the expression of P-gp.[31,262]

ECM metalloproteinases (MMPs) substrates include components of TJs and AJs as well as the ECM which surrounds endothelial cells and pericytes in the BBB. While MMP expression in a healthy brain is relatively low, both clinical and experimental studies have shown that MMP-9, −3, and −2, among others, are activated and upregulated after ischemia, which is accompanied by an increase in BBB permeability.[269]

16. Geometry

While the influence of curvature on BMECs in BBB in vitro is largely unexplored, the cylindrical geometry of microvessels imposes curvature, which may be a very intuitive effect of cylindrical geometry, but additionally limits the number of cell–cell interactions. Growing on a flat surface offers cells more cell–cell contacts than cylindrical geometries, where cells are limited to contacts with the cells either in the side in the cylinder and themselves.[270–272] BMECs in confluent monolayers were shown to resist elongation and alignment due to curvature, a trait which likely evolved to limit the total length of cell–cell junctions in a given length of a blood vessel. This was found to be different than other ECs, which minimized the effect of the curvature through elongation and alignment, further supporting this divergence between BMECs and other ECs.[272] Cylindrical geometry and shear stress are not prerequisites to achieve TJ formation, although there is potential for them to enhance or reduce the GF requirements when making an in vitro BBB model.[270–272] Overall, while cylindrical geometry increases the complexity of in vitro models, it provides some promising avenues for those brave enough to explore it, and there is space for more novel research in this area regarding the BBB.

17. Hypoxia

Hypoxia was seen as a promising method to induce barriergenesis after in vivo studies showed that barriergenesis occurred under hypoxic conditions during embryonic development.[273] In their iPSC-based BBB chip, Park et al. found that hypoxic conditions during the iPSC differentiation protocol produced a significant increase in cell–cell adhesion molecule mRNA levels and improved barrier qualities, such as higher TEER values.[272] It is thought that these improvements to barrier properties arise from the interplay between the Wnt/B-catenin signaling cascade with hypoxia-induced factor 1a (HIF1a) signaling, which Park et al. were able to show through upregulation of HIF1a in their hypoxic conditions.[274] This offers a potential work-around for the induction of hypoxic conditions, as HIF1a-mimetic or stabilizing compounds can be used in addition to or instead of the use of a hypoxic chamber.

While improved barrier properties have been seen in iPSC-based BBB in vitro models when exposed to hypoxic conditions, it should be noted that these are seen exclusively in iPSC cells, and the opposite effect has been seen in non-iPSC cell lines.[257] Fischer et al. showed VEGF/VEGF receptor-mediated permeability induced by hypoxia in primary porcine BMECs.[275] It was further shown that hypoxia-induced permeability could by inhibited by YC-1 through inhibition of HIF-1a accumulation as well as VEGF production by hypoxia-treated immortalized rat BMECs.[276] While promising, there are a lack of similar studies to support their findings on improved barrier properties after hypoxic treatment, and whether this can substantially and consistently improve and prolong barrier properties remains to be seen.

18. Conclusion and Perspective

One major conclusion from the analysis from the known barriergenic factors is that the greatest barrier that researchers in the BBB field face, especially for in vitro dynamic models, lies in benchmarking the models. As shown in **Table 1**, the dynamic BBB modeling community is the Wild West of in vitro modeling, with a wide variety of BBB metrics used, making a comparison of these models, or the large range of FSS they use, near impossible.

To combat this lawlessness, the community at large should consider standardized measurements that should be seen as a prerequisite for publication. While TJ visualization and quantification, either through Western blot or immunofluorescence, is the easiest quantification method to use in dynamic in vitro models, these are, for the purpose of comparison across models, qualitative data. In terms of quantitative barrier property visualization, TEER measurements seem to be the optimal solution, given that these are used to benchmark static models like Transwell. However, due to the different methods of measuring TEER in the papers shown in Table 1, which may not be comparable, as well as the difficulty in implementing these in dynamic models, TEER measurements may not be as useful in the dynamic in vitro model field. We consider the use of permeability assays to be an easy comparison of the barrier formation in these models. It is likely that in time, the field gravitates to certain

Table 1. Breakdown of barrier quantification in in vitro models utilizing FSS..

Measure for BBB characteristics	Reporting of BBB characteristics	References
Permeability 70 kDa fluorescent-conjugated dextran 688 Da propidium iodide TEER TJ Immunofluorescence ZO-1	Permeability Permeabilities reported as "percentage change" between static control and FSS condition. Decreased permeability in response to FSS. TEER Reported graphically, $\approx 500\,\Omega\,cm^2$ (coculture conditions). TJ Immunofluorescence Increased expression of ZO-1 in response to FSS.	[245]
Permeability 10 kDa FITC-dextran Immunocytochemistry ZO-1, occludin, claudin-5, von Willebrand factor	No reporting of relevant data on the effect on FSS on BMEC and barrier properties.	[282]
TEER	TEER TEER reported as Ω rather than $\Omega\,cm^2$ (and no surface area provided)	[249]
TJ Immunofluorescence Claudin-5, occludin, ZO-1 Western blot LAT-1, ZO-1, CLDN-5 quantitative polymerase chain reaction (qPCR) ABCN1, CDH5, CLDN5, OCLN, SLC2A1, TJP1	TJ Immunofluorescence No clear difference between 4 dyne cm^{-2} and static conditions. Western blot No significant difference between shear conditions and static for CLDN-5 and LAT-1. ZO1 expression decreased at 4 dyne cm^{-2} compared with the static condition. qPCR No significant difference between 4 and 12 dyne cm^{-2} conditions and static conditions.	[145]
Permeability Nitrazepam, nordaseoam, diazeam, flurazepam	Permeability Decreased permeability in hollow-fiber model in comparison with Transwell model.	[283]
Permeability C14 Sucrose (342 Da) TEER	Study on the effect of normoxia–normoglycemia or hypoxia–hypoglycemia and IL-6 and TNF-a release, not of the effect of shear stress. TEER Measured as % of control	[146]
Permeability Rhodamine 123 10 kDa Dextran-Aexaflour 647	Permeability Singular shear stress tested.	[262]
TEER TJ Immunofluorescence ZO-1	TEER Improved TEER from Transwell insert to BBB chip (from $28.2 \pm 1.3\,\Omega\,cm^2$ to $36.9 \pm 0.9\,\Omega\,cm^2$) TJ Immunofluorescence Used to verify the presence of BMECs in the correct channel.	[284]
Permeability D-Glucose, sucrose, mannitol, among others TEER Gene analysis	Permeability Decreased permeability of metabolic substrates. TEER Flow ($\approx 700\Omega\,cm^2$) Static ($\approx 100\,\Omega\,cm^2$) Gene analysis Increased expression of occludin, claudin-5, ZO-1, ZO-2, N-cadherin, E-cadherin (reported as fold change flow/no flow).	[284]
Permeability 40 kDa FITC-dextran TJ Immunofluorescence Claudin-5, VE cadherin	Permeability Not shown for conditions without TNF-a or IL-6 added. TJ Immunofluorescence Improved localization of claudin-5 and VE cadherin to cell–cell junctions.	[205,281]
Permeability 40 kDa FITC-dextran TJ Visualization ZO-1, Claudin--5 Western blot pTyr-Occludin	Permeability Permeability calculated as fold change from static conditions. Reduced permeability in response to shear stress compared with static conditions. TJ Visualization Greater localization to cell–cell junctions at 10 dyne cm^{-2} than in static conditions or after 24 h, it is reduced by 1 dyne cm^{-2} Western blot Decreased phosphorylation of occludin in response to chronic 10 dyne cm^{-2} shear stress compared with static condition.	[205,281]

Table 1. Continued.

Measure for BBB characteristics	Reporting of BBB characteristics	References
Permeability	Permeability	[178,205]
Gabepentin, traxoprodil, sertraline, varenicline, ethosuximide, sunitinib	Only sertraline and the unnamed drug show significant decrease in dynamic model compared with Transwell monoculture.	
TEER	TEER	
TJ Immunofluorescence	Transwell monoculture ≈25 Ω cm^2	
ZO-1	Dynamic μBBB model monoculture ≈200 Ω cm^2	
	TJ Immunofluorescence	
	Used to verify BBB characteristics of two cell lines.	
TJ Visualization	TJ Visualization	[205,281]
	Measurement of junction length in response to different curvatures.	
Western blot	Western blot	[205,281]
Claudin-5, ZO-1	Comparison of steady and pulsatile shear stress (0, 10, 20, 30, 40 dyne cm^{-2}).	
TJ Immunofluorescence	Pulsatile and high (40 dyne cm^{-2}) shear stress-downregulated TJ expression.	
ZO-1	TJ Immunofluorescence	
	Increased location of ZO-1 to cell–cell borders in response to low, steady shear stress.	
TJ Immunofluorescence	BMECs resist elongation in response to shear stress.	[205,281]
ZO-1		

molecular weights of molecules used to measure permeability of the barrier. While dynamic in vitro models of the BBB are an emerging field, the inability to compare models across papers, the effects of different conditions, does the field a great disservice.

A second conclusion is that there appear to be many avenues through which barrier formation may be induced in BMECs, and the field appears to be at an exciting precipice of discovery. This is provided experiments comparing the relative effect on barrier formation of different signaling pathway activators, BM protein coatings, scaffold stiffnesses, and FSS. Despite substantial progress and numerous inspiring in vitro models, we still lack answers to major questions.

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Conflict of Interest

The authors declare no conflict of interest.

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blood–brain barrier, endothelial cells, flows, growth factors, mechanics

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