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REVIEW
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Overview of Crosstalk Between Multiple Factor of Transcytosis in Blood Brain Barrier

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Blood brain barrier (BBB) conserves unique regulatory system to maintain barrier tightness while allowing adequate transport between neurovascular units. This mechanism possess a challenge for drug delivery, while abnormality may result in pathogenesis. Communication between vascular and neural system is mediated through paracellular and transcellular (transcytosis) pathway. Transcytosis itself showed dependency with various components, focusing on caveolae-mediated. Among several factors, intense communication between endothelial cells, pericytes, and astrocytes is the key for a normal development. Regulatory signaling pathway such as VEGF, Notch, S1P, PDGFβ, Ang/Tie, and TGF-β showed interaction with the transcytosis steps. Recent discoveries showed exploration of various factors which has been proven to interact with one of the process of transcytosis, either endocytosis, endosomal rearrangement, or exocytosis. As well as providing a hypothetical regulatory pathway between each factors, specifically miRNA, mechanical stress, various cytokines, physicochemical, basement membrane and junctions remodeling, and crosstalk between developmental regulatory pathways. Finally, various hypotheses and probable crosstalk between each factors will be expressed, to point out relevant research application (Drug therapy design and BBB-on-a-chip) and unexplored terrain.

Keywords: blood brain barrier, transcytosis, developmental, mechanical stress, cytokines, miRNA, physicochemical, tight junctions

INTRODUCTION

Blood Brain Barrier Concept and Constituents

The neurovascular unit is a complex system of blood vessels and nerves, together with neighboring cells and the extracellular matrix. There are numerous similarities, functions, interactions, and remodeling process interconnected between these two systems (vascular and nervous) in the body. A prominent example is the formation of the blood brain barrier (commonly abbreviated as BBB).

The BBB is a complex mixture of various transport systems located between blood vessels and the brain. It is predominantly composed of endothelial cells, neurons, oligodendrocytes, pericytes, astrocytes, microglia, and surrounded by the extracellular matrix which is mainly composed of collagen and laminin. The primary function of the BBB is to provide a safety mechanism to prevent potentially harmful material from entering the brain, while still enabling the transcytosis of nutrients and signaling factors. Failure to maintain the BBB integrity results in abnormalities, mainly infectious diseases such as meningitis, multiple sclerosis, neurodegenerative diseases, and a plethora of brain disorders. Understanding the exact mechanisms of transcytosis in the BBB will provide useful insights for diseases and their possible treatments.

Among this complex mixture of cells, endothelial cells (ECs) are squamous cells that form the lining wall of the vascular system. The differentiation of endothelial cells is organ and tissue specific, modified via responses of the appropriate gene expression toward hemodynamic forces, extracellular stress, interaction with adjunct cells, and matrix secretions (Marcu et al., 2018). Brain microvasculature endothelial cells (BMECs) have an adaptation to form tight barriers and also active transport protein, such as P-glycoprotein (Stebbins et al., 2015). Expression of p-glycoprotein at BMECs, which is one of the efflux transporters, shows dependency to β-catenin upregulation (Lim et al., 2008). This clearance process showed that transcytosis in the BBB can be accomplished from abluminal to luminal sides. The response and behavior of BMECs also shows dependence on cytokine stimuli (Carroll et al., 2015). The primary formation of BBB tight junction is also regulated by ECs, together with other cells. Pericyte is interconnected with ECs in microvasculature, and act as the co-regulator of ECs. In the BBB, this type of cell plays an important role as one of the supporting cell types for BBB integrity and permeability via cell to cell communication and ECM to cell communication. Pericytes have been shown to regulate the differentiation of ECs into HBMECs. These cells also interact with astrocytes to induce polarization of astrocytes surrounding the blood vessel. A lack of pericytes in the mouse model and cell culture experiment causes increased BBB permeability. Treatment of imatinib, which depletes pericytes, inhibits the release of tracers from ECs to the brain via transcytosis. Thus it can be inferred that pericytes have a role in regulating transcytosis in the BBB (Armulik et al., 2010). Astrocytes are part of the modified glial cells. The primary role of these cells is to support neurons. In the BBB complex, the foot of astrocytes encircles ECs and blood vessels. Astrocytes also enable nutrients transport and delivery to neurons. A recent study showed astrocytes may promote blood flow and microvasculature remodeling (Figley and Stroman, 2011). One of the prominent mechanisms for tight junction regulation by astrocytes is by regulating the accumulation of agrin, a heparin sulfate proteoglycan which is important for BBB integrity in the basal lamina (Abbott et al., 2006).

However, the exact mechanism whereby transcytosis may be affected by co-regulation between ECs, pericytes, and astrocytes is still unclear (**Figure 1**). Various relevant factors also still need further elucidation. This review will focus on revealing and summarizing current findings of interaction between ECs, pericytes, and astrocytes as well as the crosstalk of factors which may affect transcytosis in the BBB.

Paracellular and Transcellular BBB Permeability

Molecular transport across the BBB is highly dependent on its permeability, which is defined by the paracellular pathway (molecules cross through ECs junctions) and the transcellular pathway (endocytosis followed by endosomal rearrangement and exocytosis from the cell). These mechanism contributes to the brain's nutrition supply, as comprehensively reviewed by Lalatsa and Butt (2018). BMECs have a specialized tight junction in order to prevent undesirable paracellular transport and consequently direct the necessary molecules by a specific transcellular pathway (Zhou et al., 2019). The importance of transcytosis is emphasized under certain conditions including hypertension and strokes (Knowland et al., 2014). When pathogenesis occurs, the primary response is upregulation of caveolin, which facilitates caveolae assembly (Knowland et al., 2014). A similar finding in the case of multiple sclerosis also stresses the significant upregulation of caveolae-mediated transcytosis (Lengfeld et al., 2017). This response may be a reaction to facilitate the recovery process, which requires a higher supply of nutrients, as well as clearance of toxic materials.

Transcellular pathways in cells are categorized into clathrin mediated and non-clathrin mediated. Detail mechanism in several types of transcytosis can be studied extensively in the comprehensive review by Pulgar (2019) and Villaseñor et al. (2019). Non-clathrin mediated transport makes use of dynamin, coat proteins, small GTPases, and RhoGAP proteins. Caveolae-mediated transcytosis is one of the non-clathrin mediated pathway. Transportation between cells using extracellular vesicles (EVs) is very important during development and maintenance of the BBB. A recent study showed atheroprotective intercellular communication via EVs between ECs and smooth muscle cells through miRNAs regulation (Hergenreider et al., 2012). Based on these interactions, the transport pathway plays an essential role in intercellular communication. Which brings us to question, is there any interaction between paracellular and transcellular pathways? In the case of water transport, lack of protein transporter in the paracellular path may significantly impair the transcellular path (Kawedia et al., 2007). It has been elucidated that Cav-1 also plays a part in the regulation of TJ protein expression in HBMECs (Song et al., 2007), indicating a central role for the transcellular pathway in the BBB maintenance (**Figure 2**).

Caveolae Biosynthesis

There are two main proteins which play a major role in caveolae biosynthesis. The first is caveolin, along with multiple isomers (Cav-1, Cav-2, and Cav-3). The second one is cavin, which currently has four known isomers. These two components are an essential part of caveolae, and the absence of either one

will significantly suppress the amount and function of caveolae. Lipodystrophic phenotype has been observed both in mice and humans lacking caveolae, suggesting its importance for lipid transcytosis. A recent discovery has showed that binding of these components with phosphatidylserine plays a crucial role in caveolae formation (Hirama et al., 2017). Cav-1 and Cav-2 will form 8S oligomer on endoplasmic reticulum, followed by transport via COP II (coat protein complex II) to the Golgi. In this place, 8S Cav-1/Cav-2 will undergo oligomerization with cholesterol forming the 70S subunit, followed by transport to plasma membrane (Hayer et al., 2010).

In the context of the BBB, HBMECs have been modified to limit the number of caveolae, thus limiting leakiness and transcytosis. One reason for this regulation is the role of Mfsd2a, which is a facilitator at the cell membrane to transport the LPC-DHA supply to the brain. In contrast with the increasing lipid content, the cell will undergo suppression of caveolae amounts (Andreone et al., 2017). Meanwhile, elevated caveolae occurrence is a sign of pathogenesis (**Table 1**), indicating BBB leakiness with interaction of several other factors of transcytosis (Gu et al., 2012). However, attenuation of caveolae expression inhibits the expression of TJ proteins (Song et al., 2007) and also accelerates neurodegeneration and aging (Head et al., 2010). Keeping the balance of caveolae number for appropriate amount of transcytosis has been a challenging aspect for maintaining BBB stability.

TABLE 1 | Several diseases with abnormality concerning transcytosis, as well as abnormality of the Cav-1 as the component of endocytosis.

Pathological condition with implication for abnormality of transcytosis in BBB
➢ Focal cerebral ischemia
• Upregulation of Cav-1 and Cav-2 (Xie and Lu, 2018)
➢ Moyamoya disease
• Downregulation of Cav-1 (Chung et al., 2018)
➢ Dengue hemorrhagic fever
• Upregulation of Cav-1 (Chanthick et al., 2016)
➢ Traumatic brain injury
• Upregulation of Cav-1 phosphorylation (Nag et al., 2009)
➢ Alzheimer disease
• Upregulation of Cav-1 (Gaudreault et al., 2004)
• Downregulaton of PICALM (Ando et al., 2016)
➢ Influenza associated encelopathy
• downregulation of Cav-1 (Imakita et al., 2019)
➢ Cortical spreading depolarization
• Upregulation of Cav-1 (Sadeghian et al., 2018)
• Independent of paracellular (Sadeghian et al., 2018)
➢ Parkinson disease
• Upregulation of Cav-1 (Cha et al., 2015)
• Downregulation of TJ protein (Kuan et al., 2016)
➢ Multiple sclerosis
• Upregulation of Cav-1 (Lutz et al., 2017)
➢ Amytrophic lateral sclerosis
• Endosomal rearrangement (Rabs) instability (Farg et al., 2014)
• Exocytosis (SNARE) abnormality (Kawamata et al., 2014)

SHARED DEVELOPMENTAL PATHWAY

Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) has cytoprotective effects on ECs by preventing apoptosis, mediated through phosphatidyl inositol 3-kinase (PI3K)/Akt pathway (Ferrara et al., 2003). Action of VEGF in angiogenesis is prominent, but for maintenance at the latter stages of development, pericytes will take over this function. Instead of maintaining, VEGF reduces barrier robustness through the nitric oxide synthase (NOS)/cGMP-dependent pathway (Mayhan, 1999). The activation of eNOS in Human Umbilical Vein Endothelial Cells (HUVECs) is also crucial for caveolae formation (Bai et al., 2017). Endocytosis of VEGF receptors are also caveolae dependent, inferred from a study of leukemia cell line (Caliceti et al., 2014). *In vivo* retina study using *Macaca fascicularis* shows administration of VEGF will induce angiogenic phenotype both in ECs and pericytes, thus it might be resulting in BBB instability in adults (Witmer et al., 2004). This side effect can be neutralized by administration of Ang1, which attenuates the activity of MMP-2 and MMP-9, without disturbing the angiogenesis in mice cerebrovascular (Valable et al., 2005). Activation of VEGF/PI3K/Akt pathway may induce actin reorganization in human angioma cells (Wang et al., 2011), a process known to be crucial for endocytosis and endosomal rearrangement (Podar and Anderson, 2008; Römer et al., 2010; Coelho-Santos et al., 2016). This might be one of the ways for VEGF controlling caveolae and transcytosis in the ECs. In the early symptoms of stroke and cerebral ischemia, regions of the brain can end up in hypoxic conditions. During hypoxia, VEGF will be secreted from the pericytes which affects claudin-5 and BBB integrity via paracellular pathway (Bai et al., 2015). Other secreted cytokines such as IL-6 and G-CSF attenuates BBB transcellular robustness via an unknown mechanism. Another study also highlights astrocytes role in BBB integrity attenuation for VEGF-A secretion during pathological condition (Argaw et al., 2012). Balance between VEGF activities to properly upregulate transcytosis while maintaining BBB stability still needs further investigation.

Platelet-Derived Growth Factor (PDGF)-B/PDGF Receptor Beta (PDGFRβ)

At early stages of vessel formation, tip ECs will secrete PDGF-B to promote the recruitment of pericyte progenitor cells. This mitogen growth factor will be detected by PDGFRβ on the pericytes, leading the migration to tip ECs in the process of angiogenesis (Hellström et al., 1999). The expression will gradually decrease following vessel maturation, but irregularities will arise in the pathological conditions of several diseases as indicated by the increasing PDGF-B expression in mature vasculature (Gallini et al., 2016). This pathway still persists in the postnatal angiogenesis, indicating an important communication between pericytes and endothelial progenitor cells (EPCs) (Baumgartner et al., 2010). Lack of pericytes caused by diminished signaling of PDGF-B/PDGFRβ also showed fatality in mice phenotypes (Lindahl et al., 1997).

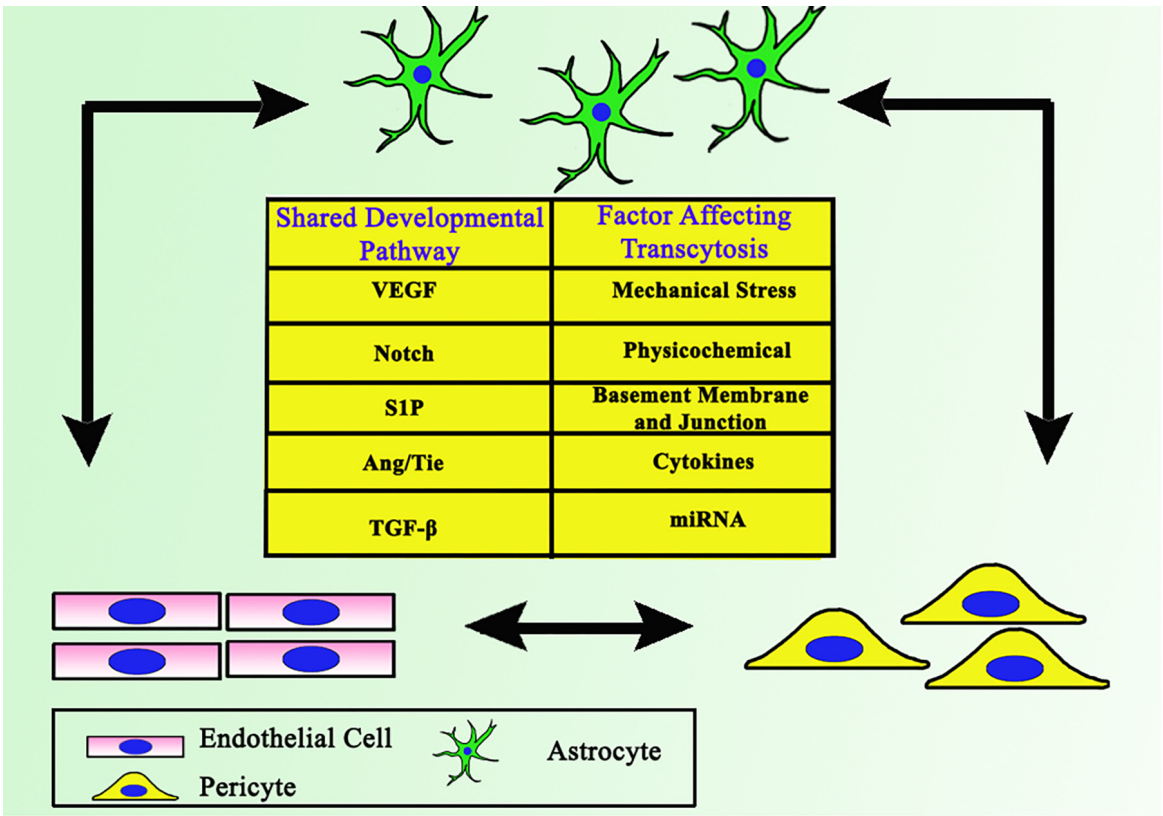

FIGURE 1 | Close interaction between ECs, astrocytes, and pericytes as the backbone of BBB. Together they share some common developmental pathways, which involves each cell to support one another.

In the neurovascular unit within adult mice, the expression of PDGFRβ exclusively persists only at pericytes (Winkler et al., 2010), differing from humans which also retain it in general ECs (Muhl et al., 2017). Transcription factor Foxf2 maintains PDGFRβ expression specifically in brain pericytes to support BBB integrity (Reyahi et al., 2015), indicating the role of the FOX family for maintaining the BBB. Endocytosis receptor Ephrin-B2 supports the internalization and also signaling of PDGFRβ in mice vascular smooth muscle cells (Nakayama et al., 2013), leaving room for further study in brain pericytes. Reactivation of PDGF-B/PDGFRβ signaling through administration of TGF-β can restore the function of the BBB after focal cerebral ischemia (Shen et al., 2018), indicating a crosstalk shared by these two pathway. *In vitro* experiment also showed protective effects of PDGF-BB on astrocytes through activation of antioxidant mechanism (Cabezas et al., 2018). Mice model also support this findings, emphasizing astrocytes roles to recover neuronal damage after hemorrhage (Zhou et al., 2019). Another complementary communication is the PDGF-D/PDGFRβ signaling which is supported by the co-receptor Neuropilin1 (NRP1) in ECs (Muhl et al., 2017). This communication involves NRP1 translocation, indicating a regulation for other pathways involving NRP1. NRP1 is also a co-receptor for the VEGF signaling pathway, indicating a crosstalk between these two pathways. NRP1 also regulates HMGB1, which induces caveolae formation in general ECs (Ma et al., 2019). Possibly PDGF signaling is able to manage transcytosis via this pathway, additionally activating a regular PI3K/AKT pathway for actin dynamic regulation.

Transforming Growth Factor-β (TGF-β)

Transforming growth factor (TGF-β) plays an important role in angiogenesis together with VEGF. These cytokines have a range of different effects on ECs depending on the conditions: TGF-β may induce apoptosis via MAPK pathway on general ECs, while VEGF will protect general ECs from apoptosis (Ferrari et al., 2009). The process of apoptosis may induce vascular remodeling, which includes vessel pruning and maturation. Thus the role of TGF-β is indispensable within normal vessels. TGF-β1 dimer starts by binding with TGF-β receptor II, followed by TGF-β receptor I. This heterotetramer complex undergoes phosphorylation, subsequently activating Smad transcription factors: Smad2/3 will be activated first, forming a heterocomplex with Co-Smad Smad4. Subsequent transport of this complex to the cell nucleus may regulate expression of target genes (Daly et al., 2008). Transport of TGF-β receptor in HeLa cells model is known to be dependent on clathrin and caveolae, including the novel endosomal fusion between two vesicles which are regulated by Rab5 (He et al., 2015). Expression of TGF-β maintains cerebrovascular integrity

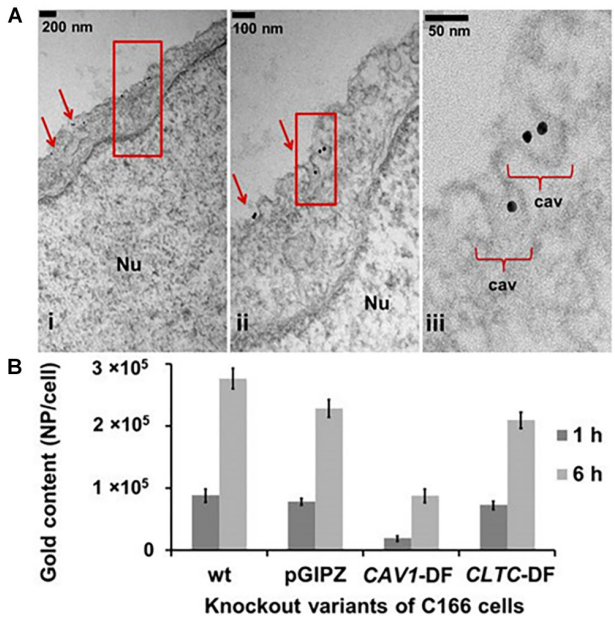

FIGURE 2 | (A) Caveolae formation in endothelial cells. Nu, nucleus. **(B)** Caveolae is the main endocytosis pathway of ECs compared to clathrin. Uptake of spherical nucleic acids (SNAs) as the nanocarrier showed 60% reduction in the Cav-1 deficient cells, also showed 10% reduction in clathrin heavy chain (CLTC) deficient cells. Reproduced with permission and courtesy of Chad Mirkin (Choi et al., 2013).

by regulating N-cadherin expression in cooperation with Notch signaling (Li et al., 2011). However, activation of TGF-β also upregulates α-SMA (Smooth Muscle Actin) and actin in the brain pericytes, as well as the VEGF, MMP-3, and MMP-9 which promotes barrier instability (Thanabalasundaram et al., 2011). This is an issue requiring further investigation. TGF-β expression in brain pericytes has showed upregulation via Foxf2 expression (Reyahi et al., 2015). Treatment of brain pericytes with bFGF (basic Fibroblast Growth Factor) may promote expression of desmin, vimentin, and nestin which suppress barrier leakiness (Thanabalasundaram et al., 2011). Both TGF-β and bFGF are secreted from astrocytes (Abbott et al., 2006), further proving their role in regulating BBB functions, as well as interaction with ECs and pericytes. ECs specific TGF-β receptor III (Endoglin) the co-receptor of TGF-βRI, activates ALK1-Smad1/5/8, which can leads to vessel destabilization. In myofibroblast model, TGF-βRI activation may suppress Cav-1 expression via p38/MAPK pathway, and it's shown to be independent to Smad activation (Sanders et al., 2015). This dual activity of TGF-β signaling should be investigated even further in BBB.

Sphingosine-1-Phosphate (S1P)

Sphingosine-1-phosphate (S1P) is synthesized by two types of sphingosine kinase (Sphk 1 and 2). The HBMECs only expressed four out of five known S1P G-protein coupled receptors. S1P exposure to ECs might induce proangiogenic gene expression, cell migration, maintenance of cell proliferation, and inhibition of apoptosis (Kimura et al., 2001; Kuwabara et al., 2003). Secretion of S1P from pericytes and astrocytes to retinal microvasculature ECs will promote barrier stability through upregulation of various junctional proteins, as well as the expression of N-cadherin which promotes cell-to-cell interaction (Paik et al., 2004; McGuire et al., 2011). Expression of S1P will induce activation of the PI3K/protein kinase B (Akt/PKB) pathway and also upregulate antiapoptotic Bcl-2 and downregulate proapoptotic Bim (Limaye et al., 2005). Upregulation and dephosphorylation of the junctional molecule PECAM-1 was also observed in HUVECs (Limaye et al., 2005). S1P and LRP1 showed synergistic effects on chemotactic migration of HBMECs (Vézina et al., 2018). Vessel carrier effects showed by the chaperone HDL-associated ApoM may deliver S1P to the S1P1 and S1P3 receptors, promoting ECs proliferation, preventing apoptosis, and also improve barrier stability at the BBB (Galvani et al., 2015; Ruiz et al., 2017). However, S1P3 receptor activation in astrocytes isolated from mice shows that it might activates RhoA which induces inflammatory cytokines and S1P expression, indicating an autocrine loop which participates in BBB breakdown (Dusaban et al., 2017). In HUVECs and mice model, S1P/S1P1R activity possibly have a vasoprotective effects by regulating the amount of proinflammatory adhesion proteins (in this case ICAM-1) (Galvani et al., 2015). Activation of S1P1R signaling was reported to induce translocation of N-cadherin (making the bond between general ECs and pericytes stronger), and it has also been proposed that it alters the adhesive property of N-cadherin. This activity in general ECs gives rise to complex cellular communication via various ligands interacting with a single receptor, but activated through different pathways (Paik et al., 2004). Loss of the S1P1R will induce BBB leakiness (Yanagida et al., 2017). Meanwhile, activation of this receptor will also contribute to the synthesis and also recovery of rat fat-pad ECs glycocalyx, which mediates vascular robustness and adsorptive-mediated transcytosis (Zeng et al., 2015). Conversely, S1P2 receptor plays a role in suppressing the PI3K pathway which is activated via S1P1R. This inhibition is achieved through the coupling mechanism of Rho-dependent activation of PTEN phosphatase. Activation of these pathways will induce vascular permeability, promoting disruption of adherens junctions and stimulates stress fibers resulting in the leaky barrier (Sanchez et al., 2007). Interestingly, activation of the PI3K/Akt pathway by VEGF has been discovered to induce transcytosis via actin dynamics and Cav-1 activation (Wang et al., 2011; Jin et al., 2015; Chen et al., 2018). Multiple responses from PI3K/Akt pathway activation or suppression is indicating another regulatory pathway is necessary for a balanced transcytosis in ECs, and the outcome of this pathway may differ depending on the cell's dynamics. A previous study using HeLa cells showed that S1P regulates transport proteins tetraspanins (CD63, CD81) and flotillin into exosomes in the process of MVEs (Multi Vesicular Endosomes) maturation (Kajimoto et al., 2013). S1P also has a protective effect on general ECs and adheren junctions, as well as actin and cytoskeleton arrangement (Kajimoto et al., 2013; Shepherd et al., 2017). In regulation of the synaptic system, sphingosine was shown to regulate the assembly of

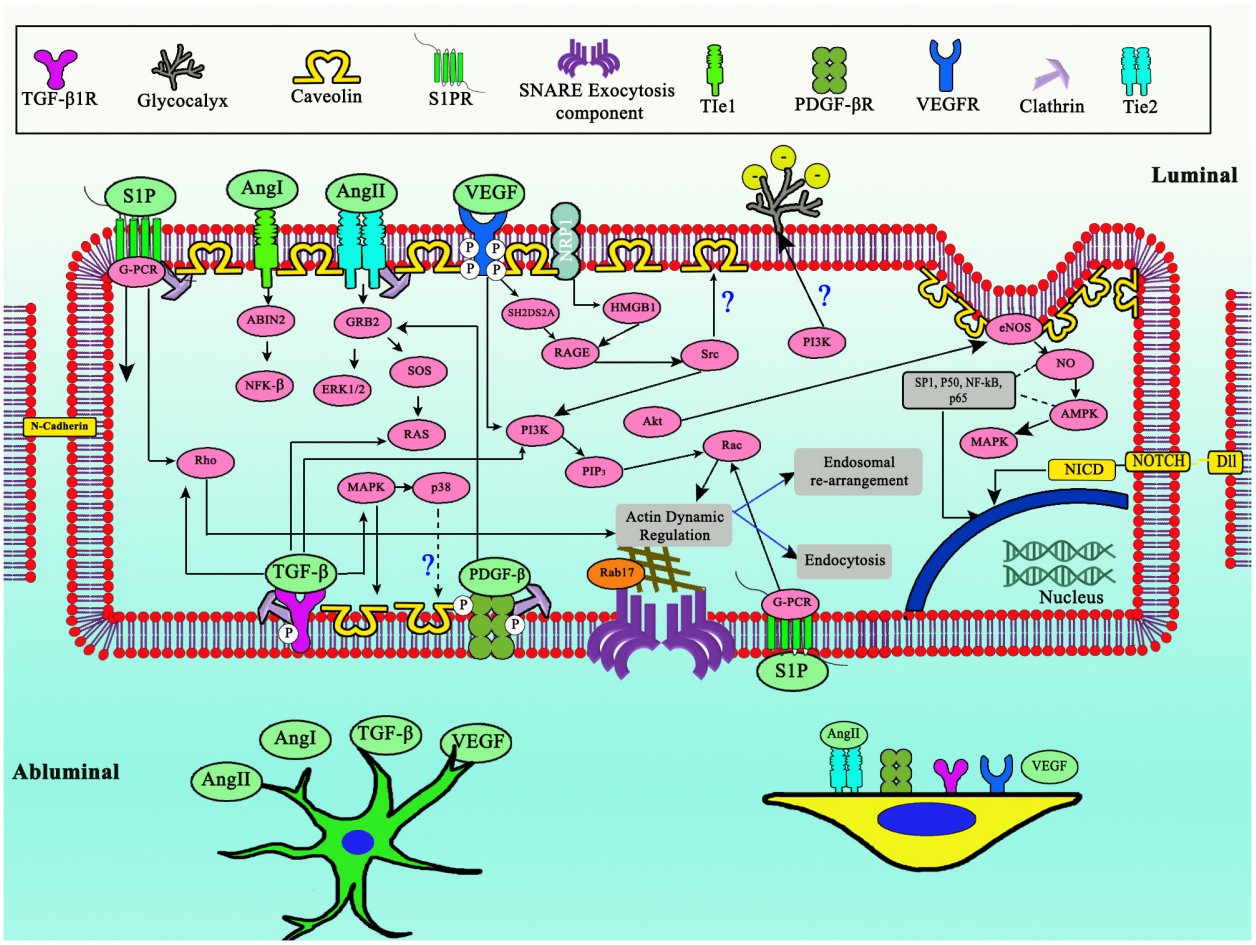

FIGURE 3 | Complex shared developmental pathway between ECs, pericytes, and astrocytes to regulate transcytosis in BBB. Notice the central role of PI3K, updated model of TIE2 expressed in pericytes, as well as glycocalyx role for negative membrane charge. Dashed arrows indicates downregulation, straight line arrows indicates upregulation.

SNARE complex via synaptobrevin (Darios et al., 2009). In neurons, S1P also regulates localization of synapsin I, showing supporting activity of exocytosis process (Riganti et al., 2016). It is currently unknown whether S1P also plays similar role in the HBMECs or BBB complex.

Angiopoietin/Tyrosine Kinase With Immunoglobulin-Like and EGF-Like Domains (Ang/TIE)

The mechanism of Ang/TIE pathway involves several angiopoietin ligands (Ang 1, 2, and 4 in humans) and TIE1/TIE2. Ang1 which is expressed from pericytes induces occludin expression in brain capillary ECs through TIE2 activation, thus promoting barrier tightness (Hori et al., 2004). Ang1 also inhibits FOXO1 activity via Akt activation in HUVECs, possibly interacting with various downstream target genes which involved in transcytosis (Daly et al., 2004). *In vitro* HUVECs study shows that after activation by Ang1, TIE2 will undergo internalization mediated by clathrin vesicles (Bogdanovic et al., 2009). Normally, Ang2 is not expressed in adult brain ECs, as Ang2 promotes barrier permeability via upregulation of Caveolin-1 (Cav-1) (Gurnik et al., 2016). Release of Ang2 showed dependency on VAMP3 in human brain ECs (Zhou et al., 2016). The expression of Ang1 and Ang2 will undergo changes during the normal aging process, whereby the former will be more expressed and meanwhile the latter will be suppressed. Expression of both receptors (TIE1 and TIE2) has been shown to be stable both in young ECs or adult ECs (Hohensinner et al., 2016). This regulation leads to vessel stability and ECs settlement in HUVECs (Hohensinner et al., 2016). Along with the senescence of HUVECs, some expression of junction proteins will be downregulated (Occludin and claudin-5), while ZO-1 will be upregulated compared to the younger ECs *in vitro* (Krouwer et al., 2012). A recent discovery has clarified that TIE2 receptors in human brain pericytes also play a vital role in the angiogenesis process (Teichert et al., 2017). Silencing of TIE2 expression in pericytes will induce pro-migratory phenotypes of ECs, indicating a close reciprocal

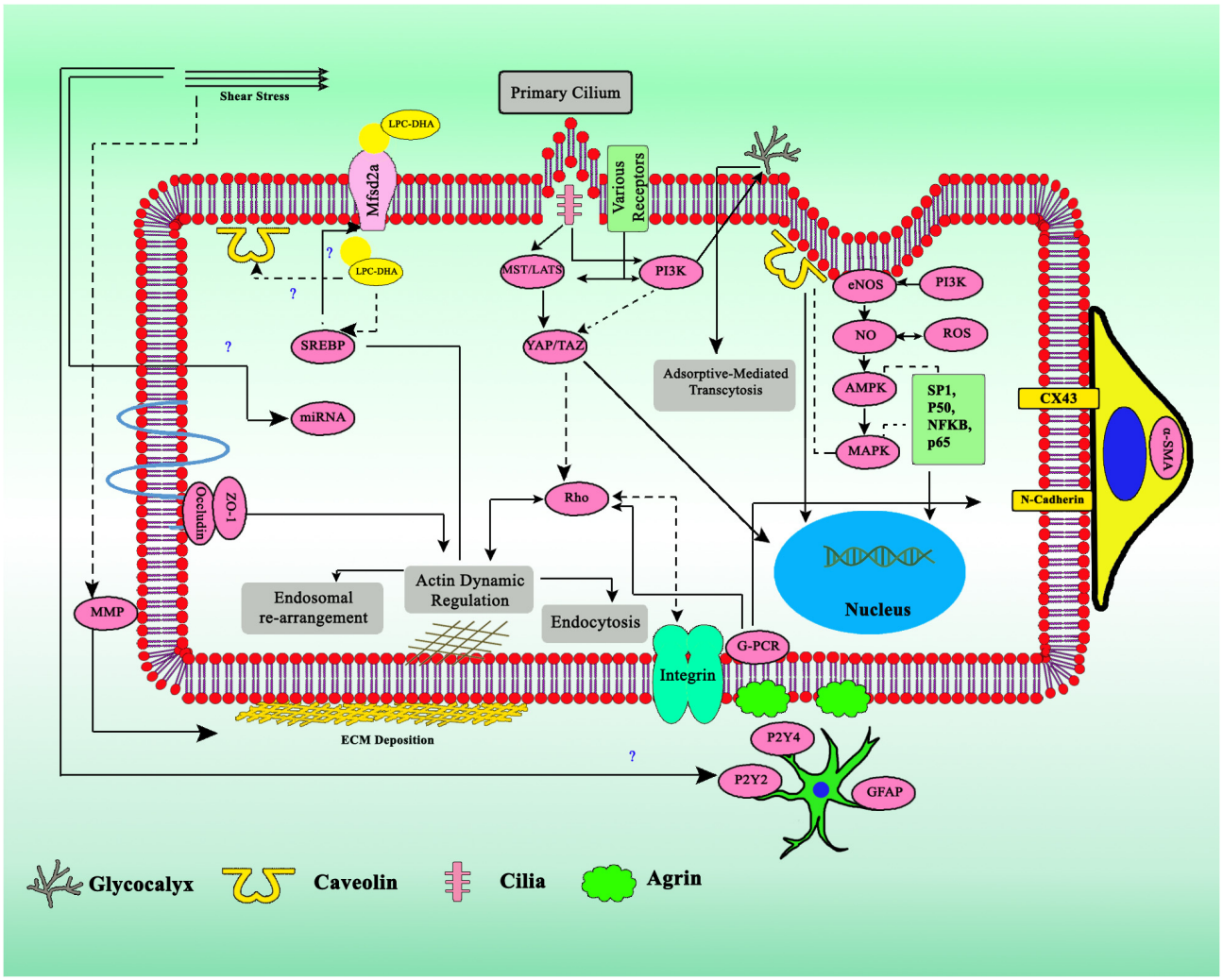

FIGURE 4 | Crosstalk of various factors involved in the regulation of transcytosis. Biomechanics flow sensing by multiple mechanosensor gives several types of regulatory signal, together with miRNA, ECM, physicochemical factors, and various cytokines. Dashed arrows indicates downregulation, straight line arrows indicates upregulation.

relationship (**Figure 3**) between pericytes and ECs (Teichert et al., 2017). This discovery also provides a hypothetical connection between astrocytes and pericytes, since astrocytes also express Ang1 (Lee et al., 2003) which could regulate TIE2 in pericyte membranes.

Notch

There are four types of Notch receptor in mammals, which showed interaction with five membrane-bound ligands, Jagged1, Jagged2, and also delta-like ligand (Dll) type 1, type 3, and type 4. Both brain pericytes and HUVECs showed expression of Jagged1 during co-culture (Kofler et al., 2015), indicating their importance in communication. Among several types of Notch receptors and ligands, only Dll4 and Notch4 specifically expressed on mammalian ECs (Shutter et al., 2000). In mice model, stimulation of Dll4 ligand will induce EphrinB2 expression in ECs. Furthermore, pericytes lacking in EphrinB2 expression will have a defects on vessel recruitment with ECs and impaired interaction with ECM (Foo et al., 2006). In a study using HUVECs, upregulation of Dll4 shows inhibition to the expression of VEGFR2 and NRP1, which regulates VEGF type A pathway (Williams et al., 2006), suggesting a mechanism to limit the number of caveolae and transcytosis across BBB. Inhibition of Notch signaling by GSI (γ-secretase inhibitor) showed its' effects to increase blood vessel diameter, but not the vessel length, indicating a local shear stress regulation (Lee et al., 2017; Davis et al., 2018). Brain ECs showed activity to regulate astrocytes' GLT-1 via Notch signaling pathway, which requires close contact between cells confirmed by *in vitro* experiment (Lee et al., 2017). This brings us to question- how does Notch signaling between ECs and astrocytes occur in the BBB when ECs are enveloped by pericytes and basal lamina? It has been discovered that Dll-4 and Jagged1 can be transported for intercellular communication, by passing through the extracellular matrix

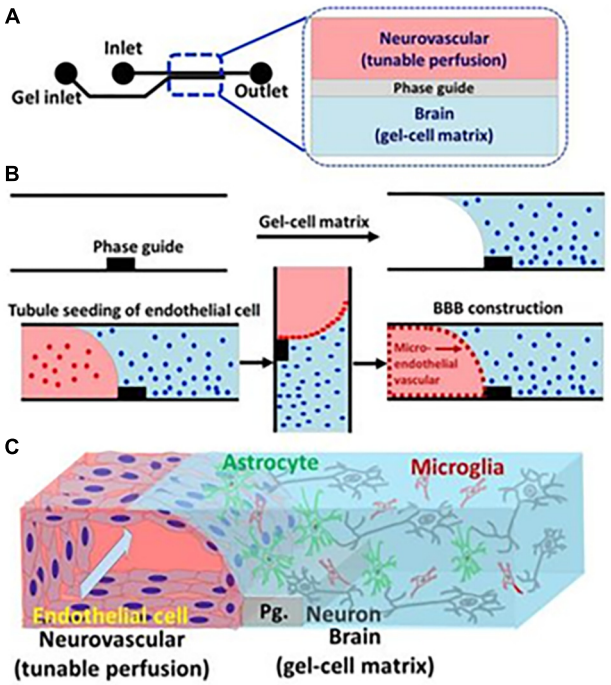

FIGURE 5 | Illustration of BBBoC for drug or toxic compound testing. Interaction between various components of the BBB will regulate transcytosis resembling *in vivo* conditions. Reproduced with permission and courtesy of Yeoheung Yun (Koo et al., 2018). **(A)** Diagram of BBBoC design with inlet and outlet for medium flow. **(B)** Steps of cell seeding and gel-cell matrix formation. **(C)** Established model of BBBoC in this study.

(Sheldon et al., 2010; Sharghi-Namini et al., 2014; Gonzalez-King et al., 2017). These discoveries bring our attention to the role of exosomes in signal trafficking, and that they possibly also regulate transcytosis.

FACTORS AFFECTING REGULATION OF TRANSCYTOSIS

Numerous factors might have a connection, either regulating directly or indirectly the transcytosis mechanism (**Figure 4**). In this review we would like to highlight some factors which have been indicated to regulate either endocytosis, endosomal rearrangement, exocytosis, or components of transcytosis.

Physicochemical (pH, Temperature, O_2, CO_2, ROS) of the Molecules and Environment

Through several discoveries, physicochemical factors have been shown to play an indispensable role in balanced transcytosis in the BBB. Transcytosis via transferrin receptor showed a dependence on pH and the polarity of proteins (Sade et al., 2014). The release of iron also utilizes pH changes in endosomes to change the affinity between iron and transferrin (Qian et al., 2002). It is undetermined whether changes in pH might alter the expression of transcytosis' components or not. A temperature shift induces membrane reorganization and actin dynamics which is cholesterol-dependent (Römer et al., 2010), indicating the role of temperature in governing transcytosis. Similar findings in neuron behavior highlights temperature-sensitive clathrin-independent endocytosis, which is mediated by dynamin and actin (Delvendahl et al., 2016). Further investigation is required to elucidate the mechanisms behind the effects of these factors. Oxygenation upregulates SSeCKS, a cytoskeleton protein which is expressed by astrocytes to invoke BBB tightness via VEGF suppression and Ang1 stimulation (Lee et al., 2003). Exposure to normobaric hyperoxia also can slow BBB damage (Liang et al., 2015). On the other hand, hyperoxia/ROS might induce the Fas-BID apoptosis signaling cascade, which is mediated by Cav-1 (Zhang et al., 2011). Signifying balanced regulation is necessary to maintain an appropriate amount of oxygen in the BBB. Hypoxic conditions might alter the content of exosomes for intercellular signaling. It has been shown that Notch ligands transport will be upregulated during hypoxia (Gonzalez-King et al., 2017). Hypoxia may induce oxidative stress, mainly caused by reperfusion (Thornton et al., 2017), triggering BBB breakdown via NOX4 activation (Casas et al., 2017). ROS which is produced by NOX is disruptive to the BBB, and has showed a dependence on cytokines, which actively downregulate junctional proteins in BBB (Rochfort et al., 2014). ROS disrupts brain ECs' tight junctions arrangement via RhoA/PI3K/PKB pathway (Schreibelt et al., 2007). ROS also enhance the transcellular migration of monocytes across BBB (Van der Goes et al., 2002), possibly due to stimulation of caveolae production via c-Src (Coelho-Santos et al., 2016). H_2O_2 from ecSOD in caveolae might also promoting VEGF activity, which causing leakiness (Oshikawa et al., 2010). These data indicate that ROS will increase BBB permeability through transcytosis regulation. Supplementation of alpha lipoic acid (ALA) and melatonin helps to alleviate ECs oxidative stress brain (Patiño et al., 2016; Badran et al., 2018), possibly explored as the treatment.

Mechanical Stress

The importance of normal blood flow for healthy brain microvasculature development since infancy has been proved (Farzam et al., 2017). Maintenance of regular blood flow by neurovascular control as well as cardiac function is prominent especially in childhood, and failure may lead to sleep-disordered breathing (Kontos et al., 2017). The maintenance of a healthy brain in adults is also closely related to normal hemodynamics, where individuals with cardiac problems will also suffer from brain aging (Sabayan et al., 2015). Hemodynamics affect neural activity and both systems are coupled and synchronized spatiotemporally, especially in excitatory neuron activity (Ma et al., 2016). One of the regulators between neural activity and HBMECs is pericytes, which control the capillary diameter within the central nervous system, depending on the neurotransmitter (Peppiatt et al., 2006). Pericyte activity as the regulator of blood flow in the neurovascular unit is also detected in the adult brain and during brain aging. Phenotypes such as BBB breakdown, neurodegeneration, and neuroinflammation were observed in pericyte-deficient model mice (Bell et al., 2010).

In vitro experiments on bovine BMECs showed some proteins related to tight junction of BBB, Occludin and ZO-1 are regulated by blood flow (Berardi and Tarbell, 2009). When there is a higher shear stress, the expression will also be upregulated, and this process is dependent on cyclic strain (Collins et al., 2005). Mechanical stress has been shown to regulate cell behavior and other factors involving transcytosis. Shear stress affects the production of NO, independent of intracellular calcium (Chen et al., 2018). A recent discovery is that there is a close reciprocal connection between Hippo pathway (mechanosensory pathway) and caveolae. It has been elucidated that caveolae are regulating the mechanosensory action of cells, and they affect the expression of YAP/TAZ which is the transcription factor of Cav-1 and Cavin1 (Rausch et al., 2019). Shear stress also affects vessel growth by regulating miRNA expression (Guan et al., 2017). Reduced blood flow will alter ion homeostasis and receptor-mediated transcytosis of insulin at the BBB, but not significantly altered the paracellular transports (Hom et al., 2001). Pericyte ability to express α-SMA indicates the cell have a contractile ability for regulating blood vessel diameter and blood flow (Alarcon-martinez et al., 2018). In some study, effects of mechanical stress to the cell permeability has been well-elucidated. One example is in the renal epithelial cells, where fluid shear stress modulated the endocytosis via mTOR pathway (Long et al., 2017). In the HUVECs, shear stress affects the endocytosis through PECAM-1 via various pathways depending on the binding of distinct epitopes (Han et al., 2015).

It is indeed a result from specialization that HBMECs behave differently under exposure to shear stress compared to HUVECs as the representative of other ECs. HBMECs can maintain a cobblestone-like appearance under high shear stress, and most likely this mechanism is to minimize the paracellular transport by minimizing the length of tight junctions (Ye et al., 2014; Reinitz et al., 2015). The detailed explanation needs to be studied even further. In HBMECs, Mfsd2a (Major Facilitator Superfamily Domain Containing 2A) has been known to facilitate the uptake of DHA into brain (Nguyen et al., 2014) as well as maintaining low rates of transcytosis in the cerebrovascular units (Zhao and Zlokovic, 2014). By transporting DHA inside the cells, the caveolae vesicles formation can be inhibited by intracellular lipid concentration, thus promoting BBB integrity (Andreone et al., 2017). Mfsd2a expression is shown to be downregulated by the metastatic brain tumor to disrupt BBB integrity and lipid metabolism (Tiwary et al., 2018). Another study also showed a lethal microcephaly phenotype was shown in the absence of Mfsd2a (Guemez-gamboa et al., 2015). The expression of Mfsd2a showed partial dependency to LXR/Srebp1 and Srebp2 (Chan et al., 2018). Interestingly, shear stress was shown to activate Srebp1 splicing mediated by integrins in EC (Liu et al., 2002). SREBP splicing is also showed dependency to shear stress through S1P and S2P activation, allowing SREBP(N) to translocates into nucleus and activating SRE-mediated genes (Lin et al., 2003). Inferred from this pathway, this hypothesis opens up a possibility for explaining how mechanotransduction may affecting transcytosis. In astrocytes, mechanical stress is positively regulating the expression of GFAP. P2Y2 and P2Y4 are the mediators of calcium signaling in astrocytes, which also colocalize with GFAP (Paniagua-Herranz et al., 2017). These calcium receptors are dependent to caveolae regulation (Pani and Singh, 2009), suggesting a crosstalk between these factors to transcytosis process in astrocytes, as well as feedback regulation for promoting caveolae formation.

Basement Membrane and Junctions Remodeling

Close contacts between neurovascular units are maintained through several ways. One of them is the peg-socket junctional complex, where the pericytes act like a peg and are inserted into EC's sockets through facilitation of proteins, such as N-cadherin and connexin 43 (CX43) hemichannels. Hemichannels are membrane protein structures which are coupled to each other in adjacent cells, providing a channel for signaling molecules and exchange of metabolites (Orellana et al., 2011). The roles of CX43 as hemichannel between pericytes and ECs has been clearly elucidated. It has a crucial part in the maintenance of intercellular communication between pericytes and ECs, consequently promoting stability of barrier properties (Li and Roy, 2009; Bobbie et al., 2010). Inactivity of CX43 expression may leads to pericytes detachment and activation of ECs apoptosis (Tien et al., 2014). Gap junction alteration as part of tissue remodeling also contributes to alteration of transcytosis. During inflammation, expression of Cx43 and Cav-3 in astrocytes will be downregulated via iNOS activity. Cx43 as the regulator of gap junctions has also showed interaction with Cav-1/Cav-2 during transcytosis, however its relation with Cav-3 still has not been elucidated (Liao et al., 2010), leaving room for further study. N-cadherin is one of the transmembrane glycoproteins that is expressed by ECs, together with VE-cadherin. While VE-cadherin is indispensable for vascular morphogenesis, N-cadherin is essential to the process of vascular maturation through pericytes recruitment (Tillet et al., 2005). The regulation of N-cadherin is closely connected to the S1P pathway, where the activation of S1P1R will promote N-cadherin-dependent of the pericyte-EC connection (Paik et al., 2004). Albumin also plays a crucial role in the transcytosis of myeloperoxidase (MPO) via caveolae-albumin binding proteins (ABPs) (Tiruppathi et al., 2004). MPO itself will be localized at fibronectin and induce nitration of ECM, thus promoting the tissue remodeling by binding with adhesion plaques (Baldus et al., 2001). The expression of Cav-1 was upregulated after the induction of juvenile traumatic brain injury (jTBI), demonstrating signs of BBB repair attempts (Badaut et al., 2015). Upregulation of MMP-2 and MMP-9 expression was the result of the decreased amount of Cav-1, together with downregulation of TJ protein ZO-1. Rescue experiment using NOS inhibitor showed reserved expression of Cav-1, inhibition of MMPs activity, and restored BBB integrity (Gu et al., 2012; Sadeghian et al., 2018). Pericytes may induce rapid localized MMP-9 activity during ischemia (Underly et al., 2017). MMP-2 is the major contributor to occludin degeneration, meanwhile Cav-1 actively redistributes claudin-5 (Liu et al., 2012). BBB stabilization through astrocytic laminin (laminins-111 and -211) secretion occurs through pericytes' integrin α2 (ITGA2)

binding. Lack of astrocytic laminins may induce pericytes into contractile form, which compromise BBB integrity (Yao et al., 2014). Consequently, all these data represent exemplary cases of how basal lamina might regulate BBB transcytosis.

Various Cytokines

The cytokine family contains the key molecules for cellular signaling. The exchange of cytokines between ECs, pericytes, and astrocytes are necessary to maintain BBB integrity, especially in the process of transcytosis (Dohgu and Banks, 2013). Discovery of the cytokines related to transcytosis is continuously studied, for example the CTRP5, which promotes LDL transcytosis (Li et al., 2018). It is indeed interesting that several pro-inflammatory cytokines are shown to be promoting transcytosis, one example is the HMGB1 which promotes albumin transcytosis through activation of Src and Cav-1 phosphorylation (Shang et al., 2016). Which bring us to question: what about dual-functioning cytokines? And also the anti-inflammatory cytokines?

Cytokine signaling has also showed a dependence on the transcytosis process. One case of this phenomenon is the trafficking of IL-11, which is known to maintain barrier function at the intestinal epithelium. Trafficking of IL-11 through IL-11R1 showed a unidirectional transcytosis process, and IL-11R1/2 also controls redirection of gp130 to the apical part of the cells (Monhasery et al., 2016). Following gp130, one of the inhibitors of the JAK/STAT pathway, the SOCS3 was showed to have another function in the stabilization of cavin-1. In return, cavin-1 also modulate SOCS3 ability to inhibit IL-6 signaling via cAMP (Williams et al., 2018). In this case we can see clearly how cytokines might alter cell-signaling processes, regulating transcytosis and at the same time was also showed dependency on the transcytosis process. Further investigation should be conducted regarding the way cytokines might alter endosomal rearrangement, including whether the quantity of cytokines exposure plays a part in transcytosis, and vice versa.

miRNA Intercellular Transport (EC, Pericytes, Astrocytes)

miRNA is another factor which is both regulating and regulated by transcytosis. It has been proven to actively contribute in cell-to-cell communication, a process heavily relying on transcytosis, mainly through EVs. During diabetic complication located on the limb, miR-503 through the shedding of microparticles (MPs) is transferred from ECs to pericytes, resulting in pericyte detachment and increased vessel permeability (Caporali et al., 2015). Cav-1 downregulation by miR-192 is also observed in synovial tissue fibroblast-like cells (Li et al., 2017), as well as another miR-199a-5p targeting clathrin in cancer cells (Huang et al., 2017). It is plausible to see whether miRNA also regulates BBB permeability and transcytosis, given the specialization of HBMECs and the neurovascular unit. An attempt has been made to characterize miR-155 effects on HBMECs (Chen et al., 2015), further research should be arranged to see the combined interaction when co-cultured with astrocytes and pericytes, or *in vivo* study. Previous study showed pericytes capability to regulate vasculogenesis by secreting miRNA targeting Fli1 (Larsson et al., 2009). Astrocytes through EVs of cytokines (TNF-α and IL-1β) also regulates miR-501-3p which disrupts tight junction (Chaudhuri et al., 2018; Toyama et al., 2018). Another study showed miR-107, which endogenously expressed in ECs but also present in the cerebrospinal fluid, may protect BBB robustness from amyloid-beta (Liu et al., 2016), effects of miRNA also supports recovery after intracerebral hemorrhage (Xi et al., 2017). In contrast, there are also miRNA with BBB disrupting activities, for example miR-155 disrupting tight junction protein expression (Zheng et al., 2017), and miR-181c which secreted by cancer cells (Tominaga et al., 2015). There have been several findings where miRNA was alternating the course of signaling pathway related to BBB transcytosis, mainly VEGF (Chamorro-Jorganes et al., 2016), Ang/Tie (Fang et al., 2016), PDGFRβ (Tanaka et al., 2013), and TGF-beta (Zhou et al., 2016). miRNA was also showed to interact with other factors concerning transcytosis, especially mechanotransduction pathway (Demolli et al., 2015) and cytokines expression (Guo et al., 2017). Altogether, miRNA functions and transport system might be one of the factors affecting and also affected by transcytosis. Further research is needed to elucidate the detail mechanism and interaction in order to design an effective treatment strategy.

CONCLUSION: FUTURE APPLICATIONS AND PERSPECTIVES

By exploring factors of transcytosis which have been described above, we can apply the knowledge to the development of drug design and also BBB-on-a-chip.

Drug Therapy Design

One of the obstacles for brain disease treatment is the special design and low permeability of the BBB which requires customized drug design. This design will enable drugs to be taken for transport, which mainly involves caveolae-dependent transcytosis (Choi et al., 2013; Piazzini et al., 2018). Several candidates have been tested as potential protagonists of Cav-1, which upregulates caveolae formation (**Table 2**). Nevertheless, specific protagonists of Cav-1 still have not been found, requiring future study. Targeting the growth factor receptor also seems promising for inducing caveolae formation, even though specificity and delivery should be considered. There are also agents targeting other receptors which may have an indirect effects to caveolae formation (**Table 2**). Targeting S1P1R might induce small molecule selective of BBB opening, indicating a possibility for drug administration (Yanagida et al., 2017). Recent study successfully create a temperature sensitive liposome (Bredlau et al., 2018) utilizing hyperthermia, and conjugated cation transporter which utilize ECs' glycocalyx negative charge (Kou et al., 2018). Inactivating P-gp remains a challenge to prevent drug efflux, and a recent study showed it can be internalized due to ROS activation dependent on Cav-1 (Hoshi et al., 2019), consequently emphasizing the control of transcytosis upregulating factors to drug therapy strategies.

TABLE 2 | Some signaling receptors, along with the characteristics of endocytosis, exocytosis (if available), protagonist, and antagonist molecules.

Signaling pathway membrane receptors	Endocytosis characteristics	Exocytosis characteristics	Protagonist	Antagonist	Relation to phenotype observed
TGF-βR I	Novel clathrin/caveolae dependent (He et al., 2015)		CTGF, SDC2 (Chang, 2016) TGF-β	SB431542 (Paonessa et al., 2019), A 83-01 (Yakoub and Sadek, 2018)	In homozygous KO mice, lethality was observed. Severe hemorrhage and abnormal vessel development was also present (Larsson et al., 2001)
VEGFR 1	Caveolae dependent (Celus et al., 2017)		VEGF-A;B	Sunitinib, Pazopanib, Axitinib (Musumeci et al., 2012)	Lack of this receptor induced by tamoxifen may cause increased angiogenesis, upregulation of VEGFR2 expression, but non-significant BBB Permeability. It also cause lethality in germline mice (Ho et al., 2012).
VEGFR 2	Caveolae dependent (Caliceti et al., 2014)		VEGF-A;C;D;E, Gremlin,	Monomeric Gremlin^{C141A}, Sorafenib, Sunitinib, Pazopanib, Vandetanib, Axitinib (Musumeci et al., 2012)	Deficient of blood-island formation and vasculogenesis was observed in the KO mice (Shalaby et al., 1995), in heterozygous KO mice, angiogenesis was perturbed (Oladipupo et al., 2018)
S1P1R	Clathrin dependent (Reeves et al., 2016), Caveolae dependent (Ephstein et al., 2013)		Fingolimod (Quancard et al., 2012), Ponesimod (Bell et al., 2018)	CYM5442 (Kim et al., 2018), NIBR-0213 (Quancard et al., 2012), AD2900 (Song et al., 2017)	Knockout mice showed lethality and severe hemorrhage since infancy (Liu et al., 2000)
TIE2	Clathrin dependent (Bogdanovic et al., 2009) Caveolae dependent (Hossain et al., 2017)		ANG1, ANG4	ANG2, ANG3	Global deletion will cause lethality to mice embryo, pericytes specific deletion may cause developmental delay and abnormal vessel maturation (Teichert et al., 2017)
Dll4	Clathrin dependent (Sheldon et al., 2010)	Exosomal markers: LAMP1 TSG101 Rab5 (Sheldon et al., 2010)		Dll4-Fc (Sheldon et al., 2010), MEDI0639 (Jenkins et al., 2012)	Heterozygous deletion will induce arteriovenous malformation and haploinsufficient lethality in mice (Krebs et al., 2004)
Notch	Clathrin dependent (Meloty-Kapella et al., 2012)			Egfl7 (Nichol et al., 2010)	Lack of Notch1 in KO mice cause lethality (Conlon et al., 1995), meanwhile artery enlargement and vein underdevelopment was observed in heterozygous KO (Kim et al., 2008)
G Protein-Coupled Receptor (GPCR)	Clathrin dependent Caveolae dependent (Zhang and Kim, 2017)		GRI977143, kynurenic acid, 3-methoxycatechol	GDP-β-S	In GPR124 KO mice, embryonic lethality, with abnormality in CNS vascular and BBB was observed (Cullen et al., 2011)
EphrinB2	Clathrin dependent (Gaitanos et al., 2016)		EphA4	Dasatinib (Barquilla and Pasquale, 2014)	Blocking of EphrinB2 may inhibit angiogenesis in brain via VEGFR2 regulation (Sawamiphak et al., 2010), global deletion causes defective angiogenesis especially in the head region, and also lethality in embryonic mice (Wang et al., 1998).
PDGFR- β	EphB2-Caveolae dependent (Nakayama et al., 2013) EphB2-null-Clathrin dependent			Sorafenib, Sunitinib (Musumeci et al., 2012)	Knockout mice showed excessive bleeding, hypoplasia of vascular smooth muscle cells in larger vessel, and lack of pericytes in microvasculature (Hellström et al., 1999)
Cav-1	-		PPARγ, Pioglitazone (Werion et al., 2016) Chlorogenic acid derivatives (Lee et al., 2017)	Lovastatin and/or Celecoxib (Shimato et al., 2013) GGTI-286 Incadronate (Iguchi et al., 2006)	Knockout mice showed loss of Cav-2 expression, endocytosis defect, hyper-proliferation and abnormal vascular development (Razani et al., 2001)
Caveolae-mediated endocytosis	-			MβCD (Moriyama et al., 2017; Wu et al., 2019) Nystatin (Burger et al., 2011)	-
Clathrin-mediated endocytosis	-			Dynasore (Wu et al., 2019)	-

BBB-on-a-Chip

The organ-on-a-chip offers a possibility to create a model closer to the human body than animal models and conventional cell culture models. One of the primary applications for BBBoC is the drug-testing field, to observe cytotoxicity and pharmacodynamics. It is also useful to study physiological interactions and responses from multiple organs (Ahadian et al., 2017). With relatively simple steps, and also time- and money-saving aspects, the organ-on-a-chip is the future model for experimentation and study models in the field of life science (Streets and Huang, 2013). Elucidation regarding transcytosis factors on the BBB will support establishment of BBBoC. For instance, a fruitful approach was taken by Koo et al. (2018), where a three-dimensional (3D) tetra-culture was made using combined construct of gel-cell matrix, phase guide, and perfusion of medium. The usage of phase guide which composed of capillary pressure barriers enable the separation of gel and fluid phases. Thus, the construction of membrane-free substrate for endothelial cell attachment was made possible, initiated by gel-cell polymerization which contained mix culture of microglia, astrocytes, and neuroblastoma combined to extracellular matrix that comprised of collagen. Cell seeding of ECs with perfused medium was done to mimics the shear stress and blood flow, which resulted in the development of neurovascular unit by ECs and gel-cell matrix (**Figure 5**). The permeability and integrity of BBBoC was tested by using AChE activity, viability, and residual organo phosphates (OPs) assay, which were known to be toxic and came across the brain through the BBB *in vivo*. Positive points of this model are the utilization of four types of cell which present *in vivo*, relatively normal permeability, and closely mimics the neurovascular unit (Koo et al., 2018). Some consideration that should be made is regarding the inability to measure TEER because of the difficulty to insert the electrodes, and another thing is the extracellular matrix which only comprised of collagen, whereas the *in vivo* extracellular matrix also comprised of fibronectin and gelatin. In conclusion, the field of BBBoC still has many possibilities for future development and integration to the body-on-a-chip system. BBBoC is a promising construct that may answer and serves as the future study model in many fields of life science.

Taken together, the whole study regarding factors of transcytosis in BBB still needs further exploration, especially regarding crosstalk between factors, context of environment and nutrition, as well as pathogenesis stimulation. With these factors in mind, application development will be more effective and efficient.

AUTHOR CONTRIBUTIONS

MT, YW, CD, and GW contributed conception and design of the study. NW and ZH organized the literature. VV performed the design of figures. MT wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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