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Regulation of brain endothelial barrier function by microRNAs in health and neuroinflammation

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ABSTRACT: Brain endothelial cells constitute the major cellular element of the highly specialized blood–brain barrier (BBB) and thereby contribute to CNS homeostasis by restricting entry of circulating leukocytes and blood-borne molecules into the CNS. Therefore, compromised function of brain endothelial cells has serious consequences for BBB integrity. This has been associated with early events in the pathogenesis of several disorders that affect the CNS, such as multiple sclerosis, HIV-associated neurologic disorder, and stroke. Recent studies demonstrate that brain endothelial microRNAs play critical roles in the regulation of BBB function under normal and neuroinflammatory conditions. This review will focus on emerging evidence that indicates that brain endothelial microRNAs regulate barrier function and orchestrate various phases of the neuroinflammatory response, including endothelial activation in response to cytokines as well as restoration of inflamed endothelium into a quiescent state. In particular, we discuss novel microRNA regulatory mechanisms and their contribution to cellular interactions at the neurovascular unit that influence the overall function of the BBB in health and during neuroinflammation. —Lopez-Ramirez, M. A., Reijerkerk, A., de Vries, H. E., Romero, I. A. Regulation of brain endothelial barrier function by microRNAs in health and neuroinflammation. FASEB J. 30, 2662–2672 (2016). www.fasebj.org

KEY WORDS: regulatory mechanisms · multiple sclerosis · stroke · neurovascular unit · inflammation

INTRODUCTION

Brain endothelial cells (BECs) constitute the major cellular element of the highly specialized blood–brain barrier (BBB) that is essential for CNS homeostasis. The cerebral microvasculature formed by BECs constitutes an elaborate network of vessels that is composed of arterioles (10–100 μm in diameter), capillaries (4–10 μm in diameter), and venules (10–100 μm in diameter), which allows transport of nutrients and gases and removal of waste products throughout the brain and spinal cord (1). Of note, BECs play an important role in maintaining blood flow and limiting entry of circulating leukocytes and blood-borne molecules into the CNS. In mammals, BECs are unique and differ from endothelial cells that are present in peripheral tissues in a number of ways, which ensures specific brain endothelial barrier properties. First, BECs have few pinocytotic vesicles and lack fenestrations that are typical of peripheral tissue capillaries. Second, BECs have a high number of mitochondria, which suggests elevated metabolic activity. Third, BECs form a metabolic barrier by containing several catalytic membrane-bound and cytosolic enzymes that are capable of regulating metabolism of endogenous and xenobiotic molecules (2, 3). Fourth, BECs allow immune surveillance of the CNS, thereby playing a fundamental role in host defense with minimal inflammation (4). Fifth, BECs are the gatekeeper of the CNS as a result of an elaborate system of transport proteins and ion channels that are present in the plasma membrane and are asymmetrically distributed on the luminal (blood-facing) and abluminal (brain- and spinal cord–facing) sides, which allows bidirectional transport of selected substances (1, 2, 5). Of importance, BECs are interconnected by complex tight junctions (TJs) between lateral plasma membranes, which results in a polarized insertion of lipids and proteins (fence function). In turn, this feature restricts paracellular diffusion of nonionic hydrophilic molecules and ionic molecules, and, consequently, confers BECs a high electrical resistance (~3000 $\Omega \cdot cm^2$ *in vivo*; barrier function) (6, 7).

The BBB is regulated and induced by the CNS-specific microenvironment, which leads to the emerging concept

ABBREVIATIONS: BBB, blood–brain barrier; BEC, brain endothelial cell; CCL2, chemokine ligand 2; EAE, experimental autoimmune encephalomyelitis; Egfl7, epidermal growth factor–like-7; ICAM-1, intracellular cell adhesion molecule 1; MS, multiple sclerosis; TJ, tight junction

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that the complex cellular structure known as the neurovascular unit is what determines its specialized function. The neurovascular unit includes dynamic communication and feedback networks between various cell types, such as BECs, pericytes, astrocytes, perivascular microglia, smooth muscle cells, and neurons, as well as the extracellular matrix, so that it may respond to physiological changes and function as a biological barrier (8, 9). It is noteworthy that gene expression programs that underline the unique brain endothelial phenotype and function are highly regulated and under the control of the CNS microenvironment under physiological conditions (10). Indeed, the complexity of the cerebrovascular network requires coordinated genetic programs that, in part, are controlled by transcriptional activity [for a review, see Minami and Aird (11)] and, in part, by chromatin remodeling [for a review, see Matouk and Marsden (12)]. Recent studies have proposed post-transcriptional gene regulation, mediated *via* small non-coding RNAs termed microRNAs, as a possible mechanism that integrates the CNS milieu and BEC phenotype and function. In this context, microRNAs would contribute to the fine tuning of the complex regulatory gene networks of BECs under physiological and pathological conditions (13, 14). Moreover, it has become evident that many aspects of BEC biology, such as angiogenesis, neuroinflammation, and barrier function, are regulated by microRNAs. Recent research has been carried out in the context of stroke (15), and this is extensively reviewed in Yin *et al*. (16). It is also worth mentioning that endothelial microRNAs regulate the permeability of the blood–tumor barrier (17, 18) and destabilize TJs in CNS tumor metastasis (19). In this review, we discuss brain endothelial microRNA regulatory mechanisms and their contribution to BBB function under both physiological and pathological states, in particular, those related to neuroinflammation.

BRAIN ENDOTHELIAL microRNA FAMILIES AND THEIR ROLES IN NEUROINFLAMMATION

To better understand the physiology of microRNAs, bioinformaticians have grouped microRNAs into broadly conserved families on the basis of sequence and structure similarity of pre-microRNAs; therefore, microRNA families can exhibit full or partial conservation among animal species on the 2–8 mature microRNA sequence (20, 21). Experimental studies demonstrate that microRNA family members execute conserved biological functions across species (22).

Our recent studies of microRNA expression in the brain endothelium, using conditions known to negatively modulate BBB function (13, 23, 24), revealed profound changes in the brain endothelial microRNA profile (**Table 1**) (13). Of these changes, several microRNA families with roles in cardiovascular biology seemed to be critically affected by cytokine treatment (**Table 2**). For instance, microRNA-30, microRNA-125, and let-7 families were remarkably negatively regulated by proinflammatory cytokines (Table 2). It is noteworthy that decreased expression of microRNA-30 family members has been associated with loss of cell adhesion, changes in cell morphology, and increased cell migration in epithelial cells by modulation of snai-1 (25), a transcription factor that silences genes that are important for TJ and adherens junction formation and stability by interacting with E-box sequences (26–28). In endothelial cells, members of the microRNA-30 family regulate vascular development and sprouting in angiogenesis by targeting the notch ligand, delta-like ligand 4 (29). Thus, members of the microRNA-30 family represent potential targets, with roles in modulation of cell-to-cell contacts, and, as a consequence, possibly barrier function in the brain endothelium; however, the role of microRNA-30 family in the regulation of BBB function *in vivo* remains to be elucidated.

Members of microRNA-125 family have been shown to play common roles that are associated with induced cell proliferation and decreased apoptosis by targeting proapoptotic genes; however, other biological effects by members of microRNA-125 family have also been reported (30). In the context of brain endothelium, we recently reported that microRNA-125a-5p supports immune quiescence and endothelial barrier function (13). Our study demonstrated that during neuroinflammation, expression of brain endothelial microRNA-125a-5p is suppressed, resulting in increased monocyte migration as a result of endothelial up-regulation of intercellular cell adhesion molecule 1 (ICAM-1) (13). In addition, members of microRNA-125 family can modulate angiogenesis by targeting transcription factors. Repression of microRNA-125a/b promotes angiogenesis, whereas overexpression of microRNA-125a/b impairs angiogenesis by targeting related transcriptional enhancer factor-1 and VE-cadherin (31, 32) in endothelial cells. MicroRNA-125b has also been shown to regulate glioma-associated angiogenesis. Mechanistically, brain endothelial microRNA-125b is suppressed by the glioblastoma microenvironment, and VEGF stimuli resulted in increased expression levels of its target myc-associated zinc finger protein, which is a transcription factor that regulates the expression of VEGF (33).

The let-7 family is another microRNA family that has been shown to support brain endothelial barrier function and quiescent state (34). The proinflammatory cytokine, TNF-α, decreased levels of let-7 family members microRNA-98 and let-7g-3p (let-7g*) in brain endothelium, which in turn increases monocyte cell adhesion and migration *in vivo* (34).

In summary, proinflammatory cytokines affect several families of brain endothelial microRNAs that have important roles in BBB function and in angiogenesis; however, it remains to be elucidated whether these families of microRNAs cooperate during neuroinflammation and whether they form a link between neuroinflammation and angiogenesis in diseases that affect the CNS.

BRAIN ENDOTHELIAL microRNA CLUSTERS AND THEIR REGULATION BY PROINFLAMMATORY CYTOKINES

More than half of all currently known microRNA genes are linked as clusters on chromosomes and are often transcribed as a single polycistronic transcript, possibly to provide the opportunity for different microRNAs to simultaneously target different genes in specific cellular pathways (35, 36). Moreover, clustered microRNAs may have similar seed sequences, thereby sharing common

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TABLE 1. *Microarray profiling of microRNAs in hCMEC/D3 cells modulated by proinflammatory cytokines*

TNF-α + IFN-γ			TNF-α + IFN-γ			TNF-α + IFN-γ		
Systematic name	*P*	Fold change	Systematic name	*P*	Fold change	Systematic name	*P*	Fold change
hsa-miR-30c	8.12389E-08	−9.581522	hsa-miR-574-5p	0.027323036	−2.3152509	hsa-miR-16-2-3p	5.52675E-05	−1.809602
hsa-miR-126-5p	0.000485535	−8.542746	hsa-let-7c	0.000616054	−2.305489	hsa-miR-362-3p	0.008895058	−1.8051215
hsa-miR-324-5p	3.54737E-07	−4.9935045	hsa-miR-30d	0.000349644	−2.280156	hsa-miR-574-3p	0.003108962	−1.7700281
hsa-miR-1826	0.003712158	−4.291751	hsa-miR-125a-5p	0.000283408	−2.2575433	hsa-miR-424	0.006721119	−1.7650859
hsa-miR-27b	1.86096E-05	−4.105959	hsa-miR-92a	0.000414864	−2.2548149	hsa-miR-125a-3p	0.00713038	−1.7644966
hsa-miR-874	3.93779E-06	−3.992484	hsa-miR-494	0.002215313	−2.252349	hsa-miR-32	1.09824E-05	−1.7617378
hsa-miR-301b	0.000479457	−3.8152556	hsa-miR-186	0.000700766	−2.2335143	hsa-let-7e	0.025587553	−1.7525474
hsa-miR-23b	6.12162E-05	−3.601834	hsa-miR-185	0.001492695	−2.228406	hsa-let-7a	0.022988936	−1.728575
hsa-miR-26a	1.13341E-05	−3.4341455	hsa-miR-923	0.004135958	−2.195537	hsa-miR-140-3p	0.012414309	−1.7161368
hsa-miR-17-3p	0.000124318	−3.4280488	hsa-miR-17	0.001421169	−2.1507244	hsa-miR-362-5p	0.01630404	−1.6898851
hsa-miR-99b	1.61812E-05	−3.2208753	hsa-miR-103	0.003778802	−2.0909104	hsa-miR-222	0.008561188	−1.6715286
hsa-miR-1290	0.002641657	−3.1863027	hsa-miR-150-3p	0.000440756	−2.0371702	hsa-miR-181b	0.010845296	−1.6529723
hsa-miR-371-5p	0.0007107	−3.0648677	hsa-miR-1915	0.001497553	−2.0087082	hsa-miR-181a	0.029396445	−1.6524701
hsa-miR-128	0.000182791	−3.0222037	hsa-miR-1246	0.009255168	−2.0042048	hsa-miR-152	0.00387907	−1.634918
hsa-miR-31-3p	0.000125563	−2.9886847	hsa-miR-23a	0.004611711	−1.9975483	hsa-miR-135a-3p	0.009624685	−1.6153176
hsa-miR-148b	0.000188	−2.9751031	hsa-miR-125b	0.005628778	−1.9848528	hsa-miR-590-5p	0.041443966	−1.6148456
hsa-miR-30b	4.00747E-05	−2.8944528	hsa-miR-630	0.018250186	−1.982934	hsa-miR-15a	0.038995758	−1.6100651
hsa-miR-361-5p	4.01654E-05	−2.8785222	hsa-miR-151-3p	0.001312037	−1.9828008	hsa-miR-34a	0.04282073	−1.5884708
hsa-miR-221	3.50817E-05	−2.8366408	hsa-miR-30a	0.002010701	−1.9754144	hsa-miR-19b	0.03343741	−1.5842471
hsa-miR-181d	0.047451783	−2.809891	hsa-miR-532-5p	0.027450703	−1.9683508	hsa-miR-20a	0.037840247	−1.5622798
hsa-miR-18a	8.78539E-05	−2.7695873	hsa-miR-19a	0.003205194	−1.9678487	hsa-miR-484	0.013314423	−1.5197021
hsa-miR-20b	6.28695E-05	−2.7563477	hsa-miR-126	0.001831075	−1.9675982	hsa-miR-1249	0.007649734	−1.5126516
hsa-miR-150	0.000877592	−2.6872363	hsa-miR-1260	0.00361547	−1.9469978	hsa-miR-1280	0.025120856	−1.5122522
hsa-miR-93	0.000146107	−2.6507607	hsa-miR-503	0.005581775	−1.9345593	hsa-miR-1914-3p	0.048061863	−1.5034409
hsa-miR-374a	0.00011589	−2.6472602	hsa-miR-30a-3p	0.003841749	−1.9253043	hsa-miR-224	0.03127229	−1.4783875
hsa-miR-30e	7.29744E-05	−2.6128151	hsa-miR-532-3p	0.000216288	−1.9232571	hsa-miR-1274a	0.034146007	−1.4665035
hsa-miR-365	5.96503E-05	−2.5604894	hsa-miR-425	0.001297808	−1.9148034	hsa-miR-1274b	0.03522798	−1.4657427
hsa-miR-374b	3.77879E-05	−2.556873	hsa-miR-361-3p	0.004850489	−1.9126633	hsa-miR-193b	0.027998302	−1.4303372
hsa-miR-324-3p	0.000386624	−2.5512288	hsa-miR-27a	0.005203042	−1.9064424	hsa-miR-155	1.28301E-06	5.6064878
hsa-miR-18b	0.00011295	−2.534564	hsa-let-7f	0.011214863	−1.9002088	hsa-let-7d-3p	0.032172572	3.5584197
hsa-miR-98	0.001585185	−2.520486	hsa-miR-130b	0.006513434	−1.8985822	hsa-miR-146b-5p	3.89186E-05	3.3043206
hsa-miR-423-5p	0.000636056	−2.4843307	hsa-miR-582-5p	0.000108982	−1.8937668	hsa-miR-21-3p	6.39822E-05	2.8556242
hsa-miR-939	0.004484594	−2.3761806	hsa-miR-130a	0.009126897	−1.8341244	hsa-miR-146a	0.001859914	1.9587377
hsa-miR-301a	0.004210644	−2.3178346	hsa-miR-29c	0.006436433	−1.8280772	hsa-let-7b-3p	0.009400772	1.9162284
			hsa-miR-338-3p	0.010069519	−1.815034	hsa-miR-1825	0.000611126	1.7643497
			hsa-miR-342-3p	6.11219E-05	−1.8118693	hsa-miR-32-3p	0.001709265	1.7560405
						hsa-miR-1281	9.31229E-05	1.6028031

hCMEC/D3 cells were stimulated with TNF-α + IFN-γ (10 ng/ml concentration of each cytokine) for 24 h. Total RNA was isolated from 3 independent biological replicates, and microarray analysis (Agilent-021827 human microRNA microarray G4470C; Agilent Technologies, Santa Clara, CA, USA) was performed to determined microRNA levels (13). miR, microRNA.

TABLE 2. *Human microRNA families and clusters in the BEC cell line hCMEC/D3*

miR represented	Members	Chromosome	Number of members	Expressed in hCMEC/D3 cells
Family				
miR-30	*miR-30a*, *miR-30b*, *miR-30c-1*, *miR-30c-2*, *miR30d*, *miR-30e*	NA	6	6
miR-130	*miR-130a*, *miR-130b*, *miR-301a*, *miR-301b*	NA	4	4
miR-221	*miR-221*, *miR-222*	NA	2	2
miR-27	*miR-27a*, *miR-27b*	NA	2	2
let	*let-7a-1*, *let-7a-2*, *let-7a-3*, let-7b, let-7c, let-7d (NE), *let-7e*, *let-7f-1*, *let-7f-2*, let-7g, let-7i, *miR-98*	NA	12	11
miR-125	*miR-125a*, *miR-125b-1*, *miR-125b-2*	NA	3	3
miR-19	*miR-19a*, *miR-19b-1*, *miR-19b-2*	NA	3	3
miR-148	miR-148a (NE), *miR-148b*, *miR-152*	NA	3	2
miR-181	*miR-181a-1*, *miR-181a-2*, *miR-181b-1*, *miR-181b-2*, miR-181c, *miR181d*	NA	6	6
Cluster				
miR-17	*miR-17*, *miR-18a*, *miR-19a*, *miR-19b-1*, *miR-20a*, *miR-92a-1*	13	6	6
miR-221	*miR-221*, *miR-222*	X	2	2
miR-125a	*let-7e*, *miR-99b*, *miR-125a*	19	3	3
let-7a	*let-7a-1*, let 7d (NE), *let-7f-1*	9	3	2
miR-130b	*miR-130b*, *miR-301b*	22	2	2
miR-30d	*miR-30b*, *miR-30d*	8	2	2
miR-23a	*miR-23a*, miR-24-2, *miR-27a*	19	3	3
miR-23b	*miR-23b*, miR-24-1, *miR-27b*, miR-3074 (NE)	9	4	3

Families and clusters were considered deregulated when levels of microRNA members changed ≥2 (italics) in the presence of TNF-α + IFN-γ (13). [Analysis was performed by using miRbase (*http://www.mirbase.org*) and miRfocus (*http://pepcyber.org/mirfocus/*) databases.] Some microRNAs with high sequence similarity (underlined) could not be distinguished by the array analysis and were considered as one. miR, microRNA; NA, not applicable; NE, not expressed.

gene targets (37). Our previous study shows that proinflammatory cytokines alter the levels of 2 important microRNA clusters in BECs, microRNA-17 and microRNA-221 clusters, both of which have been associated with several biological processes in endothelium (Table 2) (13). Certainly, one of the most studied clusters is the microRNA-17 cluster, which is formed by 6 members situated on chromosome 13 (37–40). Several members of the microRNA-17 cluster may have related functions and act as proangiogenic signals by silencing antiangiogenic genes, such as thrombospodin-1 and connective tissue growth factor, thereby leading to enhanced neovascularization (41). In addition, microRNA-17 cluster has also been shown to regulate cell sprouting and proliferation by inhibiting tissue inhibitor of metalloproteinase-1 (40, 42, 43); however, using ischemia animal models, it has been demonstrated that endothelial-specific conditional and forced overexpression of microRNA-19 and microRNA-92a, both members of the microRNA-17 cluster, negatively regulates ischemia-induced arteriogenesis (44, 45). These studies suggest that the microRNA-17 cluster might act either as a positive or negative regulator of angiogenesis depending on the cell environment.

Finally, microRNA-221 cluster and family are highly conserved in vertebrates (37). The microRNA-221 cluster regulates endothelial cell proliferation, migration, and capillary tube formation by targeting c-Kit (46). In addition, microRNA-221 cluster has been shown to indirectly modulate eNOS expression levels (47, 48), and might be implicated in endothelial cell fate commitment and cytoskeleton remodeling by targeting the Cip/Kip family of proteins (39). It should be stated that microRNA-125 and microRNA-221 might have complementary actions. Depletion of the microRNA-221 cluster indirectly reduced microRNA-125 expression in HUVECs (49). Moreover, ICAM1 is targeted by the microRNA-221 cluster, and decreased levels of this microRNA cluster result in up-regulation of ICAM1 protein expression in epithelial cells (50, 51).

Although proinflammatory cytokines induce alterations in brain endothelial microRNA cluster expression (13), whether this is mediated at the transcriptional level or by modulation of microRNA processing remains unclear.

BRAIN ENDOTHELIAL microRNAs REGULATE DIFFERENT PHASES OF THE NEUROINFLAMMATORY RESPONSE

The cerebral microvasculature plays a major role in the regulation of several phases of the neuroinflammatory response, including vascular permeability, leukocyte adhesion and migration, and restoration of activated endothelium back to a quiescent state. Indeed, the process of neuroinflammation requires a coordinated activation of immune cells, brain resident cells, and CNS vasculature to avoid collateral damage and unnecessary chronic inflammation. Depending on the strength and duration of inflammatory stimuli, brain endothelial microRNAs may either change the genetic program toward an activated inflamed vascular endothelium or regulate genes to counteract cytokine signaling to return to a quiescent state. Details of brain endothelial microRNAs that have been shown to regulate inflammation as well as their possible implication in regulating phases of the neuroinflammatory response have been summarized in the next and following sections.

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BRAIN ENDOTHELIAL microRNAs THAT PROMOTE CELL ACTIVATION IN NEUROINFLAMMATION

MicroRNA-155 promotes inflammation and activates both immune cells and brain endothelium by silencing hundreds of transcripts with modest effects, a characteristic of fine tuning regulation (14, 52, 53). MicroRNA-155 originates from an exon in a noncoding transcript, B-cell integration cluster, which is located on chromosome 21q21 (54). MicroRNA-155 is highly conserved in the animal kingdom, including mammals, chicken, lizards, zebrafish, and frogs (21, 55). Inflammatory mediators, such as bacterial LPS, polyriboinosinic-polyrobocytidylic acid, and TNF-α, induce expression of microRNA-155 in several cell types, including the brain endothelium (14, 56–58). Inhibitors of JNK, PKC, and RhoA prevent microRNA-155 up-regulation in macrophages, B cells, T cells, epithelial cells, and BECs, and prevent their ability to respond to immune challenge (59–61) (unpublished observation). In the brain endothelium, microRNA-155 seems to be an important control node for expression and organization of adhesion molecules as well as for inducing a gene expression program characteristic of inflammation (**Table 3**) (14). For instance, both treatment with TNF-α and IFN-γ and overexpression of microRNA-155 in BECs induce expression of genes associated with immune response, major histocompatibility complex, cell adhesion molecules, and the complement system, pathways that are known to contribute to the inflammatory response (14, 62, 63). It is possible that microRNA-155 up-regulation in both immune cells and brain endothelium during neuroinflammation contributes to the harmonization of cell activation as a mechanism of defense of the CNS against pathogens; however, sustained and prolonged increases in microRNA-155 levels in the brain endothelium may provoke brain endothelial barrier breakdown by altering components of the cell–cell and cell–extracellular matrix adhesion pathways, both important cellular processes that stabilize barrier function (14). Indeed, we demonstrated that brain endothelial microRNA-155 is up-regulated by proinflammatory cytokines in a time- and dose-dependent manner (14). This response coincides with cytokine-mediated increases in brain endothelial barrier permeability that is associated with redistribution of ZO1 at cell–cell contacts and with focal adhesion reorganization. Functional analysis of microRNA-155 in the brain endothelium shows that this microRNA dampened the expression of 2 interendothelial junctional complex molecules, annexin-2 and claudin-1, and 2 focal adhesion components, dedicator of cytokinesis-1 and syntenin-1, which suggests an important mechanism by which microRNA-155 exacerbates BBB breakdown (14).

Nevertheless, protective roles for microRNA-155 have also been suggested to occur in different cell systems. Two independent research groups have proposed that, in resting conditions, microRNA-155 targets caspase-3, Fas-associated death domain–containing protein, IKKε, and the receptor-interacting serine-threonine kinase-1 to maintain homeostasis and low levels of cell death (64, 65). These results suggest that microRNA-155 might not only act as a positive regulator of inflammation but can negatively regulate activation of inflammatory pathways as well, which may be dependent on the cellular context and/or strength and duration of the stimuli. In the context of endothelial cells, microRNA-155 may indeed modulate a number of biological processes. For instance, microRNA-155 has been shown to be involved in regulation of blood pressure and endothelial-dependent vasorelaxation by modulating expression of angiotensin II type 1 receptor and eNOS expression (59), respectively (66–68). Other groups recently described effects of microRNA-155 on endothelial cells, including modulation of the microcirculatory vascular tone by targeting endothelin-1 (69) and actomyosin contractility by targeting RhoA and myosin light-chain kinase (70).

BRAIN ENDOTHELIAL microRNAs THAT INHIBIT INFLAMMATION

MicroRNA-146a/b

The human genome contains 2 members of the microRNA-146 family, microRNA-146a (chromosome 5q34) and microRNA-146b (chromosome 10q24), that are regulated by proinflammatory cytokines (Table 1) (71). MicroRNA-146a and microRNA-146b are coded from noncoding transcripts units (71). The microRNA-146 family seed sequence is evolutionary conserved (21) but the mature forms of microRNA-146a and microRNA-146b differ by 2 nucleotides at the 3′ region (71) that might play a role in compensatory target recognition (72). In the context of their physiological roles, microRNA-146a and microRNA-146b are positively regulated in immune cells by microbial components and proinflammatory cytokines and function as negative feedback regulators of inflammatory response (71). Pharmacologic studies suggest that cytokine-induced microRNA-146a is dependent on NF-κB and JNK-1/2 activity, whereas increased levels of microRNA-146b are mediated by MEK-1/2 and JNK-1/2 (73). Similar to immune cells, brain endothelial microRNA-146 seems to be an inhibitor of the activated state of the endothelium during neuroinflammation (74, 75). The molecular mechanism that underlies this biological process in BECs and in immune cells include a negative feedback regulatory loop on NF-κB signaling by silencing IL-1 receptor–associated kinase 1/2 and TNF receptor–associated factor 6 protein genes (71, 74–77). We also observed that brain endothelial microRNA-146 represses nuclear factor of activated T cells 5 and the RhoGTPase RhoA, which, in turn, prevents NF-κB nuclear translocation and activity (74). Gain- and loss-of-function analysis of microRNA-146 in BECs during inflammation *in vitro* shows that this microRNA suppresses Jurkat T-cell adhesion to the brain endothelium as a result of indirect inhibitory effects on protein levels of the cell adhesion molecule, VCAM1, and a chemokine implicated in mononuclear cell infiltration, chemokine ligand 2 (CCL2) (74). Altogether, these studies support a role for brain endothelial microRNA-146 as a molecular

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TABLE 3. *Up-regulation of mRNA transcripts in hCMEC/D3 cells after overexpression of microRNA-155*

Term	Count	%	*P*	Genes	List total	Fold enrichment	Bonferroni	Benjamini
Immune response	53	1.61	9.85E-16	IFI44L, HLA-DMA, IL10, NOD2, HLA-H, EXOSC9, SP100, LY96, TNFRSF14, HLA-E, HLA-G, PDCD1LG2, HLA-F, IL18BP, TNFSF13B, HLA-DPA1, GBP4, GBP2, GBP1, PSMB10, IFIH1, IFITM2, IFITM3, RSAD2, OAS1, C1R, OAS2, CX3CL1, C1S, CCL5, CD74, IFI35, GCH1, TNFRSF1B, FCN3, SQSTM1, TAP2, TAP1, DHX58, IL18R1, IL1RL1, CFB, TRIM22, PSMB8, PSMB9, DDX58, TNFSF10, OASL, PLCG2, C1RL, TGFBR3, IFI6, DMBT1	290	3.583128436	1.90E-12	1.90E-12
Response to virus	19	0.58	1.47E-11	IFIH1, RSAD2, IFI44, IFI16, STAT1, CCL5, TRIM22, IFI35, ISG20, STAT2, DDX58, IRF9, PLSCR1, ISG15, IRF7, EIF2AK2, MX1, MX2, DMBT1	290	8.131350838	2.79E-08	1.40E-08
Host–virus interaction	21	0.64	6.68E-07	PSMB10, CFLAR, IFIH1, SP100, HTATIP2, TNFRSF14, SP110, STAT1, TRIM22, PSMB8, PSMB9, STAT2, ISG15, CD93, ITGA5, TAP2, IRF7, TAP1, RBCK1, EIF2AK2, DMBT1	369	3.840964199	2.47E-04	6.17E-05
Nucleosome	11	0.34	7.38E-07	H1F0, HIST1H2AC, HIST2H2AA3, HIST1H2BD, HIST1H1C, HIST1H2BK, HIST2H2BE, HIST2H2AC, HIST3H2A, H2AFJ, HIST1H4H	272	8.205065359	1.85E-04	1.85E-04
Antigen processing and presentation	12	0.37	1.44E-06	HLA-H, TAP2, IFI30, HLA-DPA1, HLA-E, TAPBPL, HLA-DMA, CD74, PSMB8, HLA-G, PSMB9, HLA-F	290	6.74432904	0.002730866	5.47E-04
Histone core	9	0.27	2.90E-06	HIST1H2AC, HIST2H2AA3, HIST1H2BD, HIST1H2BK, HIST2H2BE, HIST2H2AC, HIST3H2A, H2AFJ, HIST1H4H	338	9.85739645	0.001916069	0.001916069
Chromatin assembly	11	0.34	1.59E-05	H1F0, HIST1H2AC, HIST2H2AA3, HIST1H2BD, HIST1H1C, HIST1H2BK, HIST2H2BE, HIST2H2AC, HIST3H2A, H2AFJ, HIST1H4H	290	5.898057868	0.029684206	0.003759627
DNA replication, recombination, and repair	10	0.30	4.06E-05	DDX58, IFIT1, IFIH1, DDX60, PRIC285, POLQ, MMRN1, DHX58, DHX32, TOP3B	49	5.527210884	6.90E-04	6.90E-04
Peptide transport	4	0.12	2.30E-04	SLC15A1, TAP2, TAP1, SLC15A3	369	29.7870693	0.08124383	0.010535918
Lysosome	11	0.34	6.03E-04	HYAL1, CD68, LAMP3, GUSB, IFI30, ACP2, SLC15A3, HLA-DMA, CD74, CTSL1, GLB1	369	3.848329423	0.199669631	0.022026854
Cell adhesion molecules	8	0.24	0.038480818	ICAM2, PECAM1, HLA-DPA1, HLA-E, HLA-DMA, PDCD1LG2, HLA-G, HLA-F	123	2.505543237	0.990624458	0.404795422
Complement and coagulation cascades	5	0.15	0.082928761	CFB, SERPINA5, TFPI, C1R, C1S	123	2.995758218	0.999966429	0.52090005

Identified up-regulated gene transcripts determined by microarray (Illumina HumanRef-8, v3.0; Illumina, Inc., San Diego, CA, USA) analysis after overexpression of microRNA-155 (using 30 nM pre-microRNA-155) in hCMEC/D3 cells (14). Analysis was performed by using DAVID Bioinformatics (*https://david.ncifcrf.org*) (98). All genes with a small but significant fold change in mRNA levels (>1.17-fold increase) were included in the analysis above; analysis from 3 independent biological replicates. $P < 0.01$.

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break during the neuroinflammatory response to harmonize a gradual down-regulation of inflammatory signal in brain endothelium.

In prostate carcinoma cells, microRNA-146 regulates the expression of Rho kinase 1 (78), a key mediator in endothelial stress fiber formation and contractility that precedes cell–cell junction delocalization and increased barrier leakage during inflammation (79). However, brain endothelial microRNA-146 does not affect Rho kinase 1 protein levels during neuroinflammation (74), which suggests that microRNA-146 confers accuracy in response in a cell type–specific manner. Moreover, consistent with a role for endothelial microRNA-146 as a negative regulator of NF-κB signaling by repressing multiple targets, overexpression of microRNA-146 in endothelial cells negatively regulates the thrombin-induced increase in leukocyte adhesion to the endothelium by targeting caspase recruitment domain family 10 (80, 81), a key scaffold protein of GPCR-mediated NF-κB signaling (80). In addition, a recent study demonstrated that endothelial microRNA-146 participates in the resolution of cytokine-induced activated endothelium. Indeed, Cheng *et al*. (75) demonstrated that whereas microRNA-146a and microRNA-146b transcript (pri-microRNA-146a/b) expression occurs early after treatment with proinflammatory cytokine IL-1β, the mature forms, microRNA-146a/b, did not increase until after long-term treatment with IL-1β, which coincides with the resolution of inflamed endothelium into a quiescent state. Mechanistically, endothelial microRNA-146 post-transcriptionally regulates RNA binding protein HuR, a protein that is involved in endothelial activation and leukocyte recruitment in response to the proinflammatory cytokine IL-1β (75).

MicroRNA-126/microRNA-126-5p

MicroRNA-126 and microRNA126-5p are highly enriched in endothelial cells and play key roles in endothelial integrity and function (82, 83). Cytogenic location of microRNA-126 and microRNA-126-5p is on chromosome 9q34.3, and both originate from an intron of the gene that codes for epidermal growth factor–like-7 (Egfl7) (82–84). Expression of microRNA-126 has also been detected in the hematopoietic system, including bone marrow stem cells, bone marrow samples from patients with acute promyelocytic leukemias (85), and $CD4^+$ cells of patients with multiple sclerosis (MS) (86). MicroRNA-126 has been more extensively studied than microRNA-126-5p and has been implicated in several important aspects of vascular biology, including cell migration, cytoskeletal organization, capillary formation, and vascular inflammation (43, 87). Functional roles of microRNA-126 were first reported by using knockout animal models that showed their essential role in blood vessel formation and integrity (82, 83). Indeed, before these studies, it was observed that Egfl7-knockout phenotype was associated with failure in angiogenesis and blood vessel sprouting and formation, and only 50% of embryos survived *in utero* (88); however, selective floxed $Egfl7^{\Delta}$ (removal of exon 5–7 without disruption of microRNA-126) and microRNA-126 Δ (removal of a segment of intron 7 without disruption of Egfl7) alleles demonstrated that $Egfl7^{\Delta/\Delta}$ mice were phenotypically normal, whereas microRNA-$126^{\Delta/\Delta}$ mice presented abnormalities in the vasculature (83, 84) similar to those previously reported after complete removal of Egfl7 (88). Phenotypic abnormalities in microRNA-126-knockout mice include lethality in ~50% of embryos because of loss of vascular integrity (remarkably in the brain) accompanied by edema and hemorrhage (83, 84). Surviving mutant neonates showed defects in endothelial sprouting, proliferation, radial migration, and angiogenesis, leading to defective retinae vascularization and cardiac neovascularization after myocardial infarction (83, 84). It has been suggested that the partial lethality of the microRNA-126 mutant mice might be a result of modulation of the gene expression program in endothelial vascularization, rather than a complete shut-down of the angiogenic process (83). Indeed, microRNA-126 has been shown to regulate responses of endothelial cells to VEGF and basic fibroblast growth factor *via* MAPK and PI3K activity (82–84). The proangiogenic actions of microRNA-126, in part, may be a result of reduced expression of negative regulators of angiogenesis, including sprouty-related EVH1 domain-containing protein 1 that binds and inhibits RAF-1 involved in the Ras/RAF-1/MAP/ERK signaling pathway, and by silencing PIK3R2/p85-b, a regulatory subunit of PI3K that results in reduced AKT/PKB activity (83, 84).

MicroRNA-126 is also involved in vascular inflammation and is highly regulated by proinflammatory cytokines (Table 1). Indeed, microRNA-126 is an inhibitor of inflammation by negative modulation of VCAM-1 expression in the resting endothelium (89). Moreover, BEC activation by cytokines decreases levels of microRNA-126, which is associated with loss in VCAM-1 post-transcriptional control and promotes leukocyte adherence to the luminal side of brain endothelium (89) (unpublished observation). Furthermore, Ets-1 and Ets-2, important regulators of immune response and of angiogenesis, interact with Ets binding elements upstream of Egfl7/microRNA-126 (90) and mediate microRNA-126 expression. In addition, Ets-1 can be induced by TNF-α in the endothelium and promotes transcription of genes that are involved in vascular inflammation, including CCL2, VCAM-1, and matrix metallopeptidase 9 (87). Harris *et al*. (89, 90) have proposed that microRNA-126 might act as a negative feedback loop in TNF-α signal transduction. Furthermore, the net effect of Ets-1 on vascular inflammation might, in part, depend on the balance between Ets-1–induced proinflammatory factors, such as CCL2 and VCAM-1, and Ets-1–induced anti-inflammatory factors, such as microRNA-126.

OTHER microRNAs WITH ROLES IN CEREBRAL VASCULATURE HOMEOSTASIS AND PATHOPHYSIOLOGY

High-throughput studies indicate that the brain endothelial microRNA milieu facilitates the capacity of cells to respond to stress and healthy environmental factors. Indeed, lupus serum and complement protein C5a, which are known to

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negatively regulate brain endothelial integrity (91), as well as caloric restriction, which is known to positively regulate brain endothelial integrity (92), induce changes in the levels of brain endothelial microRNAs (91, 92). This suggests that brain endothelial microRNA expression not only orchestrates accurate tuning of gene expression that contributes to cell homeostasis and BBB integrity, but also might enforce new gene expression patterns that can influence the pathophysiology of disorders that affect CNS vasculature. In this context, when BECs are treated with homocysteine, a factor known to disrupt the BBB *in vitro* (93) and to play a role in brain damage (94), expression of microRNA-29 family members is significantly increased (93). Mechanistically, the authors suggested that microRNA-29b suppresses DNA (cytosine-5)-methyltransferase 3β, which leads to increased levels of matrix metallopeptidase 9, a known factor that disrupts BBB integrity (93). In addition, a recent study demonstrated that HIV-1 Tat protein up-regulates expression of brain endothelial microRNA-101, leading to suppression of VE-cadherin protein expression and to increased brain endothelial barrier permeability (95). microRNA-150 is another microRNA with roles in brain endothelial barrier function. Enhanced BBB breakdown is observed by overexpression of microRNA-150 during permanent middle cerebral artery occlusion, an animal stroke model (15). It was reported that microRNA-150 induces enhanced BBB breakdown by directly targeting brain endothelial tyrosine-protein kinase receptor TIE-2, a factor involved in maintaining vascular homeostasis and barrier function (15). Moreover, intracerebroventricular injection of microRNA-150 inhibitor significantly ameliorates BBB disruption in middle cerebral artery occlusion (15). Of note, brain endothelial microRNAs might also contribute to molecular processes that contribute to brain vascular normalization after brain injury. Ge *et al.* (96, 97) suggested that increased levels of microRNA-21 could exert protective roles during BBB damage as a result of a traumatic brain injury animal model by modulating several pathways concurrently. During traumatic brain injury, increased levels of microRNA-21 promoted both VEGF and angiopoietin-1/TIE-2 expression simultaneously, factors known to increase angiogenesis and cerebrovascular integrity (96, 97); therefore, microRNA-21 could be used as a therapeutic strategy to promote neovascularization with barrier properties during brain injury.

THERAPEUTIC POTENTIAL OF BRAIN ENDOTHELIAL MICRORNAS FOR CNS DISEASES

MS is a condition with a central neuroinflammatory component in which brain endothelial barrier function is compromised. Furthermore, MS is a chronic autoimmune CNS disease that is characterized by demyelination, axonal degeneration, and, ultimately, brain and spinal cord atrophy. Studies using gadolinium imaging and MRI analysis show that BBB disruption occurs in localized brain areas and is an early event that precedes development of MS lesions and disease progression (98, 99); however, the critical molecular events in the initiation of cerebrovascular endothelial dysfunction during MS are largely unknown. It is noteworthy that recent work demonstrates that microRNA-155 is focally increased in the active inflammatory MS plaques at the neurovascular unit (14, 56) and activated infiltrative leukocytes (100–102) and might contribute to the pathophysiology of MS. Our pioneering work shows that microRNA-155 is highly expressed in confined areas with MS lesions in the cerebrovascular endothelium. Our study further suggests that increased levels of brain endothelial microRNA-155 contribute to early BBB impartment observed during neuroinflammation. Indeed, microRNA-155 levels are rapidly up-regulated in experimental autoimmune encephalomyelitis (EAE), an animal model for MS, during the relapsing-remitting paralysis course, which are clinical stages with compromised BBB integrity (14). Moreover, mice that are deficient in microRNA-155 show partial resistance to the development of relapsing-remitting EAE and its associated increases in BBB permeability, observations that are consistent with the effect of microRNA-155 as a microRNA that promotes inflammation in T cells, astrocytes, and brain endothelium in the CNS (14, 56, 100, 101). However, it still remains elusive whether the functional consequences of inflammation-associated microRNA-155 are specific to each of the cell types involved or whether it represents a conserved generalized feedback mechanism of the neurovascular unit leading to cellular uncoupling during neuroinflammation.

Moreover, a recent study further supports brain endothelial microRNA-155 as a potential molecular target for improving BBB breakdown (103). The authors investigated therapeutic manipulation of microRNA-155 by using a specific anti-microRNA-155 that was injected systemically and observed that microRNA-155 inhibition at an early subacute stage in an animal model of stroke led to preservation of brain endothelial TJs and barrier integrity *in vivo* (103). These studies suggest that brain endothelial microRNA-155 plays an essential role in neuroinflammatory cerebrovascular pathologies that are associated with disruption of the BBB, and modulation of microRNA-155 in brain resident cells and brain endothelium may constitute a novel therapeutic approach for CNS neuroinflammatory vascular diseases.

Another microRNA therapeutic target for CNS neuroinflammatory disorders is brain endothelial microRNA-146. This microRNA was found predominately in the cerebral microvasculature of active lesions of MS and is highly abundant in the brain endothelium that is surrounded by perivascular inflammatory cuffs in the spinal cord of an EAE relapsing-remitting mice model (74). Mice that are deficient for microRNA-146 are developmentally normal at birth but acquired chronic inflammation around 5–6 mo of age (75) and had enhanced expression of several inflammatory genes, including Vcam-1, Icam-1, Sele, Mcp-1, and Egr1/3 after challenge with the proinflammatory cytokine IL-1β (75). Wu *et al.* (74) demonstrated that microRNA-146 negatively modulated the NF-κB signaling pathway in cultured human brain endothelium *via* stifling multiple genes, including IL-1 receptor–associated kinase 1, TNF receptor–associated factor 6 protein, nuclear factor of activated T cells 5, and RhoA, which regulates VCAM1 and CCL2 protein expression, and, therefore, directly resulted in decreased leukocyte adhesion to inflamed blood vessels.

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Another study has analyzed the therapeutic manipulation of brain endothelial microRNA-98 and Let-7g-3p to prevent BBB dysfunction in neuroinflammation. In this study, the authors identified 2 potential targets, CCL2 and CCL5, by which microRNA-98 and Let-7g-3p may regulate leukocyte adhesion (34). These studies suggest that a brain endothelial microRNA therapeutic strategy aimed at ameliorating BBB dysfunction during neuroinflammation may be beneficiated as a result of microRNAs concomitantly modulating several molecular and cellular processes implicated in neuroinflammation.

CONCLUSIONS

Cytokines regulate the expression of brain endothelial microRNAs that either promote or inhibit inflammatory pathways to orchestrate neuroinflammation at the cerebrovascular bed. Depending on the strength and duration of the inflammatory stimuli, brain endothelial microRNAs may either regulate gene expression to counteract cytokine signaling to maintain the quiescent state or promote a gene expression profile toward an activated cerebrovascular endothelium that could lead to BBB breakdown. Here, we presented that some cytokine-responsive brain endothelial microRNAs can be grouped into coexpressed microRNA clusters and families previously implicated in molecular and cellular processes of angiogenesis. Nevertheless, it remains to be determined whether these altered microRNA clusters and families might participate in increased angiogenesis that has been observed in neurologic conditions affected by neuroinflammation.

Further research in brain endothelial microRNAs will no doubt increase our understanding of the molecular processes that regulate BBB integrity in health and disease, which will tremendously benefit the development of microRNA therapeutic strategies aimed at ameliorating BBB dysfunction during neuroinflammation. Indeed, several preclinical and clinical trials have started microRNA-based therapeutics for many types of disease, including cancers that affect the CNS such as glioblastomas (104). Use of systemic administration of locked nucleic acid–modified oligonucleotide might be a promising form for the delivery of inhibitory microRNAs to the CNS (105), including the brain vasculature (103), which could be used to ameliorate or prevent CNS disorders in which microRNAs play a key pathogenic role (106). In the near future, a major challenge will be to define the spatio-temporal activities of brain endothelial microRNAs and whether microRNA therapeutic strategies are targeted to ameliorate BBB dysfunction during transient or chronic CNS disorders. FJ

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