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Blood–brain barrier (BBB) dysfunction is a major hallmark of many neurological diseases, including multiple sclerosis (MS). Using a genomics approach, we defined a microRNA signature that is diminished at the BBB of MS patients. In particular, miR-125a-5p is a key regulator of brain endothelial tightness and immune cell efflux. Our findings suggest that repair of a disturbed BBB through microRNAs may represent a novel avenue for effective treatment of MS.

Introduction

The blood–brain barrier (BBB) tightly controls the homeostasis of the CNS and actively limits entry of blood-borne molecules and circulating leukocytes. In essence, the BBB is formed by specialized endothelial cells sealed together by intercellular tight junction protein complexes. Although mature astrocytes are not needed for BBB induction (Daneman et al., 2010), these cells are essential in the maintenance of the barrier function through the release of soluble mediators (Abbott et al., 2006; Alvarez et al., 2011). Disruption and immune activation of the BBB are central and early features of multiple sclerosis (MS), a chronic inflammatory disorder of the CNS (Neuwelt et al., 2008, 2011; Zlokovic, 2008). Compromised function of the BBB is also a key early event in the pathogenesis of several neurodegenerative disorders with an inflammatory component, such as Alzheimer’s disease and Parkinson’s disease. Proinflammatory cytokines, such as TNFα and IFNγ secreted by activated leukocytes and/or CNS-resident cells, are considered to mediate changes in gene expression in brain endothelial cells toward an “inflamed” phenotype. Indeed, increased permeability and expression of cell-adhesion molecules on the brain endothelium allow the trafficking of inflammatory agents and circulating leukocytes into the CNS, leading to demyelination and axonal loss (Lassmann et al., 2007; Larochelle et al., 2011). An understanding of the underlying mechanisms of barrier disruption in MS may lead to the development of novel and selective routes of intervention to prevent the influx of inflammatory cells into the CNS.

MicroRNAs, which are endogenous noncoding small RNAs, are now recognized to play a critical role in key cellular functions by specifically repressing gene expression (Filipowicz et al., 2008). Importantly, altered microRNA expression levels have been demonstrated in a number of CNS pathologies, including brain tumors, neurodegeneration, and MS (Hébert and De Strooper, 2009; O’Connell et al., 2010; Junker et al., 2011; Smits et al., 2012). Several microRNAs have been identified in endothelial cells and they have been implicated in primary endothelial cell function and angiogenesis (for review, see Suárez and Sessa, 2009; Bonauer et al., 2010). Thus far, it is not known whether microRNAs play a role in BBB function, which was hypothesized in the current study. Our novel data show that a set of microRNAs modulates BBB function under normal and inflammatory conditions. Most importantly, levels of BBB-associated microRNAs were diminished in isolated MS patient capillaries. Together, our findings uncover an unprecedented and
exciting regulatory mechanism of brain endothelial cell barrier function in health and disease and provide novel opportunities to treat neurovascular-dependent brain diseases through microRNAs.

Materials and Methods

Cells, cell culture, and materials. The human cerebral microvascular endothelial cell line hCMEC/D3 (Weckler et al., 2005) was grown in Endothelial Cell Basal Medium-2 (EGM-2, Lonza) supplemented with human EGF, hydrotorsione, GA-1000, FBS, VEGF, human fibroblast growth factor-B, R3-IGF-1, ascorbic acid, and 2.5% fetal calf serum. To obtain astrocyte cultures, fetal tissue (cerebral hemispheres) was obtained at 17–23 weeks of gestation following Canadian Institute of Health Research-approved guidelines. Human fetal astrocytes were used between postnatal days 2 and 4, and cultures were determined to be >90% pure, as determined by GFAP immunostaining. Astrocyte-conditioned media (ACM) was harvested once a week from confluent flasks of human fetal astrocyte cultures. Anti-vascular endothelial-cadherin (anti-VE-cadherin; clone F8) was from Santa Cruz Biotechnology. The tight junction protein zona occludens-1 was stained with rabbit anti-zona occludens-1 from Zymed. Secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 555 goat anti-mouse IgG were from Invitrogen.

MicroRNA profiling of inflamed brain endothelial cells. Human brain endothelial (hCMEC/D3) cells were grown on collagen-coated six-well plate tissue culture until confluence and then maintained for 72 h. Subsequently, culture media was changed to EGM-2 with all the supplements mentioned above with the exception of VEGF. After stimulation with TNFα and IFNγ, cells were plated tissue culture until confluence and then maintained for 72 h. Subsequently, culture media was changed to EGM-2 with all the supplements mentioned above with the exception of VEGF. After stimulation with TNFα and IFNγ, cells were

qPCR analysis of microRNA expression. The Universal cDNA Synthesis Kit (Exiqon) was used for cDNA synthesis. Extracted RNA was diluted to 5 μg/μl using RNAse-free water. For each reaction, 2 μl of diluted RNA was added to a mixture of 2 μl of 5x buffer, 4.5 μl of RNAse-free water, 1 μl of enzyme mix, and 0.5 μl of RNA spike-in. The samples were incubated at 42°C for 1 h followed by heat inactivation at 95°C for 5 min. Samples were cooled to 4°C and stored at −20°C thereafter. The qPCR was performed using the Pick-And-Mix microRNA PCR Panel using the LNA microRNA PCR primer sets for the indicated 11 microRNAs (Exiqon). Three independent samples per plate were tested. The cDNA was diluted 1:100 in RNAse-free water. Per well, 5 μl of sample and 5 μl of 2x SYBRgreen mastermix were added and plates were then sealed and centrifuged. qPCR was performed on an Applied Biosystems 7700 machine for 40 cycles, which consisted of two steps at 95°C for 10 s and 60°C for 1 min. Specificity of the PCR product was determined by a melting curve at the end of the qPCR.

Lentiviral delivery of miR-125a-5p. The miR-125a-5p sequence was obtained from the miR-Vec library (Voorhoeve et al., 2006) and ligated between the NdeI and EcoRI restriction sites of pRRL-cPPT-CMV-X2-PRE-SIN-IRES-eGFP vector (kindly provided by Dr. J. Seppen, Department of Experimental Hepatology, Academic Medical Center, Amsterdam, The Netherlands). Recombinant lentviruses were produced by cotransfecting subconfluent HEK293T cells with the specific expression plasmids and packaging plasmids (pMDL/gp, pRRE, pRSV-Rev, and pMD2G) using calcium phosphate as a transfection reagent. HEK293T cells were cultured in DMEM, supplemented with 10% FCS and 1% penicillin/streptomycin, in a 37°C incubator with 5% CO2. Infectious lentiviruses were collected 48 h after transfection. The supernatant was centrifuged at 2000×g for 40 min. Specificity of the PCR product was determined by a melting curve at the end of the qPCR.

Isolation of MS capillaries. The tissues were obtained from The Netherlands Brain Bank (NNB, Netherlands Institute for Neuroscience, Amsterdam. For all material, NNB obtained from donors a written informed consent for brain autopsy and the use of the material and clinical information for research purposes. Brain capillaries were isolated from periventricular non-neurological patient tissue, periventricular normal-appearing white matter, and periventricular MS lesions from postmortem MS patients. After resection, the tissues were homogenized using pestles and capillaries were separated from myelin and single cells using diethylaminoethyl–dextran gradient (15%; Sigma-Aldrich) centrifugation for 25 min at 2500 rpm without brake. Isolated capillaries were washed on a 40 μm meshed filter (BD Falcon) to remove red blood cells. Finally, RNA was extracted with Trizol and stored in −80°C. The capillaries were characterized by qPCR analysis of brain endothelial cell markers zona occludens-1, claudin-5, VE-cadherin, and P-glycoprotein; the pericyte marker platelet-derived growth factor receptor-β; and astrocyte marker GFAP.

Transfections. hCMEC/D3 cells were transfected using Amaxa Technology (Lonza). Per condition, 15 cm² of 80–90% confluent cells were trypsinized and transfected with siRNA against miR-125a-5p (2.5 μg of 25 μm miRCURY LNA microRNA Inhibitor, Exiqon) or scrambled RNA (2.5 μg of 25 μm miRCURY LNA microRNA Inhibitor Negative Control, Exiqon). The transfection was performed with the 4D-Nucleofector System and P5 Primary Cell Solution Kit (Amaxa, Lonza), according to the manufacturer’s protocol. After transfection, the cells were resuspended in RPMI-1640 medium supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) for 5 min, and subsequently seeded on collagen-coated electrical cell-substrate impedance-sensing (ECIS) arrays with additional supplemented EGM-2 medium. Knockdown of miR-125a-5p was assessed by qPCR.

ECIS assay. ECIS Model 1600R (Applied BioPhysics) was used to measure the transendothelial electric resistance of hCMEC/D3 cell monolayers in real time as described previously (Keese et al., 2004). We seeded 100,000 cells onto each well of an 8W10+ ECIS array coated with collagen. Impedance was measured at 6000 Hz in real time. Cells were treated with a 1:1 mixture of ACM and EGM-2 containing 2.5% FCS or with 10 ng/ml TNFα and IFNγ immediately after seeding. Lentiviral transduction of microRNA-125a was performed directly after seeding in the ECIS array.
Statistical analysis. Statistical analysis was performed with the Student’s t test (Prism 4.0; GraphPad Software), and results were considered significant if \( p < 0.05 \).

Results

Here we explored whether microRNAs control BBB function and its immunoquiescence in MS. First, microRNA expression profiling indicated that exposure of cultured brain endothelial cells to inflammatory mediators significantly changed the expression level of 107 microRNAs (Fig. 1A). Of note, most (98 of 107) of the differentially expressed microRNAs were downregulated by TNFα/IFNγ treatment, which was also shown to impair barrier function (Fig. 1B). Next, as a means to identify barrier-related microRNAs, we cultured brain endothelial cells in the presence of astrocyte-released factors. Continuous treatment of brain endothelial monolayers with conditioned media of cultured astrocytes for 48 h induced a tighter barrier in cultured brain endothelial cells (Fig. 1D) and significantly changed the expression level of 365 microRNAs (Fig. 1C). In this case, 278 of 365 of the differentially expressed microRNAs were increased in the brain endothelial cells by astrocyte-released factors, suggesting that microRNA-mediated induction of BBB properties is largely due to repression of protein synthesis within the brain endothelium. Remarkably, the overlay of the microRNA expression profiles between TNFα/IFNγ (i.e., barrier-reducing) and astrocyte factor (i.e., barrier-inducing) treatments revealed that the expression of 50 of the 55 microRNAs regulated by both treatments changed in opposite directions. MicroRNAs decreased by barrier-reducing TNFα/IFNγ treatment were enhanced by barrier-inducing astrocyte factors and vice versa (Fig. 1E). These data strongly suggest a potential and important function of a set of microRNAs controlling the balance between a tight and leaky or inflamed BBB.

To specifically identify microRNAs involved in the immune-activated and dysfunctional BBB as observed in MS, we next assessed the expression level of this particular set of BBB-related microRNAs. Quantitative PCR analyses of 11 microRNAs with the highest differential in expression levels when comparing barrier-enhancing versus barrier-reducing stimuli (Fig. 1E, colored dots) was done using freshly isolated brain capillaries from MS lesion areas and normal-appearing white matter in MS patients (Fig. 2A, patient characteristics). Freshly isolated brain capillaries comprised brain endothelial cells as indicated by zona

Figure 1. Brain endothelial microRNAs are deregulated in inflammation in vitro. A, C, Heat map of differential microRNA expression in (A) TNFα and IFNγ (10 ng/ml for 24 h) and (C) astrocyte factor-treated (ACM, 50% v/v, 48 h) hCMEC/D3 cells. Purple indicates relatively higher expressed microRNAs. Yellow indicates relatively lower expressed microRNAs. B, ECIS analysis of control and TNFα/IFNγ-treated hCMEC/D3 cells. Maximal resistance in control: 1014 ± 41.2 Ω; TNFα/IFNγ: 667.6 ± 41.2 Ω, \( n = 4 \), \( p = 0.001 \) by Student’s t test. D, ECIS analysis of control and astrocyte-derived factor-treated hCMEC/D3 cells. Maximal resistance in control: 1673 ± 1.1 Ω; ACM (50% v/v): 2008 ± 58.8 Ω, \( n = 3 \), \( p = 0.001 \) by Student’s t test. Error bars represent the mean ± SEM. E, Representation of common microRNAs regulated after ACM and TNFα/IFNγ-treatment in hCMEC/D3 cells.
occludens-1, claudin-5, P-glycoprotein, and VE-cadherin expression and contained minor amounts of astrocytes (GFAP and GFAPβ) and pericytes (platelet-derived growth factor receptor-β) (Fig. 2B). Most strikingly, all the microRNAs selected according to their opposing regulation in TNFα/IFNγ and astrocyte factor-treated brain endothelial cells were reduced in brain capillaries in MS lesions (Fig. 2C). These findings indicate that the currently identified microRNAs may represent potential and novel mechanisms by which BBB function is altered in MS. Of note, it was reported before that miR-125a-5p in particular can regulate inflammatory processes (Chen et al., 2009; Li et al., 2010; Zhao et al., 2010). The decreased expression of miR-125a-5p in MS lesions was corroborated in brain endothelial cells obtained from normal-appearing white matter and active lesions of MS patients through a laser capture approach (Fig. 2D).

Normally, the barrier function of the brain endothelium is instigated by the presence of specific cell–cell junction complexes and a high endothelial resistance to paracellular trafficking of ions and other molecules. We therefore set out to assess the contribution of miR-125a-5p to the formation of a tight brain endothelial barrier. Using cell lines overexpressing miR-125a-5p, we determined that miR-125a-5p significantly increased brain endothelial cell barrier function, thereby mimicking the function of astrocytes (Fig. 3A). Conversely, specific knockdown of miR-125a-5p reduced the barrier-enhancing effect of astrocytes without influencing barrier formation under control conditions (Fig. 3B). Subsequent confocal microscopy analyses revealed that cells with increased levels of miR-125a-5p formed thicker and more continuous junctional complexes of VE-cadherin and zona occludens-1, whereas the opposite was found in cells with reduced levels of miR-125a-5p (Fig. 2C). The altered expression levels of VE-cadherin was confirmed by Western blotting (data not shown).

In general, the brain endothelial cell barrier maintains immune quiescence of the brain through the low expression of adhesion molecules, such as intracellular cell adhesion molecule (ICAM)-1, a cell adhesion molecule involved in vascular permeability and leukocyte infiltration. However, under inflamed conditions, as apparent during MS, brain endothelial cells strongly upregulate cell adhesion molecules that in turn mediate the migration of leukocytes into the brain. Treatment of the brain endothelium with the proinflammatory mediators TNFα/IFNγ caused a strong reduction of miR-125a-5p levels (Fig. 3D), which in turn was associated with enhanced expression of the endothelial cell adhesion molecule ICAM-1 and increased diapedesis of leukocytes (Fig. 2E, F). Interestingly, overexpression of miR-125a-5p in the brain endothelial cells reduced TNFα-mediated ICAM-1 expression and monocyte transmigration through the brain endothelial cell barrier (Fig. 3E, F).

Discussion
Here, we are the first to show that microRNAs contribute to modulation of BBB function. We assessed microRNA expression in brain endothelial cells treated either with proinflammatory cytokines known to impair BBB function or with astrocyte-released factors that can strengthen BBB function. We found that while a large number of microRNAs were downregulated in brain endothelial cells with impaired BBB function, strengthening BBB function was generally associated with increased microRNA expression. Thus, we identified a microRNA signature that is cen-
Brain endothelial miR-125a-5p regulates barrier tightness and prevents leukocyte passage.

**A.** Overexpression (A) and knockdown (B) of miR-125a-5p in hCMEC/D3 cells significantly modulates barrier tightness in control (A) and ACM-treated cells (B) as determined by ECIS (control: 809.3 ± 48.9 Ω; miR-125a-5p: 948.5 ± 18.7 Ω). C, VE-cadherin and zona occludens-1 (ZO-1) subcellular distribution in control cells, in miR-125a-5p-overexpressing hCMEC/D3 cells, and in miR-125a-5p-knockdown hCMEC/D3 cells. Scale bars, 25 μm. D, TNF-α treatment of hCMEC/D3 decreases the expression of miR-125a-5p as measured by qPCR. Data show the abundance of miR-125a-5p normalized to the small nuclear RNA U6 relative to levels in control cells ± SEM, n = 3, *p < 0.05, **p < 0.005 by Student’s t test. E, Transendothelial migration of monocytes was studied in control hCMEC/D3 cells and in cells with enhanced expression of miR-125a-5p previously treated or not with TNF-α. Data are expressed as the percentage of transmigrated monocytes and show the mean ± SEM. F, ICAM-1 expression in control hCMEC/D3 cells and in cells with enhanced expression of miR-125a-5p in the presence or absence of TNF-α as measured by flow cytometry. Data are expressed as the mean fluorescence intensity and show the mean ± SEM.
2009). Such findings indicate that MS is the effect of (auto)immune responses mediated, at least partially, by microRNAs. In 2010, a study published in *Nature* (Du et al., 2009) showed that the expression of a TH-17 cell-associated microRNA, miR-326, is highly correlated with disease severity in patients with MS and in mice with experimental autoimmune encephalomyelitis, a model for brain inflammation widely used to study CNS demyelinating diseases, such as MS.

Although the mechanism of action and specific mRNA targets of miR-125a-5p are largely unknown, others have shown that miR-125a-5p has a role in different tumor types and inflammation. miR-125a-5p is decreased in non-small cell lung cancer (Jiang et al., 2010), hepatocellular carcinoma (Murakami et al., 2006), breast cancer (Guo et al., 2009), and medulloblastoma (Ferretti et al., 2009). Low expression levels of miR-125a-5p were associated with enhanced malignant potential of gastric cancer, possibly through repression of ERBB2, leading to reduced extracellular-signal regulated kinase 1/2 and Akt signaling (Scott et al., 2007; Nishida et al., 2011). Other cancer-related mRNA targets of miR-125a-5p are TrkC (Ferretti et al., 2009), the oncoproteins (IL-2, IL-6, TNF-α) that miR-125a decreases the secretion of some inflammatory diapedesis (Reijerkerk et al., 2012). Others previously showed expression in vascular endothelial cells (Li et al., 2010).

It is of interest that miR-125a-5p inhibits endothelin-1 expression in vascular endothelial cells (Li et al., 2010). Endothelin-1 is a proinflammatory protein involved in monocyte diapedesis (Reijerkerk et al., 2012). Others previously showed that miR-125a decreases the secretion of some inflammatory cytokines (IL-2, IL-6, TNF-α, and TGF-β) by targeting the oxysterol binding protein-like 9 (Chen et al., 2009). Moreover, miR-125a contributed to elevated inflammatory chemokine RANTES (regulated on activation, normal T-cell expressed and secreted) levels via targeting of KLF13 in systemic lupus erythematosus (Zhao et al., 2010). The mechanism of action of miR-125a-5p in the inflamed BBB warrants further study. Finally, deregulation of microRNAs contributes to many human diseases and there is great interest in microRNAs as novel therapeutic targets or tools. The direction toward therapeutic application has been set in animal models to suppress liver cancer (Hébert and De Strooper, 2009) or chronic hepatitis C virus infection (Kota et al., 2009). Importantly, our current data point to a significant and novel role of microRNAs in BBB functioning. Many neurological disorders are associated with profound alterations in the vasculature of the brain. Therapeutic application of microRNAs, such as miR-125a-5p, potentially could re-establish normal functioning of the brain vasculature in endothelial cell-based neurological diseases, in particular MS.

References


Reijerkerk, Lopez-Ramirez et al. • MicroRNAs and the Inflamed Blood–Brain Barrier


