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miRNA-27a-3p and miRNA-222-3p as Novel Modulators of Phosphodiesterase 3a (PDE3A) in Cerebral Microvascular Endothelial Cells

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Abstract

Endothelial dysfunction is a key element in cerebral small vessel disease (CSVD), which may cause stroke and cognitive decline. Cyclic nucleotide signaling modulates endothelial function. The cyclic adenosine monophosphate-degrading enzyme phosphodiesterase 3 (PDE3) is an important treatment target which may be modulated by microRNAs (miRNAs) important for regulating gene expression. We aimed to identify PDE3-targeting miRNAs to highlight potential therapeutic targets for endothelial dysfunction and CSVD. PDE3-targeting miRNAs were identified by in silico analysis (TargetScan, miRWalk, miRanda, and RNA22). The identified miRNAs were ranked on the basis of TargetScan context scores and their expression (log2 read counts) in a human brain endothelial cell line (hCMEC/D3) described recently. miRNAs were subjected to co-expression meta-analysis (CoMeTa) to create miRNA clusters. The pathways targeted by the miRNAs were assigned functional annotations via the KEGG pathway and COOL. hCMEC/D3 cells were transfected with miRNA mimics miR-27a-3p and miR-222-3p, and the effect on PDE3A protein expression was analyzed by Western blotting. Only PDE3A is expressed in hCMEC/D3 cells. The in silico prediction identified 67 PDE3A-related miRNAs, of which 49 were expressed in hCMEC/D3 cells. Further analysis of the top two miRNA clusters (miR-221/miR-222 and miR-27a/miR-27b/miR-128) indicated a potential link to pathways relevant to

Highlights

- Cyclic nucleotides are essential in endothelial cell function.
- The cAMP degrading phosphodiesterase 3 (PDE3) is a target in stroke treatment.
- PDE3A is expressed in cerebral microvascular endothelial cells.
- PDE3A-associated miRNAs likely regulate cerebral microvascular endothelial function.
- PDE3A expression is reduced by specific miRNAs important for endothelial function.

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cerebral and vascular integrity and repair. hCMEC/D3 cells transfected with miR-27a-3p and miR-222-3p mimics had reduced relative expression of PDE3A protein. PDE3A-related miRNAs miR-221/miR-222 and miR-27a/miR-27b/miR-128 are potentially linked to pathways essential for immune regulation as well as cerebral and vascular integrity/function. Furthermore, relative PDE3A protein expression was reduced by miR27a-3p and miR-222-3p.

Keywords Small vessel disease · microRNA · PDE3 · Stroke · Endothelial cells

Introduction

Cerebral endothelial dysfunction is an important factor in the pathophysiology of cerebral small vessel disease (CSVD) [1]. Cyclic nucleotides play a key role in endothelial function and integrity [2, 3]. Phosphodiesterase 3 (PDE3) is a cyclic adenosine monophosphate (cAMP)-specific enzyme present in cerebral arteries [4]. The effects of PDE3 can be modulated by cilostazol, a selective PDE3 inhibitor used for the secondary prevention of stroke and peripheral artery disease [5–7]. The roles of PDE3 in endothelial dysfunction and stroke remain to be clarified, but the use of cilostazol has gained interest, as it may improve cerebral endothelial function and integrity by increasing cAMP levels [8–10]. Currently, cilostazol is being tested for effects in CSVD stroke (NCT02481323, clinicaltrials.gov). In addition, a recent genome-wide association study showed an association between stroke and PDE3A and designated it as a risk locus [11].

CSVD represent a quarter of cerebral ischemic strokes which increase the risk for subsequent strokes, progressive cognitive decline, and high mortality [12, 13]. No specific treatment is available for patients with CSVD, which is characterized by white matter hyperintensitites, microbleeds and lacunes in deeply located cerebral arterioles, in basal ganglia, and other subcortical regions [14]. One treatment involves the use of cilostazol; however, the associated side effects such as headache and cardiac arrhythmias limit its use. Therefore, other means of modulating the expression of PDE3 are warranted, such as the use of specific microRNAs (miRNAs). miRNAs are endogenously expressed small noncoding RNAs 18–22 nucleotides long. They act as post-transcriptional gene function and expression modulators and have recently been suggested as potential biomarkers in disease detection and staging [15] and as therapeutics [16]. miRNAs are known to modulate processes involved in endothelial function and the associated inflammatory response and angiogenesis [17, 18]. Stroke-specific miRNAs are an attractive focus for both disease risk monitoring and therapeutic drug development, as early prevention and management of CSVD risk factors are essential to reduce subsequent functional deficits.

There are two isoforms of PDE3, which show different expression patterns. PDE3A is expressed in the brain, heart, platelets, endothelial cells, and vascular smooth muscle cells, and PDE3B is expressed in adipocytes, hepatocytes, and beta cells [19, 20]. Mutations in PDE3A result in increased enzyme activity and a susceptibility to hypertension [21, 22]. PDE3B in beta cells regulates cAMP-mediated insulin signaling [23]. As diabetes and hypertension are risk factors for CSVD, both PDE3 isoforms may play a role in CSVD disease management. Despite the associated side effects, cilostazol improves endothelial integrity response, inhibits the proliferation of vascular smooth muscle cells [24], and improves cerebral blood flow [6, 25]. At present, cilostazol and other available PDE3 inhibitors do not discriminate between the two isoforms of PDE3. Hence, PDE3 isoform-specific modulators may be better able to enhance cerebral endothelial function [9, 26–28]. Notably, miRNAs can target the specific 3′ untranslated regions (UTRs) of each of the PDE3 isoforms. The specificity of miRNAs and their regulation of target gene expression represents their potential for finding a therapeutic target or specific disease biomarker. Abnormal expression of miRNAs may, however, also contribute to other diseases of the central nervous system [29–31].

In the search for treatment targets for CSVD, we aimed to investigate if PDE3 isoform-specific miRNAs are related to cerebral endothelial cell signaling. Such miRNA-based modulators would be potential drug targets to manage disease progression and development. Therefore, we evaluated the expression of PDE3 isoforms in an in vitro cerebral endothelial cell model (hCMEC/D3). We found that these cells only express PDE3A. A systematic bioinformatics analysis was conducted to identify PDE3A-targeting miRNAs and their downstream pathways to infer their possible roles in processes important to CSVD and endothelial signaling. The results showed that the relative PDE3A protein expression was reduced by miR-27a-3p and miR-222-3p mimics.

Materials and Methods

Identification of miRNAs Targeting PDE3A

In silico miRNA target prediction analysis was performed to identify miRNAs targeting PDE3A through binding to its 3′ UTR. TargetScan version 6.2 human miRNA target predictions were retrieved from http://targetscan.org [22]. We used the TargetScan context scores and evolutionary conservation scores aggregated per gene and miRNA. The miRNAs targeting PDE3A were selected based on a TargetScan context score cutoff of < -0.01. The predicted target module

in miRWalk version 2.0 was used to retrieve predicted miRNAs targeting the 3′ UTR of PDE3A. For miRWalk analysis, target predictions by three algorithms (miRWalk, miRanda, and RNA22) were included [32]. For further analysis, we used the miRNAs that were predicted by both TargetScan and miRWalk analysis (Supplementary Table 1). Evidence of expression for these predicted miRNAs was retrieved from a published study on small RNA sequencing of the hCMEC/D3 cell line (a model of the human blood brain barrier (BBB) http://bioinformaticstools.mayo.edu/bbbomics [1]).

miRCo and COOL/KEGG Analysis of PDE3A-Related miRNAs

Predicted PDE3A miRNAs were subjected to miRCo analysis in CoMeTa [33] to create miRNA clusters. CoMeTa is built on the principle that the targets of a given miRNA are likely to be co-expressed and, therefore, belong to the same miRNA network. It integrates expression data from hundreds of cell types and tissues and creates clusters of miRNAs based on their co-expressed targets. Representative miRNAs from the top two clusters were identified and subjected to further analysis. The miRNAs in the top two clusters were used for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and COOL analysis to assign functional roles to the networks using CoMeTa [33].

Cell Line Culture

Supplements to endothelial basal medium (EBM-2, catalogue no. CC3156; Bionordika, Herlev, Denmark) included HEPES, 10 mM (catalogue no. H0887); bFGF, 1 ng/ml (catalogue no. H0887); penicillin-streptomycin, 1% (catalogue no. P0781); ascorbic acid, 5 μg/ml (catalogue no. A4555); hydrocortisone, 1.4 μM (catalogue no. H0135); fetal bovine serum (FBS), 5% (catalogue no. 0679); calf skin collagen type I (catalogue no. C8919) (all from Sigma-Aldrich, Broendby, Denmark); and chemically defined lipids, 1% (catalogue no. 11905-031; Life Technologies, Naerum, Denmark).

hCMEC/D3 cells (catalogue no. SCC066; Merck, Hellerup, Denmark) were seeded at a density of ~25,000 cells/cm^2 and cultured to confluence in EBM-2 in tissue culture flasks coated with calf skin collagen type I (10 μg/cm^2) before experiments were performed. Cell cultures were maintained in a 5% CO_2 incubator at 37 °C.

Transfection with miRNA mimics

Approximately 300,000 hCMEC/D3 cells were seeded per well a day before transfection. At ~80% confluence, the cells were transfected in EBM-2 (minus penicillin-streptomycin) using 2 μg/ml Lipofectamine® 2000 (catalogue no. 11668030) in Opti-MEM®I-reduced serum medium (catalogue no. 31985-062) at concentrations of 5 or 10 nM of *miR*Vana™ miRNA mimics of hsa-miR-222-3p (catalogue no. MC11376), hsa-miR-27a-3p (catalogue no. MC10939), and mirVana™ miRNA mimic negative control no. 1 (catalogue no. 4464058) (all purchased from Life Technologies, Naerum, Denmark). Transfection efficiency (~90%) was confirmed using 30 nM siGLO green transfection indicator (catalogue no. D-001630-01-05; Dharmacon, GE healthcare, Little Chalfont, Buckinghamshire, UK). A mock transfection was included as control. After 48 h of incubation, the cells were harvested for further analysis. The addition of miRNAs to transfected cells was confirmed by RT-qPCR (Supplementary Fig. 1).

Note that miRNA mimics were tested at 5 nM and 10 nM, but 5 nM did not alter PDE3A protein levels relative to those with the negative control and mock transfections (Supplementary Fig. 2).

Western Blotting

The following antibodies were used: polyclonal rabbit anti-PDE3A (catalogue no. SC-20792; Santa Cruz Biotechnology, USA); polyclonal rabbit anti-PDE3B (catalogue no. ab99290) and monoclonal mouse anti-GAPDH (catalogue no. 8245) (both Abcam, Cambridge, UK); and horseradish peroxidase-conjugated secondary anti-mouse IgG (catalogue no. 7072S) and anti-rabbit IgG (catalogue no. 7074S) (both Cell Signaling Technology, Herlev, Denmark).

Protein samples were adjusted to 15 μg/25 μl and separated using 4–12% Bolt™ Bis-Tris gels (catalogue no. NW04122BOX; Life Technologies, Naerum, Denmark). Proteins were blotted onto 0.45-μm nitrocellulose membrane (catalogue no. 8025; Life Technologies) and blocked with 5% skim milk in TBS-T 0.1%) for 1 h at room temperature before incubating with rabbit-anti-PDE3A (1:300) and mouse anti-GAPDH (1:5000) antibodies on a mixer at 4 °C overnight. For PDE3B detection, protein lysates adjusted to 30 μg hCMEC/D3 and 20 μg of INS-1E cells (rat pancreatic beta cells used as a positive control) were separated according to the same procedure as described above and incubated with anti-PDE3B antibody (1:1000) overnight at 4 °C. After a brief wash, the membranes were incubated with secondary antibody for 1 h on an orbital shaker and developed with the Amersham ECL Supersignal chemiluminescence detection kit (catalogue no. RPN2232; GE health care, Life Technologies) using an LAS4000 imager. Western blotting data from transfection experiments were initially normalized to an internal standard (GAPDH), and relative values were calculated. Quantification was performed with ImageQuantTL software (GE healthcare, Life Sciences, Broendby, Denmark). Four independent experiments were performed, and representative blots are shown.

Extraction of RNA and RT-qPCR

RT-qPCR was performed with Primetime standard TaqMan qPCR assays for *PDE3A*, *PDE3B*, and *HPRT1* (Hs.PT.58.26521785, Hs.PT.58.2413391, and Hs.PT.58v 45621572, respectively; Integrated DNA Technology, Leuven, Belgium). Reactions were run in triplicates with 5 ng of hCMEC/D3 cDNA, 10-μM primer, 2.5-μM probe (final concentration), and Brilliant III Ultra-Fast QPCR Master Mix (catalogue no. 600880; Agilent Technologies Inc., Glostrup, Denmark). Reaction mixtures were in a volume of 10 μl on CFx 384 Real-Time thermal cycler (Bio-Rad, Hercules, CA, USA). Relative amounts of mRNA were quantified by normalizing to the internal control *HPRT1* gene. Values are the means ± SEMs of delta cycle threshold (ΔC_T) values from three independent experiments.

miRNA Isolation and RT miRNA qPCR

Total RNA was extracted from the transfected cells using a miRNeasy extraction kit (Qiagen) and Trizol according to the manufacturer's instructions except for the addition of 200-μl chloroform. cDNA was synthesized from 10 ng of RNA in a total reaction volume of 10 μl using a miRCURY LNA™ Universal RT cDNA synthesis kit II (catalogue no. 2033551; Exiqon, Vedbaek, Denmark) according to the manufacturer's instructions and run on a GeneAmp PCR system 9700 (Applied Biosystems, CA, USA) for 60 min at 42 °C, 5 min at 95 °C, and then held at 4 °C. The qPCR was set up using ExiLENT SYBR Green master mix (catalogue no. 203421; Exiqon) with 80× diluted cDNA using specific LNA™ PCR primer assays (Qiagen, Copenhagen E, Denmark) for hsa-miR-27a-3p (catalogue no. YP00206038) and hsa-miR-222-3p (catalogue no. YP00204551). Relative quantification of miRNA levels was calculated after normalization with an internal control, the small nuclear RNA U6 (*RNU6*; catalogue no. YP00203907) [34]. All the miRNA RT-qPCR reactions were performed in duplicates at 95 °C for 10 min, with 45 cycles of 95 °C for 10 min and 1 min at 60 °C on a CFx 384 Real-Time thermal cycler (Bio-Rad, Hercules, CA, USA) [35]. Four independent experiments were performed, and means ± SEMs were plotted.

Statistical Analysis

All data from transfection experiments are presented as the means ± SEMs from four independent experiments. We used two-tailed, paired student's *t* tests for comparisons. Results were considered statistically significant when the *p* value was < 0.05.

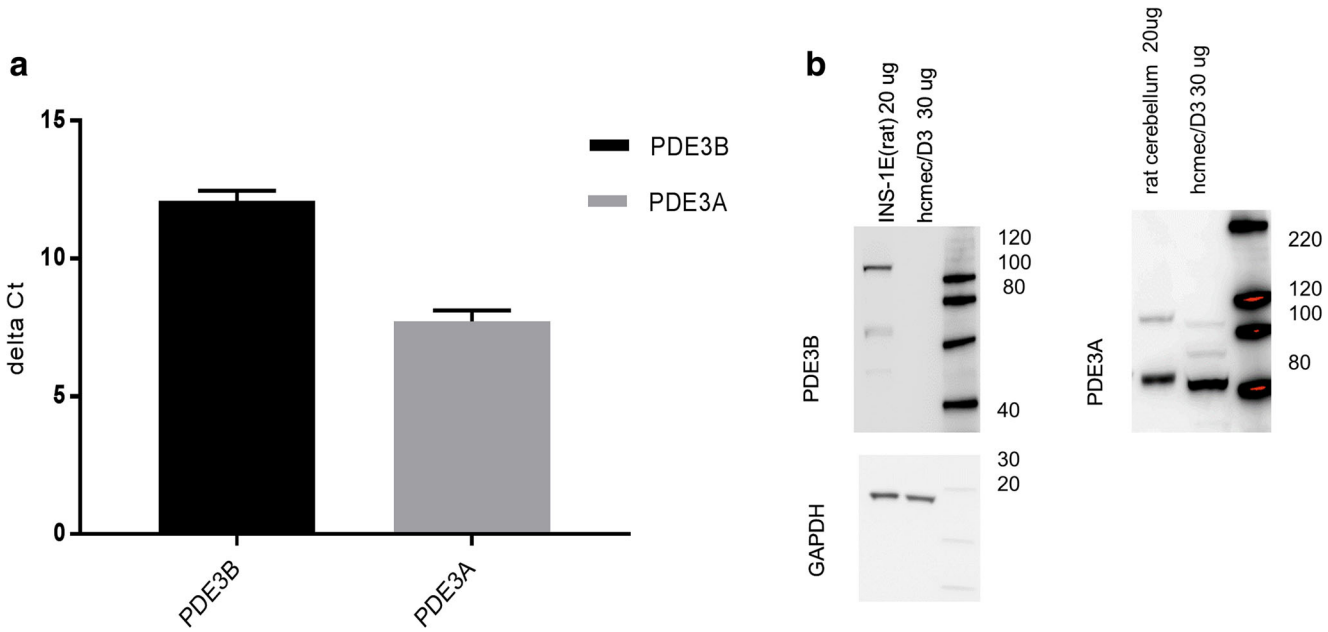

Fig. 1 **a** Five nanograms of cDNA was used to run qPCR with TaqMan assays for PDE3A and PDE3B. Relative levels of mRNA are quantified with internal housekeeping *HPRT1* and means ± SEMs of $n = 3$ are presented ($\Delta C_T = C_T$ *PDE3* $- C_T$ *HPRT1*). **b** Expression of PDE3A and PDE3B protein in hCMEC/D3 analyzed by Western blotting. Cell lysates from hCMEC/D3 (30 μg) and from rat cerebellum (20 μg; positive control) were stained with anti-PDE3A antibody. To check expression of PDE3B in hCMEC/D3, 30 μg hCMEC/D3 lysate was separated along with lysates from INS-1E beta cells from rat (20 μg; positive control) and stained with anti-PDE3B antibody. *GAPDH* was included as housekeeping in western blot. $n = 3$ independent experiments. Representative blot is shown

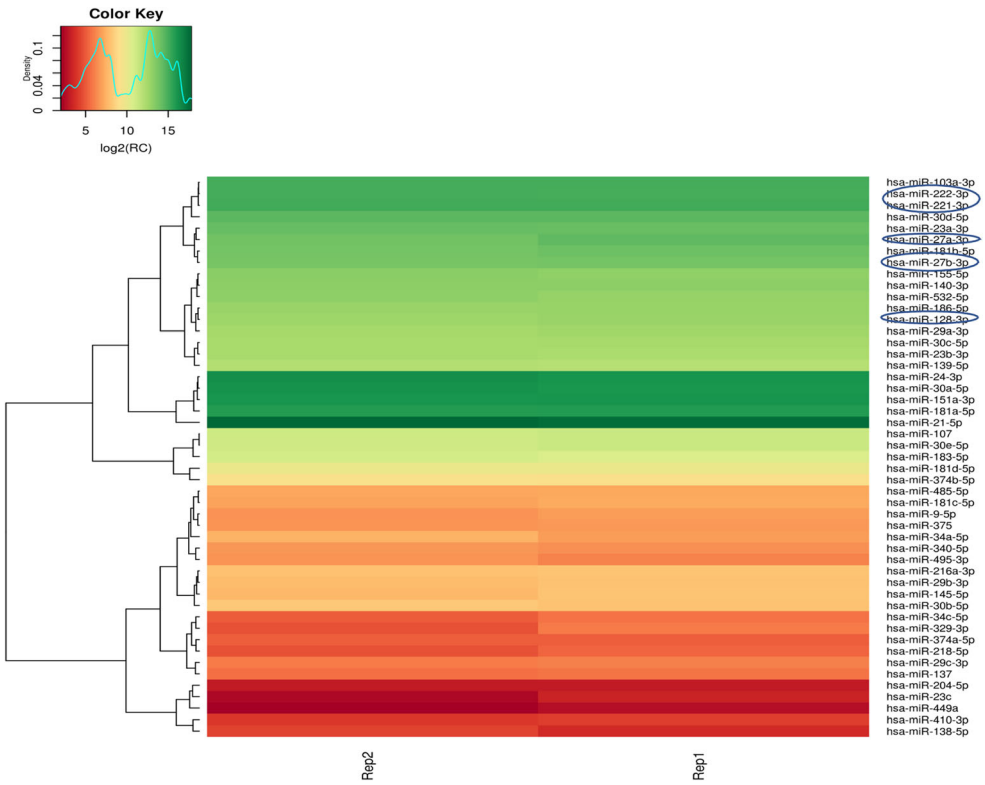

Fig. 2 Heat map showing hierarchical clustering of log2-transformed read counts (RC) of the 49 PDE3A miRNAs expressed in hCMEC/D3 cells. Rep1 and Rep2 are the two replicates from a recent study from [38]. The red and green colors represent the lowly and highly expressed miRNAs, respectively

Results

Expression of PDE3 in the hCMEC/D3 Cell Line

The expression of PDE3 isoforms was evaluated in the hCMEC/D3 cell line model of cerebral endothelial microvessels [36]. Immunoblotting and gene expression analyses were performed to determine protein and mRNA levels, respectively. While the expression of PDE3A, although low, was confirmed at both protein and mRNA level in hCMEC/D3 cells, PDE3B was not detected at the protein or mRNA level (Fig. 1a and b). The mean raw C_T values were 35 and 38.6 for PDE3A and for PDE3B, respectively ($n = 3$ independent experiments).

Prediction of miRNAs Targeting PDE3A

miRNAs targeting PDE3A were predicted using TargetScan (version 6.2) [37], and we identified a total of 67 miRNAs targeting the 3′ UTR of PDE3A and selected those for further analysis (Supplementary Table 1). A recent study revealed that 49/67 of these miRNAs are expressed in hCMEC/D3 cells [38]. A heat map profile based on hierarchical clustering of the 49 PDE3A miRNAs expressed in the hCMEC/D3 cell line was produced (Fig. 2) [38]. Log2-transformed raw read counts were plotted on the basis of RNA-seq data from two replicates. The miRNAs with low expression are depicted in red and highly expressed miRNAs are in green. We observed that some of the endothelial specific miRNAs such as miR-21-5p and miR-24-3p were highly expressed in this endothelial cell line.

miRCo Analysis of miRNAs Related to PDE3A Expressed in hCMEC/D3

miRNAs with similar functions form a network and target co-expressed genes with similar and overlapping biological functions. miRNA functions can be classified based on the targets they regulate. The 49 miRNAs related to PDE3A and expressed in hCMEC/D3 cells were subjected to miRCo analysis [33] to cluster the miRNAs based on their shared co-expressed gene targets. We noted a total of seven miRNA clusters/communities for the 49 PDE3A miRNAs (Table 1). Next, the miRNAs were ranked and prioritized based on their context scores and expression values in the hCMEC/D3 cell line. The top two clusters containing five endothelial miRNAs which were predicted to target PDE3A were selected for further analysis: cluster 1, hsa-miR-221-3p/hsa-miR-222-3p; and cluster 2, hsa-miR27a-3p/hsa-miR-27b-3p/hsa-miR-128-3p.

Top miRNA Clusters May Target Pathways in Immune Regulation, Neuronal Function, and Survival

The five miRNAs in the top two clusters (hsa-miR-221-3p, hsa-miR-222-3p, hsa-miR27a-3p, hsa-miR-27b-3p, and hsa-

Table 1 miRNAs related to PDE3A and expressed in hCMEC/D3 were subjected to miRCo analysis. miRNAs with overlapping functions are co-expressed and exist together in networks/communities. Context score ++ describe the complementarity to the target PDE3A and log2(RC) is quantification of miRNAs. Data shown for two replicates indicating its expression in the hCMEC/D3 cells. The top two clusters (cluster 1 and 2) contain the highly expressed miRNAs and have a high complementarity to PDE3A. *NA*, no mirCo miRNA community detected in CoMeTa. *RC*, read counts. miRNA names for clusters with > 10 members are not listed

miRNA	UTR start	UTR end	Context++ score	Log2(RC) Rep 1	Log2(RC) Rep2	CoMeTa (miRCo community)	Cluster
hsa-miR-221-3p	7998	8005	−0.399	15.14	15.12	miR-221, miR-222	Cluster1
hsa-miR-222-3p	7998	8005	−0.399	15.03	15.09	miR-221, miR-222	Cluster1
hsa-miR-27a-3p	387	393	−0.144	14.40	13.94	miR-128, miR-27a, miR-27b	Cluster2
hsa-miR-27b-3p	387	393	−0.144	13.86	13.84	miR-128, miR-27a, miR-27b	Cluster2
hsa-miR-128-3p	386	393	−0.242	12.84	12.70	miR-128, miR-27a, miR-27b	Cluster2
hsa-miR-139-5p	83	90	−0.264	11.96	12.11	> 10	
hsa-miR-29a-3p	7417	7423	−0.234	12.67	12.51	miR-29a, miR-29b, miR-29c	Cluster3
hsa-miR-151a-3p	3810	3816	−0.128	16.19	16.11	NA	
hsa-miR-21-5p	259	265	−0.02	17.55	17.81	miR-21, miR-590, miR-1251	
hsa-miR-24-3p	1505	1511	−0.059	16.19	16.34	NA	
hsa-miR-30a-5p	2079	2086	−0.096	16.06	16.25	miR-30a, miR-30b, miR-30c, miR-30d, miR-30e	Cluster4
hsa-miR-181a-5p	947	954	−0.03	15.86	15.81	miR-181a, miR-181b, miR-181c, miR-181d	Cluster5
hsa-miR-103a-3p	1825	1832	−0.035	15.09	15.04	NA	
hsa-miR-30d-5p	2079	2086	−0.096	14.53	14.48	miR-30a, miR-30b, miR-30c, miR-30d, miR-30e	Cluster4
hsa-miR-23a-3p	6752	6759	−0.03	14.19	14.24	miR-23a, miR-23b	Cluster6
hsa-miR-181b-5p	947	954	−0.03	14.05	13.90	miR-181a, miR-181b, miR-181c, miR-181d	Cluster5
hsa-miR-140-3p	3023	3029	−0.079	13.21	13.19	hsa-miR-140-3p,hsa-miR-485-3p,hsa-miR-126,hsa-miR-487a,hsa-miR-487b	
hsa-miR-155-5p	521	527	−0.02	13.10	13.30	> 50	
hsa-miR-532-5p	3714	3720	−0.02	12.95	13.14	> 50	
hsa-miR-186-5p	1019	1026	−0.03	12.90	12.83	hsa-miR-186,hsa-miR-1276,hsa-miR-1248,hsa-miR-1178,hsa-miR-189,hsa-miR-1282,hsa-miR-1279,hsa-miR-103,hsa-miR-1244	
hsa-miR-30c-5p	2079	2086	−0.096	12.44	12.38	miR-30a, miR-30b, miR-30c, miR-30d, miR-30e	Cluster4
hsa-miR-23b-3p	6752	6759	−0.03	12.27	12.40	miR-23a, miR-23b	Cluster6
hsa-miR-107	1825	1832	−0.035	11.32	11.15	hsa-miR-107,hsa-miR-195,hsa-miR-503,hsa-miR-16,hsa-miR-15b,hsa-miR-497,hsa-miR-424,hsa-miR-15a	
hsa-miR-30e-5p	2079	2086	−0.112	11.32	11.12	miR-30a, miR-30b, miR-30c, miR-30d, miR-30e	Cluster4
hsa-miR-183-5p	3082	3088	−0.226	10.64	10.98	> 50	
hsa-miR-181d-5p	947	954	−0.03	9.95	9.94	miR-181a, miR-181b, miR-181c, miR-181d	Cluster5
hsa-miR-374b-5p	1561	1567	−0.02	9.14	9.23	> 50	
hsa-miR-30b-5p	2079	2086	−0.096	8.03	8.20	miR-30a, miR-30b, miR-30c, miR-30d, miR-30e	Cluster4
hsa-miR-216a-3p	386	393	−0.25	8.07	7.94	> 10	
hsa-miR-29b-3p	7417	7423	−0.234	8.02	7.79	miR-29a, miR-29b, miR-29c	Cluster3
hsa-miR-145-5p	8168	8174	−0.109	8.08	7.64	> 50	
hsa-miR-34a-5p	7786	7792	−0.144	6.83	7.48	miR-34a, miR-34c	Cluster7
hsa-miR-485-5p	899	906	−0.048	7.18	7.07	NA	
hsa-miR-181c-5p	947	954	−0.03	7.23	6.97	miR-181a, miR-181b, miR-181c, miR-181d	Cluster5
hsa-miR-9-5p	7793	7799	−0.122	6.87	6.52	> 40	
hsa-miR-375	719	725	−0.037	6.73	6.61	> 10	
hsa-miR-340-5p	1661	1667	−0.01	6.49	6.66	hsa-miR-205,hsa-miR-1262,hsa-miR-340,hsa-miR-1261,hsa-miR-490-5p,hsa-miR-1246	
hsa-miR-495-3p	1458	1464	−0.073	6.09	6.63	> 90	
hsa-miR-29c-3p	7417	7423	−0.234	6.04	5.95	miR-29a, miR-29b, miR-29c	Cluster3
hsa-miR-137	7769	7775	−0.2	5.73	5.64	> 50	
hsa-miR-34c-5p	7786	7792	−0.181	5.67	5.04	miR-34a, miR-34c	Cluster7
hsa-miR-329-3p	251	257	−0.012	5.93	4.75	miR-329, miR-362, miR-1228	
hsa-miR-374a-5p	1561	1567	−0.02	5.13	5.09	> 50	
hsa-miR-218-5p	62	68	−0.117	5.29	4.75	> 10	
hsa-miR-410-3p	1669	1675	−0.039	4.17	4.00	> 10	
hsa-miR-138-5p	208	214	−0.408	3.58	4.39	> 50	
hsa-miR-204-5p	1648	1655	−0.273	3.00	3.00	miR-204, miR-188, miR-211	
hsa-miR-23c	6752	6759	−0.03	3.32	2.32	NA	
hsa-miR-449a	7786	7792	−0.156	2.58	2.00	NA	

NA, no mirCo miRNA community detected in CoMeTa

miRNA names for clusters with > 10 members are not listed

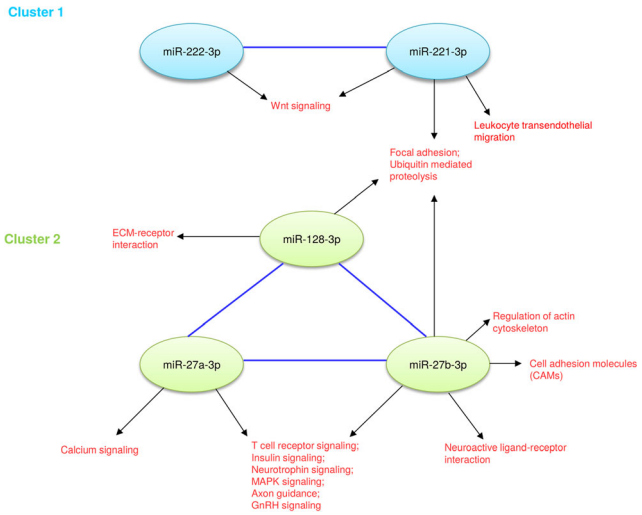

Fig. 3 KEGG annotated network pathways for the top two miRNA clusters targeting PDE3A: cluster 1, miR-221-3p/222-3p; and cluster 2, miR-27a-3p/27b-3p/128-3p. The miRNAs in each cluster are connected by blue lines. Only the most relevant and overlapping pathways between the cluster members are shown, which includes T cell receptor signaling, Wnt signaling, ubiquitin-mediated proteolysis, cellular adhesion, and pathways involved in vascular integrity, neuronal development, and repair processes

miR-128-3p) were subjected to KEGG analysis to assign functional roles to the networks they possibly target. The analysis revealed that the miRNAs in both clusters are significantly associated ($p \leq 0.05$) with pathways related to Wnt signaling, T cell receptor signaling, cellular adhesion, ubiquitin-mediated proteolysis, and neuronal homeostasis (Fig. 3 and Supplementary Table 2).

Expression of hsa-miR-27a-3p and hsa-miR-222-3p in hCMEC/D3 Cell Line

The basal expression of the miRNAs miR-27a-3p and miR-222-3p was investigated in hCMEC/D3 cells. Total RNA was extracted from the cells and enriched for small RNAs followed by RT-qPCR. miRNA-specific primers were used to measure the transcripts and were normalized to the expression of *RNU6*. The results showed basal expression of both miR-27a-3p and miR-222-3p in hCMEC/D3 cells, with ΔC_T values of ~7 and ~5, respectively (Fig. 4).

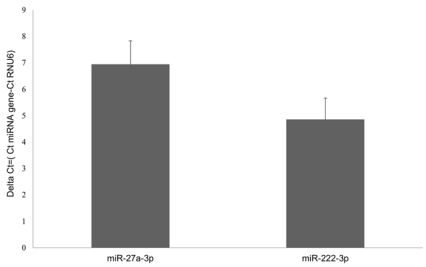

Fig. 4 miRNA-27a-3p and miR-222-3p expression in hCMEC/D3 normalized with RNU6 via RT-qPCR. ΔC_T =(C_T miRNA − C_T *RNU6*) values from three independent experiments were plotted. Mean ($n = 3$) raw C_T values: miR-27a-3p, 29.09; and miR-222-3p, 27.4

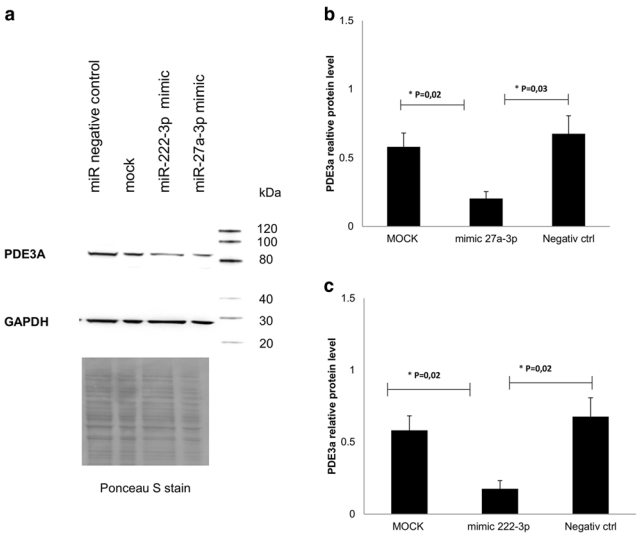

Fig. 5 **a** Western blotting performed with protein lysates (15 μg/μl) from hCMEC/D3 cells treated with mimics hsa-miR27a-3p and hsa-miR-222-3p (10 nM) on 4–12% Bis-Tris gels. Both miRNAs reduced PDE3A levels compared to that in miR-negative control (10 nM) and mock-transfected cells. GAPDH is used as a loading control. Representative blot from four independent experiments. **b** PDE3A relative protein levels were reduced significantly with mimic miR-27a-3p compared to that in mock-transfected ($p = 0.02$) and negative control ($p = 0.03$) cells. **c** PDE3A relative protein levels were reduced with mimic miR-222-3p compared to that in mock-transfected ($p = 0.02$) and negative control ($p = 0.02$) cells. $n = 4$, means ± SEMs, two-tailed paired t test. Quantification of Western blot data performed with ImageQuantTL software based on pixel density

Mean raw Ct value for miR-27a-3p and miR-222-3p was 29 and 27.4, respectively ($n = 3$ independent experiments).

PDE3A Protein Expression Is Reduced by hsa-miR-27a-3p and hsa-miR-222-3p

For a proof of concept, we focused on one miRNA form each of the top two clusters: miR-222-3p and miR-27a-3p. Each of these miRNAs has a high context score and high expression in the hCMEC/D3 cell line. Furthermore, accumulating evidence supports their association with diabetes and hypertension [39–45], both significant risk factors for CSVD. hCMEC/D3 cells were transfected with mimics of miR-27a-3p and miR-222-3p to investigate whether they target PDE3A. The overexpressed miRNA was quantified in the cells transfected with the miRNA mimics, which showed higher levels of the miRNAs versus the negative control and mock-transfected cells (Supplementary Fig. 1). hCMEC/D3 cells transfected with either miR-27a-3p or miR-222-3p miRNA had reduced relative PDE3A protein levels compared with those in the negative control and mock-transfected cells (Fig. 5). Quantification of the blots confirmed that the relative PDE3A protein levels in the cells overexpressing miR-27a-

3p or miR-222-3p were significantly lower than that in the negative control or mock-transfected cells (miR-27a-3p: versus mock, $p = 0.02$; versus negative ctrl, $p = 0.03$; miR-222-3p versus mock and negative ctrl, $p = 0.02$) (Fig. 5). In line with our hypothesis, the expression of miR-27a-3p and miR-222-3p reduced relative PDE3A protein levels in hCMEC/D3 cells.

Discussion

In the present study, we demonstrated that the relative PDE3A protein levels were reduced by miR-27a-3p and miR-222-3p in cerebral microvascular endothelial cells. Furthermore, a pathway analysis of the miRNAs in two clusters (miR-221-3p/miR-222-3p and miR-27a-3p/miR-27b-3p/miR-128-3p) suggests possible involvement in pathways essential to immune regulation, endothelial integrity, and neurogenesis.

We found that even though PDE3A was lowly expressed, and present at mRNA and protein level in the hCMEC/D3 cells, PDE3B was not expressed at protein nor mRNA level. An in silico analysis predicted 67 miRNAs targeting PDE3A, 49 of which were shown, in a recent study, to be expressed by hCMEC/D3 cells [38]. Further ranking and clustering of PDE3A-regulating miRNAs on the basis of context score and expression in hCMEC/D3 cells resulted in seven clusters, the top two of which contained miR-221-3p/miR-222-3p (cluster 1) and miR-27a-3p/miR-27b-3p/miR-128-3p (cluster 2). These miRNAs were subjected to KEGG pathway analyses to assign putative biological functions, which identified T cell receptor and Wnt signaling (important for BBB development), neurogenesis, cellular adhesion, and ubiquitin-mediated proteolysis pathways as potential targets. We validated the presence of the relevant miRNAs in hCMEC/D3 cells in the in vitro experiments and further confirmed that PDE3A was targeted by miR-27a-3p and miR-222-3p. Several studies have suggested these miRNAs are involved with diabetes and hypertension, which are the major risk factors for CSVD stroke [39–45].

The results of this study suggest that the targeting of PDE3A may modulate events accompanying the pathophysiological mechanisms associated with CSVD. To rescue the ischemic penumbra and minimize CSVD-induced damage, an emphasis should be on targets with neuroprotective and neurorestorative outcomes, which may improve cerebral blood circulation and endothelial function. Endothelial dysfunction is a preliminary marker of CSVD [46, 47]. Modifying endothelial function and cerebral blood flow by enhancing cyclic nucleotide signaling reduces the reactivity of the endothelium [24, 25]. Inhibition of PDE3 enhances cAMP signaling, which augments the expression of tight junction proteins; modifies the actin cytoskeleton distribution; and, thus, improves the barrier integrity of brain capillary endothelial cells [9, 48, 49]. Furthermore, PDE3 inhibition leads to a tighter endothelial barrier and decreased permeability [10].

The PDE3 inhibitor cilostazol is used for the secondary prevention of stroke, though mainly in Asian countries [50, 51]. However, it is used worldwide for treatment of peripheral artery disease [46]. Cilostazol inhibits platelet aggregation and produces vasodilatory effects on arteries reported to be beneficial in both animal and human studies [10, 50, 51]. As PDE3 inhibitors available for clinical use do not discriminate between PDE3 isoforms and may contribute to side effects, such as cardiac arrhythmias, nausea, and headache, the targeting of specific isoforms is likely to be of great clinical interest. Our data suggest that the endothelial specific miRNAs targeting PDE3A, such as miR-222-3p and miR-27a-3p, could be new therapeutic targets, as they are highly expressed in endothelial cells and are involved in regulating PDE3A expression [11]. miR-222-3p was previously shown to be expressed in vascular smooth muscle cells and endothelial progenitor cells [52], in which it maintains a state of quiescence, thus reflecting its role in vascular endothelium homeostasis [53]. In addition, it regulates vascular remodeling in response to vascular injury by targeting proangiogenic genes [53–55]. Likewise, miR-27a-3p affects angiogenic signaling pathways that promote endothelial cell proliferation, migration and promote angiogenesis [56, 57]. Patients with acute stroke exhibit increased expression of miR-27a-3p and decreased expression of miR-222-3p [58]. Reduced expression was also observed after experimental traumatic brain injury [59]. Importantly, miR-27a-3p targets proapoptotic proteins in neurons and may be essential to reduce neuronal cell death [59]. Recently, miR-27a was shown to regulate genes that control endothelial sprouting and assist angiogenesis [56]. Furthermore, miR-27a-3p targets the vascular endothelial cadherin junctional protein, which plays a key role in maintaining tight control of vascular leakiness of endothelial cells [61]. The involvement of miR-27a-3p in other neurological diseases characterized by a dysfunctional BBB emphasizes its importance in maintaining the BBB. Frigerio et al. reported that cerebrospinal fluid of Alzheimer's patients had lower miR-27a-3p expression than those from controls [60]. In multiple sclerosis, the level of miR-27-3p is higher in active brain lesions [61]. In support of the present data indicating that miR-27a-3p and miR-222-3p may be therapeutic targets to amend cerebral endothelial cell function/reactivity, previous studies also suggest that miR-27a-3p and miR-222-3p could regulate neovascularization in response to vascular injury.

As evidenced by numerous miRNA expression profiling studies, miRNAs are suggested to be involved in various biological processes underlying stroke pathophysiology [62–65]. A recent genome-wide association study identified PDE3A as novel risk locus for stroke, which further supports our hypothesis that PDE3A is a potential drug target for stroke [66]. Our approach of inferring PDE3A-related miRNAs and their

possible roles in signaling networks vital to cerebral microvascular disorders may lead us to new therapeutic targets for CSVD. miRNA-based therapies for some diseases are currently or have been in clinical trials, i.e., miR-34 mimics for cancer are in phase I clinical trials [67], anti-miR122 for hepatitis has reached phase II clinical trials [68], and a patent for miR-23a-3p/miR27a-3p mimics for neuronal injury has been filed [59].

However, caution is needed while developing miRNA-based drugs, since the miRNA-mRNA relationship is not one miRNA to one target mRNA, such that one miRNA can modulate the expression of many genes in a network resulting in off-target effects. This adds to the complexity in developing miRNA-based therapeutic approaches, since undesired indirect effects can be detrimental. To evaluate the beneficial capacity of miR-27a-3p/miR-222-3p-based drugs, in vivo studies are warranted, and such studies may reveal strategies to develop clinical applicability.

Conclusions

We identified 49 miRNAs expressed in hCMEC/D3 cells that potentially target PDE3A. Two clusters of miRNAs expressed in hCMEC/D3 cells, namely, miR-221-3p-miR-222-3p and miR-27a-3p-27b-3p-128-3p, were predicted to involve possible pathways regulating immune response, neurogenesis, and signaling pathways relevant for cell survival, repair processes, and endothelial integrity. This suggests possible neurorestorative and immunoregulatory roles for these miRNAs in stroke pathology. hCMEC/D3 cells transfected with miR-27a-3p or miR-222-3p had lower levels of PDE3A protein than control cells, implying possible effects on PDE3A expression. Modulation of PDE3A by specific endothelial miRNAs is likely a suitable therapy for CSVD because of the ability of miRNAs to concomitantly influence multiple pathways relevant for cerebral small vessel disease.

Author Contributions Study concept and design: CK, AHM, SY, SK, and FP; acquisition of data: SK, AHM and SY; analysis and interpretation of data: SY, SK, CK, FP and BB; drafting of the manuscript: SY; critical revision of the manuscript for important intellectual content: SY, SK, CK, FP, AHM, and BB; approval of final manuscript: CK, SY, SK, FP, BB and AHM; obtained funding: SY and CK; study supervision, CK, FP and BB.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflict of interest.

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