Supplementary Information

MiR-34a regulates blood-brain barrier permeability and mitochondrial function by targeting cytochrome c

Mimi Bukeirat[1], Saumyendra N. Sarkar[1], Heng Hu[1,2], Dominic D. Quintana[1], James W. Simpkins[1,2], Xuefang Ren[1,2]

[1]Department of Physiology and Pharmacology, [2]Experimental Stroke Core, Center for Basic and Translational Stroke Research, West Virginia University, Morgantown, West Virginia, 26506 USA

***Correspondence to**

Xuefang Ren, Dr. Med.

Address: Biomedical Research Center Rm.109, One Medical Center Dr. PO Box 9229, Morgantown, WV 26506 USA

Fax: 1-304-293-3850

Tel: 1-304-581-1892

E-mail: xuren@hsc.wvu.edu

The PDF contains

Supplemental Materials and Methods

Supplemental Figures and Figure Legends

Supplemental Materials and Methods

Cell culture and transfection

We grew cerebrovascular endothelial cells (CECs, bEnd.3 cell line, CRL-2299 from ATCC, Manassas, Virginia) in high glucose Dulbecco's modified Eagle's medium (DMEM, ATCC) supplemented with 10% FCS and 1% penicillin/streptomycin (Hyclone, South Logan, Utah) at 37 °C in 5% CO2 humid atmosphere. We used passages 25-30 for experiments in the study. For cell transfection, we replaced with non-antibiotics medium and transfected miRNA-Alxea-Fluo®-555 (Life technologies, New York, USA), miR-34a plasmid, or vector control with lipofectomine RNAiMAX Reagent (Life technologies) per the manufacturer's protocol.

RNA extraction and real-time PCR

We extracted total RNA from serum using miRNeasy Serum/plasma kit (Cat. No. 217184, Qiagen, Germany), and from tissue or cells using miRNeasy Micro kit (Cat. No. 217184, Qiagen) according to the manufacturer's instructions. We conducted RNA reverse transcription using micript RT kit (Cat. No. 218161, Qiagen). We performed quantitative RT-PCR in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, USA) with SYBR Green Ready Mix (Qiagen) and miR-34a primers (Qiagen). We analyzed the expression of miR-34a and housekeeping genes defined from the threshold cycle (Ct), and calculated the relative expression levels using the 2^{-Ct} method after normalization with reference to the expression of miR-39 (serum) or RNU6 (tissue or cells) and normalization with naïve controls.

MiR-34a plasmid

We followed the standard molecular cloning protocols and engineered miR-34a plasmid. BamHI/HindIII sites of U6 promoter were used for sub-cloning. Plasmids were amplified in DH5a

Escherichia coli and purified using the Endo-free Mega Preg kit (Qiagen) in accordance with the manufacturer's instructions. Plasmid DNA was dissolved in endotoxin-free TE buffer and quantified by Nanodrop2000 (Thermo Scientific, Hemel Hemstead UK); the plasmid DNA was free of major protein and RNA contamination with an A260/A280 ratio between 1.8 and 2.0. To test the transfection efficiency, we used fluorescence labeled miRNA-Alexa Fluo® 555 purchased from Life technologies.

Antibodies used for flow cytometry

We stained the cells with anti-rabbit-NADH-dehydrogenase-ubiquinone-1-alpha-subcomplex-assembly-factor-1 (NDUFAF1, sc-292085, 1:100, Santa Crutz, Texas, USA), anti-mouse-NADH-dehydrogenase-ubiquinone-1-subunit-C2 (NDUFC2, sc-393771, 1:100, Santa Crutz), anti-mouse-NADH-dehydrogenase-ubiquinone-ironsulfur-protein-2 (NDUFS2, sc-390596, 1:100, Santa Crutz), anti-rabbit-Succinate-dehydrogenase (SDH, cat.11998, 1:100, Cell Signaling, Massachusetts, USA), anti-rabbit-cytochrome reductase (CYB, sc-33174, 1.;100, Santa Cruz), anti-rabbit-Cytochrome c (CYCS, cat.4280, 1:100, Cell Signaling), anti-mouse-Cytochrome c oxidase (COX IV, cat. 4850, 1:100, Cell Signaling) antibodies for 30 min and labeled the cells with a proper second antibody, PE-anti-rabbit (Cat.8885, 1:100, Cell Signaling) or PE-anti-mouse (Cat.8887, 1:500, Cell Signaling).

Immunohistochemistry staining

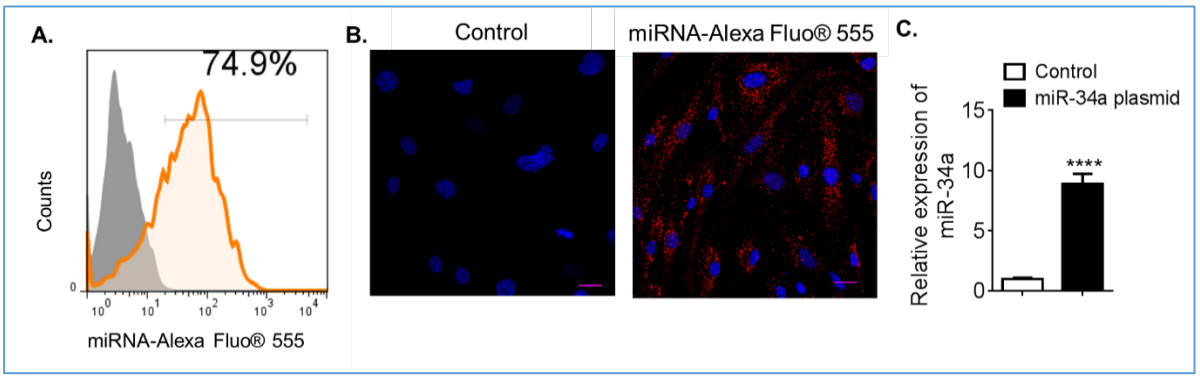
We performed the immunohistochemistry staining of tight junction protein ZO-1 on cover slips. Briefly, we cultured CECs on cover slips until monolayer confirmed microscopically, washed cells with PBS then fixed CECs with 2% paraformaldehyde (PFA, Polysciences, Inc.) for 10 minutes at 37 °C. We treated CECs with 5% goat serum staining buffer and stained cells with anti-rabbit-ZO-1 (1:500, Life technologies) overnight, then washed cells with PBS and stained with goat-

anti-rabbit-FITC (1:500, Life technologies) for 2 hours. The cells were further washed with PBS and mounted on glass slides using prolong gold anti-fade reagent (Life technologies). The slides were photographed with confocal LSM 510 microscope Zeiss (Zeiss, Oberkochen, Germany) using software ZEN 2012.

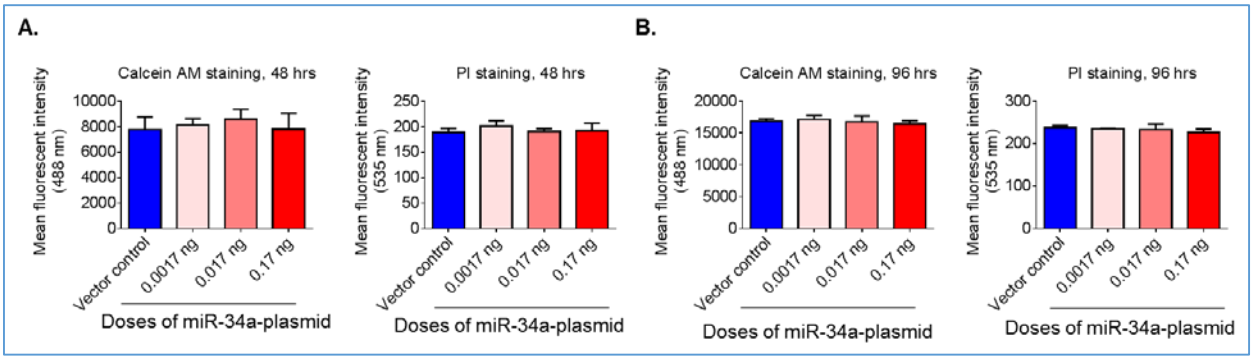
Cell viability assay

We performed the cell viability assay simultaneously with the oxygen consumption assay. We seeded cells in 96 black well plates and transfected cells with miR-34a plasmid. We added PI (2 µg/ml) to evaluate cell death on plate reader (Ex. 535 nm and Em. 617 nm), washed with PBS, added Calcein AM (Life technology) and stained for 30 minutes to measure cell viability (Ex. 490 nm and Em. 520 nm).

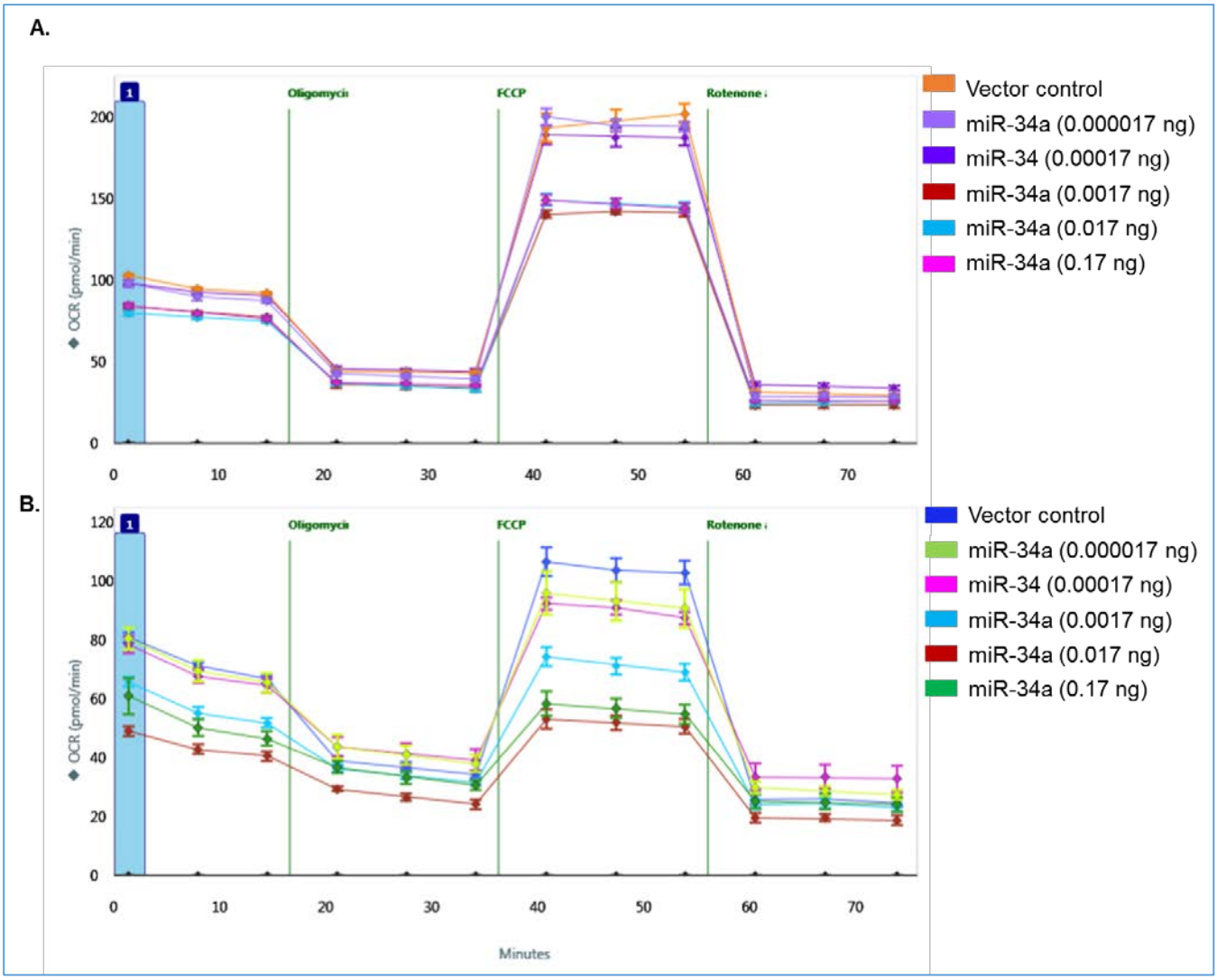
Supplemental Figures and Figure Legends



Supplemental Figure 1. Overexpression of miR34a in cerebrovascular endothelial cells. Transfection efficiency of miRNA-Alxea-Fluo®-555 in CECs was assessed by flowcytometry (**A**) and confocal microscope (**B**). Blue: DAPI, stained for Nuclei; Red: miRNA-Alexa Fluo® 555. (**C**) Overexpression of miR34a (0.017 ng) in CECs was confirmed by RT-PCR after 48 hours transfection of the miR-34a plasmid. Results are from 3 independent experiments. Data are expressed as mean±SD; Student's t test; N=3; ****, P<0.0001.



Supplemental Figure 2. Overexpression of miR34a in cerebrovascular endothelial cells. Cell viability by calcein AM staining and cell death by propidium iodide (PI) staining in CECs that transfected with various doses of miR-34a plasmid for (**A**) 48 hours and (**B**) 96 hours. 1-way ANOVA followed by post hoc Tukey's test. Results are from 3 independent experiments. Data are expressed as mean±SD. Overexpression of miR-34a did not change cell viability or induce significant cell death.



Supplemental Figure 3. Raw data of bioenergetics functional assay in CECs transfected with miR-34a plasmid vs. control at (A) 48 hours and (B) 72 hours.