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Aging-Induced Dysregulation of Dicer1-Dependent MicroRNA Expression Impairs Angiogenic Capacity of Rat Cerebromicrovascular Endothelial Cells

Zoltan Ungvari,[1,2] Zsuzsanna Tucsek,[1] Danuta Sosnowska,[1] Peter Toth,[1] Tripti Gautam,[1] Andrej Podlutsky,[1] Agnes Csiszar,[1,3] Gyorgy Losonczy,[4] M. Noa Valcarcel-Ares,[1,5] William E. Sonntag,[1,2] and Anna Csiszar[1,2]

[1]Reynolds Oklahoma Center on Aging, Department of Geriatric Medicine and
[2]The Peggy and Charles Stephenson Cancer Center, University of Oklahoma Health Sciences Center.
[3]Institut für Bio- und Nanosysteme, Forschungszentrum Jülich GmbH, Germany.
[4]Department of Pulmonology, Semmelweis University, Budapest, Hungary.
[5]Aging and Inflammation Research Lab, INIBIC - CHU A Coruña, Spain.

Address correspondence to Zoltan Ungvari, MD, PhD, Reynolds Oklahoma Center on Aging, Department of Geriatric Medicine, University of Oklahoma HSC, 975 N. E. 10th Street - BRC 1303, Oklahoma City, OK 73104. Email: zoltan-ungvari@ouhsc.edu

Age-related impairment of angiogenesis is likely to play a central role in cerebromicrovascular rarefaction and development of vascular cognitive impairment, but the underlying mechanisms remain elusive. To test the hypothesis that dysregulation of Dicer1 (ribonuclease III, a key enzyme of the microRNA [miRNA] machinery) impairs endothelial angiogenic capacity in aging, primary cerebromicrovascular endothelial cells (CMVECs) were isolated from young (3 months old) and aged (24 months old) Fischer 344 × Brown Norway rats. We found an age-related downregulation of Dicer1 expression both in CMVECs and in small cerebral vessels isolated from aged rats. In aged CMVECs, Dicer1 expression was increased by treatment with polyethylene glycol–catalase. Compared with young cells, aged CMVECs exhibited altered miRNA expression profile, which was associated with impaired proliferation, adhesion to vitronectin, collagen and fibronectin, cellular migration (measured by a wound-healing assay using electric cell–substrate impedance sensing technology), and impaired ability to form capillary-like structures. Overexpression of Dicer1 in aged CMVECs partially restored miRNA expression profile and significantly improved angiogenic processes. In young CMVECs, downregulation of Dicer1 (siRNA) resulted in altered miRNA expression profile associated with impaired proliferation, adhesion, migration, and tube formation, mimicking the aging phenotype. Collectively, we found that Dicer1 is essential for normal endothelial angiogenic processes, suggesting that age-related dysregulation of Dicer1-dependent miRNA expression may be a potential mechanism underlying impaired angiogenesis and cerebromicrovascular rarefaction in aging.

Key Words: Angiogenesis—Capillary density—Vascular aging—Cerebrovascular—Cardiovascular aging—Epigenetics.

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VASCULAR cognitive impairment (VCI) is the second most prevalent type of age-associated cognitive dysfunction in the United States today (1). Numerous clinical and experimental studies demonstrate that a large portion of the clinical picture of VCI is due to pathological microvascular alterations (2). Importantly, there is convincing evidence that aging is associated with cerebromicrovascular rarefaction and that decreases in cerebromicrovascular density contribute to the age-related decline in cerebral blood flow (3–16). The resulting mismatch between metabolic demand and blood flow has been shown to be important contributing factors to aging-induced cognitive impairment in the absence of or preceding neurodegeneration in the elderly individuals (1,4,7,17,18).

The process of angiogenesis, new capillary formation from existing blood vessels, is critical for maintenance of the cerebromicrovasculature. Previous studies in laboratory rodents demonstrate that aging is associated with a progressive deterioration of microvascular homeostasis in many organs due to age-related impairment of angiogenic processes (19–23). It is assumed that these changes have a key role in age-related microvascular rarefaction (24), decreasing tissue blood supply, and impairing adaptation to hypoxia (25–27). Yet, the underlying mechanisms by which aging impairs endothelial angiogenic processes remain elusive.

MicroRNAs (miRNAs) are short, endogenous, noncoding transcripts that negatively regulate the expression of specific mRNA targets. Recent studies demonstrate that angiogenesis is regulated by miRNAs (28–30). Dicer1 (ribonuclease III) is a key enzyme of the miRNA machinery, which is responsible for synthesis of mature functional miRNAs. There is evidence that Dicer1 in endothelial cells may play a role in regulation of angiogenic processes (30–33). miRNAs control life span and the pace of aging in model

organisms (34–36), and there is preliminary evidence that miRNA expression and Dicer1 expression in the liver may be altered also in mammalian aging (37,38). However, no studies have investigated age-related alterations in Dicer1 and miRNA expression profile in cerebromicrovascular endothelial cells (CMVECs). Furthermore, there are no studies to our knowledge investigating the role of Dicer1-dependent pathways in impaired endothelial angiogenic capacity in aging.

This study was designed to elucidate how aging and reactive oxygen species (ROS) alter Dicer1 expression, how dysregulation of Dicer1 in aging affects the angiogenic miRNA signature and what is the causal role of dysregulation of Dicer1 in age-related impairment of angiogenesis. Using CMVECs isolated from young and aged Fischer 344 × Brown Norway (F344 × BN) rats, we tested the hypothesis that overexpression of Dicer1 in aged endothelial cells improves angiogenic capacity, including proliferation, adhesion, migration, and ability to form capillary-like structures. We also determined whether siRNA knockdown of Dicer1 impairs angiogenic processes in young CMVECs, mimicking the cerebromicrovascular aging phenotype.

Materials and Methods

Animals and Vessel Isolation

F344 × BN rats were used as a model of aging, as this strain has a lower incidence of age-specific pathology than other rat strains. Thus, in F344 × BN rats, the primary effects of aging can be studied uncomplicated by compensatory effects caused by age-related pathology. Male F344 × BN rats (3 and 24 months old; $n = 15$ in each group) were obtained from the National Institute on Aging. All animals were disease free with no signs of systemic inflammation and/or neoplastic diseases. The rats were housed in an environmentally controlled vivarium under pathogen-free conditions with unlimited access to food and water and a controlled photoperiod (12 h light; 12 h dark). All rats were maintained according to National Institutes of Health guidelines, and all animal use protocols were approved by the Institutional Animal Care and Use Committees of the participating institutions. The animals were euthanized with CO_2. From the first cohort of animals, branches of the middle cerebral arteries and the hippocampi were isolated using sterile microsurgery instruments. From the second cohort of animals, the brains were rapidly dissected to establish primary CMVEC cultures.

Establishment and Characterization of Primary CMVEC Cultures

Brains were removed aseptically, rinsed in ice-cold phosphate-buffered saline (PBS) and minced into ≈1 mm squares. The tissue was washed twice in ice-cold 1× PBS by low-speed centrifugation (50*g*, 2–3 minutes). The diced tissue was digested in a solution of collagenase (800 U/g tissue), hyaluronidase (2.5 U/g tissue), and elastase (3 U/g tissue) in 1 mL PBS/100 mg tissue for 45 minutes at 37°C in rotating humid incubator. The digested tissue was passed through a 100-µm cell strainer to remove undigested blocks. The single-cell lysate was centrifuged for 2 minutes at 70*g*. After removing the supernatant carefully, the pellet was washed twice in cold PBS supplemented with 2.5% fetal calf serum, and the suspension was centrifuged at 300*g*, for 5 minutes at 4°C.

To create an endothelial cell enriched fraction, the cell suspension was gradient centrifuged using OptiPrep solution (Axi-Shield, PoC, Norway). Briefly, the cell pellet was resuspended in Hanks' balanced salt solution (HBSS) and mixed with 40% iodixanol thoroughly (final concentration: 17% (w/v) iodixanol solution; ρ = 1.096 g/mL). Two milliliters of HBSS were layered on top and centrifuged at 400*g* for 15 minutes at 20°C. Endothelial cells, which banded at the interface between HBSS and the 17% iodixanol layer, were collected. The endothelial cell–enriched fraction was incubated for 30 minutes at 4°C in dark with anti-CD31/PE (BD BD Biosciences, San Jose, CA) and anti-MCAM/fluorescein isothiocyanate (FITC) (BD Biosciences). After washing the cells twice with MACS Buffer (Milltenyi Biotech, Cambridge, MA, USA), anti-FITC magnetic bead–labeled and anti-PE magnetic bead–labeled secondary antibodies were used for 15 minutes at room temperature. Endothelial cells were collected by magnetic separation using the MACS LD magnetic separation columns according to the manufacturer's guidelines (Milltenyi Biotech). The endothelial fraction was cultured on fibronectin coated plates in Endothelial Growth Medium (Cell Application, San Diego, CA) for 10 days.

Endothelial cells were phenotypically characterized by flow cytometry (GUAVA 8HT, Merck Millipore, Billerica, MA). Briefly, antibodies against five different endothelial-specific markers were used (anti-CD31-PE, anti-erythropoietin receptor-APC, anti-vascular endothelial growth factor (VEGF) R2-PerCP, anti-intercellular adhesion molecule-fluorescein, and anti-CD146-PE) and isotype-specific antibody–labeled fractions served as negative controls. All antibodies were purchased from R&D Systems (Minneapolis, MN).

Western Blotting

To analyze protein expression of Dicer1 in homogenates of young and aged CMVECs, Western blotting was performed as described (39), using the following primary antibody: anti-Dicer1, #3363S Cell Signaling Technology (Beverly, MA; 1:2000 in 5% milk). All polyvinylidene fluoride membranes were incubated in primary antibody overnight at 4°C. A donkey anti-rabbit polyclonal secondary antibody was used (Abcam, ab16284; 1:2000 in 5% milk). Equal amounts of protein were loaded in each lane. Because among the housekeeping genes tested the relative

mRNA expression of β-actin was not statistically different in young and aged CMVECs (β-actin:hypoxanthine phosphoribosyltransferase [HPRT] ratio; young: 1 ± 0.16, aged: 0.73 ± 0.02, ns), for normalization purposes we used mouse anti-β-actin (Abcam, ab6276, 1:10,000 in 5% milk) with a sheep anti-mouse IgG horseradish peroxidase–linked secondary antibody (NA931V GE Healthcare UK, 1:10,000).

Measurement of Cellular ROS Production

To assess cellular peroxide production, we used the cell-permeant oxidative fluorescent indicator dye CM-H_2DCFDA (5 [and 6]-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate-acetyl ester, Invitrogen, Carlsbad, CA) as we previously reported (40,41). In brief, cells were washed with warm PBS and incubated with CM-H_2DCFDA (10 µM, at 37°C, for 30 minutes). CM-H_2DCFDA fluorescence was assessed by flow cytometry (40,41).

Assessment of the Effects of Oxidative Stressors and Antioxidants on Dicer1 Expression in CMVECs

To assess the effect of scavenging of H_2O_2 on Dicer1 expression, we treated young and aged CMVECs with polyethylene glycol–catalase (200 U/mL for 24 h). To assess the effects of oxidative stressors on Dicer1 expression, young CMVECs were treated with H_2O_2 (0.1–10 µmol/L, for 24 h), high glucose (30 mmol/L, for 24 h), and TNFα (10 ng/mL, for 24 h). To assess the effects of activators of Nrf2 signaling, aged CMVECs were treated with two structurally different potent inducers of Nrf2: resveratrol (10^{-7} to 10^{-5} mol/L, for 24 h) and sulforaphane (10^{-6} mol/L, for 24 h). Expression of Dicer1 mRNA was assessed by quantitative real-time RT-PCR (described in the following section).

miRNA Expression Profiling

The expression profile of 373 unique rat miRNAs in hippocampi of aged and young rats and CMVECs derived from young and aged rats was analyzed using the TaqMan Array Rodent MicroRNA A+B Cards Set v3.0 (Applied Biosystems, Life Technologies, Carlsbad, CA).

Knockdown and Overexpression of Dicer1

To disrupt Dicer1 signaling in young CMVECs, Dicer1 was downregulated (by ~50%) by RNA interference using four proprietary shRNA sequences (OriGene Technologies, Rockville, MD) and the electroporation-based Amaxa Nucleofector technology (Amaxa, Gaithersburg, MD), as we have previously reported (42–45). Experiments were performed on Day 2 after the transfection, when gene silencing was optimal. Overexpression of Dicer1 (approximately twofold) was achieved in CMVECs by transfection with a Dicer1 full-length cDNA encoding plasmid, pDEST-mycDICER (Addgene plasmid 19873, Addgene, www.addgene.org, provided by the laboratory of Dr. Thomas Tuschl (46)). Overexpression and knockdown of Dicer1 were confirmed by Western blotting. The negative controls were vector only and scrambled shRNA (Origen), respectively. To induce angiogenic processes, CMVECs were treated with recombinant human (VEGF, 100 ng/mL; R&D systems).

Quantitative Real-Time RT-PCR

A quantitative real-time RT-PCR technique was used to analyze mRNA expression of Dicer1 and selected miRNAs, as previously reported (39,47–51). In brief, total RNA was isolated with a Mini RNA Isolation Kit (Zymo Research, Orange, CA) and was reverse transcribed using Superscript III RT (Invitrogen). A real-time RT-PCR technique was used to analyze mRNA expression using a Strategen MX3000, as reported (39,47–51). Amplification efficiencies were determined using a dilution series of a standard vascular sample. Quantification was performed using the efficiency-corrected ΔΔCq method. The relative quantities of the reference genes *HPRT*, *GAPDH*, and *ACTB* were determined, and a normalization factor was calculated based on the geometric mean for internal normalization. Oligonucleotides used for quantitative real-time RT-PCR are listed in Table 1. To assess expression of MIR193-b, MIR214, MIR574, MIR744, MIR532, MIR672, MIR145, and MIR146, primer sequences proprietary to Applied Biosystems were used.

Cell Adhesion Assays

Angiogenesis is a multistep process involving cell adhesion, proliferation, migration, and morphogenesis (52). To determine the effects of Dicer1 in regulation of the adhesion capacity of CMVECs, cells were transfected with control plasmid, Dicer1 siRNA, or Dicer1 cDNA. After 48 hours, they were collected, washed, counted, and

Table 1. Oligonucleotides for Real-Time RT-PCR

mRNA or miRNA Targets	Description	Sense	Antisense
Dicer1	Ribonuclease type III	CACTACAACACTATTACTGATT	GTGCTTGGTTATGAGGTA
Hprt	Hypoxanthine phosphoribosyltransferase 1	AAGACAGCGGCAAGTTGAATC	AAGGGACGCAGCAACAGAC
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	CCAAGGAGTAAGAAACCC	TTGATGGTATTCGAGAGAAGG
Actb	β-Actin	GAAGTGTGACGTTGACAT	ACATCTGCTGGAAGGTG

Note. RT-PCR = reverse transcription-polymerase chain reaction.

labeled with the fluorescent CyQuant dye (Invitrogen; incubation time: 60 minutes at 37°C). Equal amounts of cells, stimulated with VEGF (100 ng/mL), were seeded in 96-well plates previously coated with 50 µL of either vitronectin (1.6 µg/mL), collagen (50 µg/mL), fibronectin (50 µg/mL), laminin (50 µg/mL), or bovine serum albumin (BSA, 12 µg/ml), which was used as the negative control. After 3 hours of incubation at 37°C, unattached cells were removed by rinsing the wells three times with warm PBS. The ratio of adhering cells was quantified by assessing the background-corrected fluorescence (excitation/emission: 508/527 nm, respectively) using an Infinite M200 plate reader (Tecan, Research Triangle Park, NC).

As an additional measurement, we used electric cell–substrate impedance sensing (ECIS) technology (Applied Biophysics, Troy, NY) to monitor adhesion of CMVECs to collagen. Briefly, VEGF (100 ng/mL) stimulated cells were seeded in collagen coated 96-well array culture dishes containing gold film surface electrodes (ECIS 96W1E). The same numbers of cells were added to each well in complete cell culture medium (2.5×10^5 cells/well). The arrays were placed in an incubator and the time course for changes of capacitance (at 60 kHz) due to the adhesion of cells to the active electrode was obtained. The inverse of the time to reach 50% cell adhesion (100% change corresponds to the maximum level of cell coverage reached on the active electrode) was used as an index of adhesiveness.

Cell Proliferation Assay

Cell proliferation capacity was assessed in CMVECs transfected with Dicer1 siRNA, Dicer1 full-length cDNA encoding plasmid, or the respective scrambled control plasmids using the flow cytometry-based Guava CellGrowth assay (Guava Technologies, Hayward, CA) as recently reported (51). Briefly, cells were collected, resuspended in PBS containing 0.1% BSA, and stained with 16 µmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE) for 15 minutes at 37°C. This dye diffuses into cells and is cleaved by intracellular esterases to form an amine-reactive product that produces a detectable fluorescence and binds covalently to intracellular lysine residues and other amine sources. Upon cell division, CFSE divides equally into the daughter cells halving the CFSE concentration of the mother cell; therefore, there is an inverse correlation between the fluorescence intensity and the proliferation capacity of the cells. After incubation, unbound dye was quenched with serum-containing medium. Then, cells were washed three times and incubated for 24 hours with 100 ng/mL VEGF. Finally, cells were collected, washed, stained with propidium iodide (to gate out dead cells), and analyzed with a flow cytometer (Guava EasyCyte 8HT; Millipore, Billerica, MA). The inverse of the fluorescence intensity was used as an index of proliferation.

Assessment of Cell Migration by ECIS-Based Wound-Healing Assay

The ECIS technology was used to monitor migration of CMVECs in a wound-healing assay as reported (51). Briefly, CMVECs (2.5×10^5 cells/well) were seeded in 96-well array culture dishes (ECIS 96W1E), placed in an incubator (37°C), and changes in resistance and impedance were continuously monitored. When impedance reached a plateau, cells in each well were subjected to an elevated field pulse ("wounding") of 5 mA applied for 20 seconds at 100 kHz, which killed the cells present on the small active electrode due to severe electroporation. The detachment of the dead cells was immediately evident as a sudden drop in resistance (monitored at 4,000 Hz) and a parallel increase in conductance. VEGF (100 ng/mL) was immediately added to each well. CMVECs surrounding the active electrode that had not been subjected to the wounding then migrated inward to replace the detached dead cells resulting in resistance recovery (continuously monitored at 4,000 Hz for up to 24 hours). Time to reach 50% resistance recovery (corresponding to 50% confluence on the active electrode) was determined for cells in each experimental group and this parameter, and the known physical dimensions of the electrode were used to calculate the migration rate (expressed as µm/h).

Tube Formation Assay

To investigate the influence of age and Dicer1 on tube formation ability, 24 hours after transfection with Dicer1 siRNA or Dicer1 full-length cDNA encoding plasmid or the respective scrambled control plasmids, young and aged CMVECs were plated on Geltrex Reduced Growth Factor Basement Membrane Matrix (Invitrogen) in Medium 200PRF (Invitrogen). Briefly, 150 µl/well of Geltrex was distributed in ice-cold 24-well plates. The gel was allowed to solidify while incubating the plates for 30 minutes at 37°C. CMVECs were then seeded at a density of 5×10^4 cells/well and placed in the incubator for 24 hours. Microscopic images were captured using a Nikon Eclipse Ti microscope equipped with a ×10 phase-contrast objective (Nikon Instruments, Melville, NY). The extent of tube formation was quantified by measuring total tube length in five random fields per well using NIS-Elements microscope imaging software (Nikon Instruments), as recently reported (51). The mean of the total tube length per total area imaged (µm tube/mm^2) was calculated for each well. Experiments were run in quadruplicates. The experimenter was blinded to the groups throughout the period of analysis.

Data Analysis

Statistical analyses were performed using one-way analysis of variance. $p < .05$ was considered statistically significant. Data are expressed as means ± SEM.

Results

Age-Related Decline in Dicer1 Expression in the Rat Cerebrovasculature

A quantitative real-time RT-PCR technique was used to analyze the effect of aging on Dicer1 expression in the hippocampus and in microdissected branches of the middle cerebral arteries of F344 × BN rats. We found that both hippocampal (Figure 1A) and cerebrovascular (Figure 1B) Dicer1 expression progressively declined with age. To analyze Dicer1 expression specifically in CMVECs, we isolated primary CMVECs from young and aged rats using an immunomagnetic isolation technique. To evaluate the specificity of immunomagnetic isolation technique, flow cytometry was performed in each cell strain (five independent strains were individual isolates from five different brains per age group). Flow cytometric analysis showed that after the third cycle of immunomagnetic selection, there were virtually no CD31−, CD146−, EpoR−, and VEGFR2− cells in the resultant cell populations (Figure 1B). We found that both young and aged CMVECs showed comparable and abundant expression of CD31, CD146, EpoR, and VEGFR2 (Figure 1B). Western blotting showed that CMVECs derived from aged rats exhibit a significantly decreased expression of Dicer1 (Figure 1C and D).

Age-Associated Oxidative Stress Is Associated With Downregulation of Dicer1 in CMVECs

Using a CM-H_2DCFDA fluorescence-based method, we demonstrated that aging results in increased H_2O_2 production in CMVECs (Figure 2A). The finding that scavenging

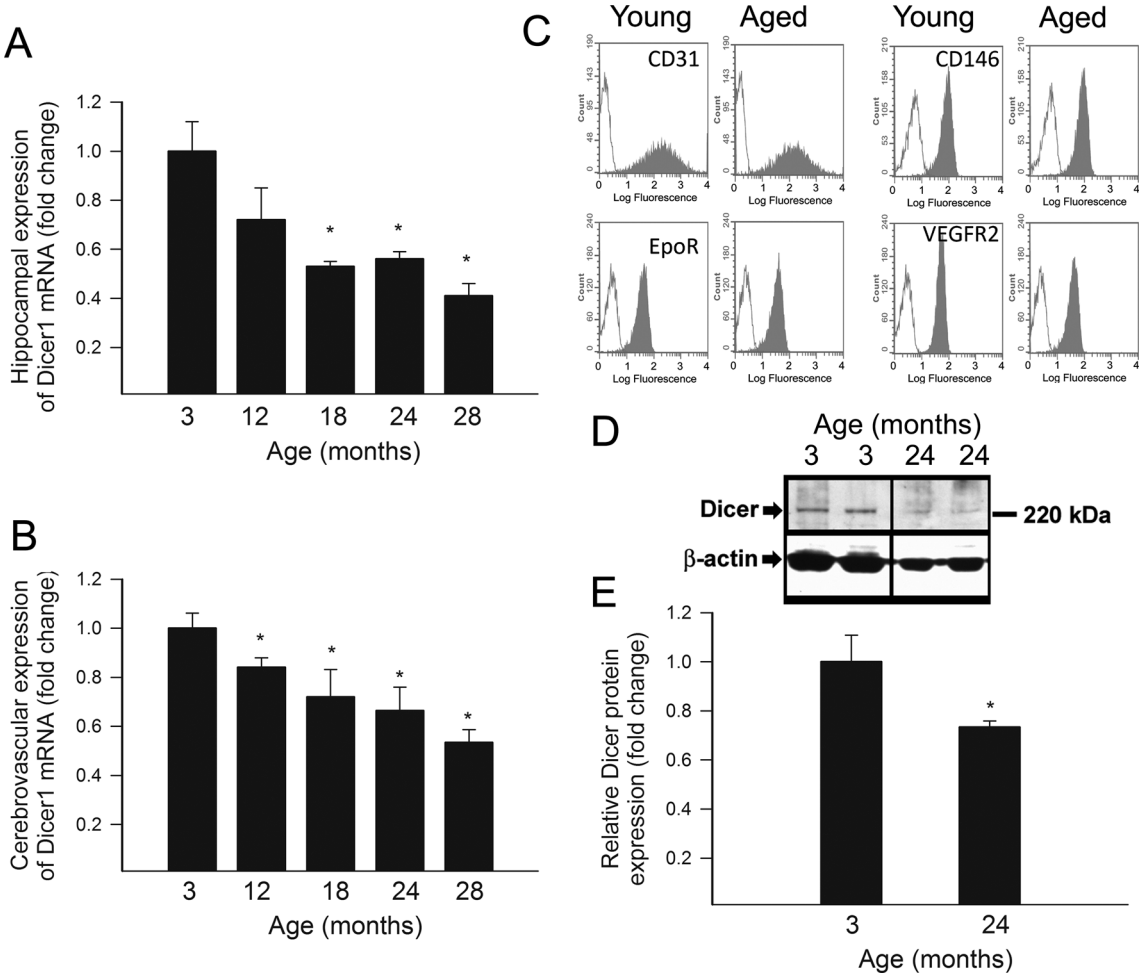

Figure 1. (A) Quantitative real-time reverse transcription-polymerase chain reaction data showing age-related downregulation of mRNA expression of Dicer in microdissected small branches of the middle cerebral arteries in F344 × BN rats. Data are mean ± *SEM* ($n = 5–7$). *$p < .05$ vs 3-month-old controls. (B) Flow cytometric analysis of endothelial markers in primary cerebromicrovascular endothelial cells (CMVECs) derived from young (3 months old) and aged (24 months old) F344 × BN rats. Analysis of anti-CD31, anti-CD146, anti-EpoR, and anti-VEGFR2 reactivity (filled areas) shows the high purity of endothelial cell cultures. Appropriate isotype controls (blank areas) are also shown. (C) Original Western blots showing protein expression of Dicer in CMVECs isolated from 3- and 24-month-old F344 × BN rats. β-Actin was used for normalization purposes. Bar graphs (Panel D) are summary densitometric values. Data are mean ± *SEM*. *$p < .05$ vs 3-month-old control.

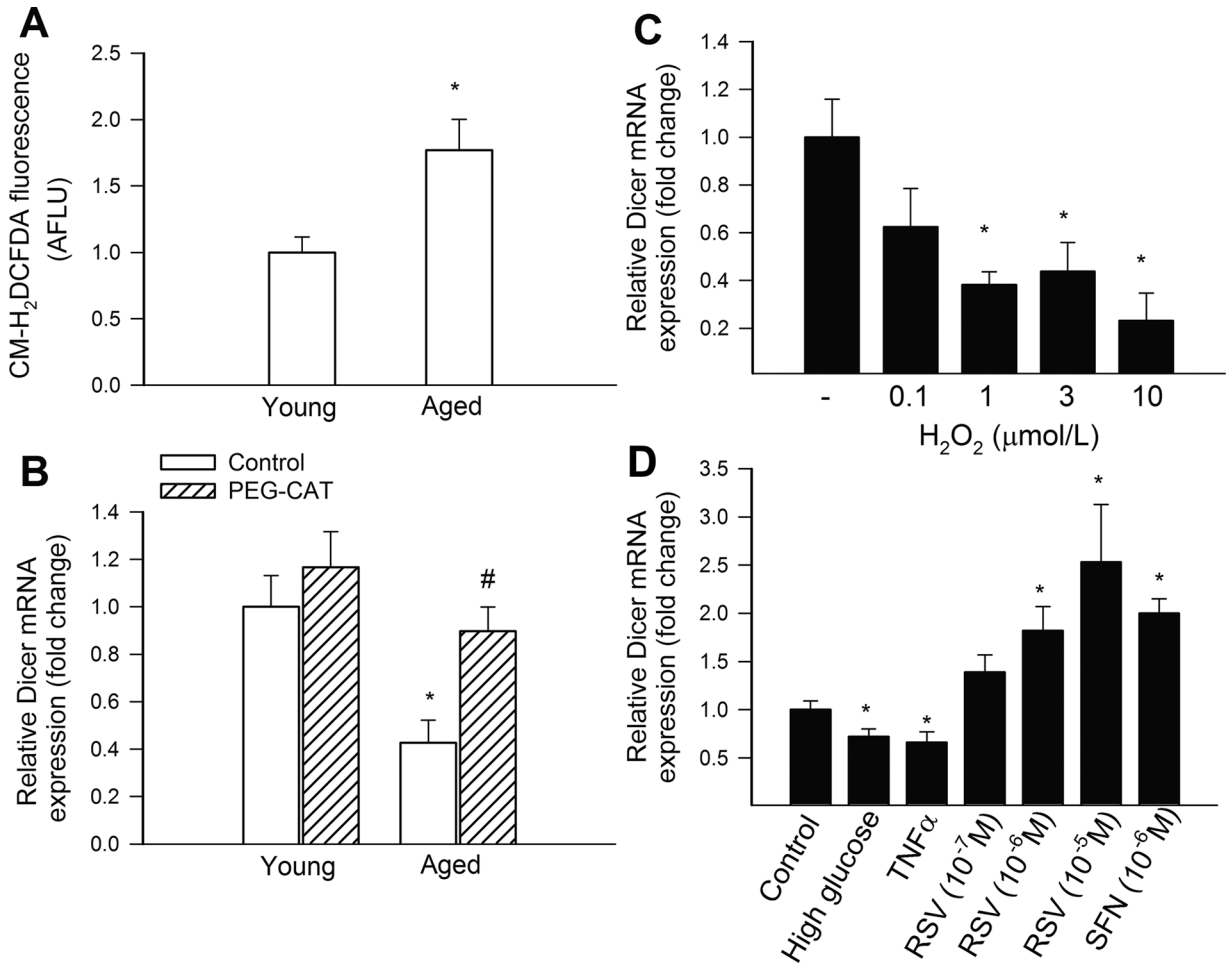

Figure 2. (A) Flow cytometry data showing that compared with young cells, aged cerebromicrovascular endothelial cells (CMVECs) exhibit increased peroxide production, as indicated by the significantly increased CM-H_2DCFDA fluorescent signal. Data were obtained using $n = 5$ cell strains (samples run in triplicates) in each group. Data are mean ± *SEM*. *$p < .05$ vs 3-month-old control. (B) Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) data showing the effect of treatment with polyethylene glycol–catalase on mRNA expression of Dicer1 in CMVECs isolated from young and aged F344 × BN rats. Data are mean ± *SEM* ($n = 5$ in each group). *$p < .05$ vs young controls; #$p < .05$ vs untreated aged CMVECs. (C and D) Quantitative real-time RT-PCR data showing the effect of treatment with the oxidative stressors H_2O_2 (Panel C), TNFα and high glucose (Panel D) and the Nrf2 activators resveratrol and sulforaphane (Panel D) on mRNA expression of Dicer1 in young CMVECs. Data are mean ± *SEM* ($n = 5$ in each group). *$p < .05$ vs untreated controls.

of H_2O_2 significantly increases Dicer1 expression in aged CMVECs suggests that age-related oxidative stress and dysregulation of Dicer1 are causally related (Figure 2B). Further support for this concept is provided by the experiments showing that administration of exogenous H_2O_2 results in significant downregulation of Dicer1 expression in young CMVECs (Figure 2C), mimicking the aging phenotype. We found that experimental hyperglycemia and treatment with TNFα (for 24 hours), which are also known to increase cellular ROS production (53), also result in significant downregulation of Dicer1 in young CMVECs (Figure 2D). Interestingly, short-term exposure (2–4 hours) to high glucose resulted in an upregulation of Dicer1 (data not shown).

Treatment of CMVECs with two structurally different inducers of Nrf2 activation, resveratrol (44), and sulforaphane, resulted in a significant upregulation of Dicer1 (Figure 2D). Yet, presently, it is unclear whether the upregulation of Dicer1 expression in CMVECs upon resveratrol and sulforaphane treatment is mediated directly by Nrf2 binding to the Dicer1 promoter. We used rVISTA (http://rvista.dcode.org/), a tool that combines transcription factor-binding sites database search with a comparative sequence analysis (54), to confirm the presence of an ARE consensus sequence in the 5′ flanking region of the human Dicer1 gene (ATGACTGAGCA) and the mouse Dicer1 gene (TGCTGGATCAC). However, we could not identify a conserved ARE consensus sequence in the promoter region of the rat Dicer1 gene.

Downregulation of Dicer1 Is Associated With Alterations in miRNA Expression Profile of CMVECs

To determine whether age-related downregulation of Dicer1 is associated with alterations in miRNA expression

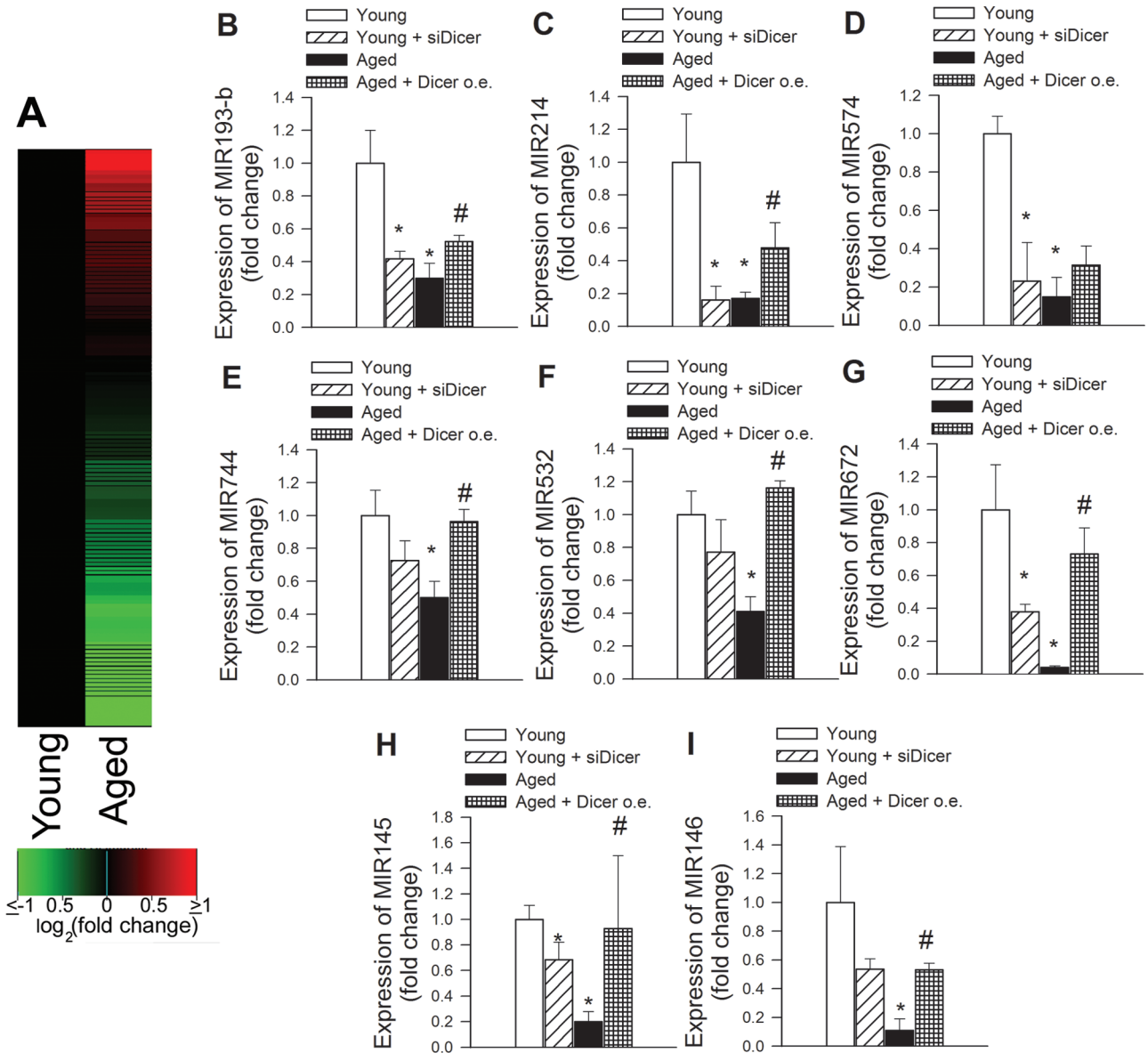

Figure 3. Effects of aging and downregulation of Dicer on miRNA expression in cerebromicrovascular endothelial cells (CMVECs). (A) miRNA expression patterns of five young and five aged CMVEC strains were examined using microfluidic cards containing TaqMan probes and primer pairs for mature rodent miRNAs. A total of 138 miRNAs were expressed at a significant level in CMVECs. The two columns correspond to the expression profiles of young and aged CMVECs, and each row corresponds to a miRNA. The color in each cell reflects the mean level of expression of the corresponding miRNA in the cells, relative to the mean level of expression in young CMVECs. The increasing intensities of green mean that a specific miRNA has a lower mean expression in aged CMVECs and the increasing intensities of red mean that this miRNA has a higher expression in aged CMVECs. The scale reflects mean miRNA abundance ratio relative to the mean level in young CMVECs. Panels B–I: Quantitative real-time reverse transcription-polymerase chain reaction data showing expression of MIR193-b, MIR214, MIR574, MIR744, MIR532, MIR672, MIR145, and MIR146 in young CMVECs, young CMVECs with siRNA knockdown of Dicer (siDicer), aged CMVECs, and aged CMVECs with overexpression (o.e.) of Dicer. Data are means ± *SEM* ($n = 5$ in each group). $^{*}p < .05$ vs young control CMVECs, $^{\#}p < .05$ vs untreated aged CMVECs.

in CMVECs, we first determined global miRNA expression profiles of five independent strains of young CMVECs and five independent strains of aged CMVECs using microfluidic cards containing TaqMan primers and probes for mature rodent miRNAs. Using the Rodent MicroRNA A+B Cards Set v3.0 TaqMan Array, we detected the expression of 148 miRNAs in young and aged CMVECs. We found that the majority (89%) of miRNAs that exhibited significant age-related changes in their expression level were downregulated (Figure 3A). To assess the role of Dicer1 in age-related alterations in miRNA expression patterns, we chose a subset of miRNAs that were underexpressed by more than 50% in aged CMVECs. This subset of miRNAs is known to be expressed in various endothelial cell strains and are thought regulate the expression of a number of genes regulating vital cell cycle processes, such as apoptosis, proliferation,

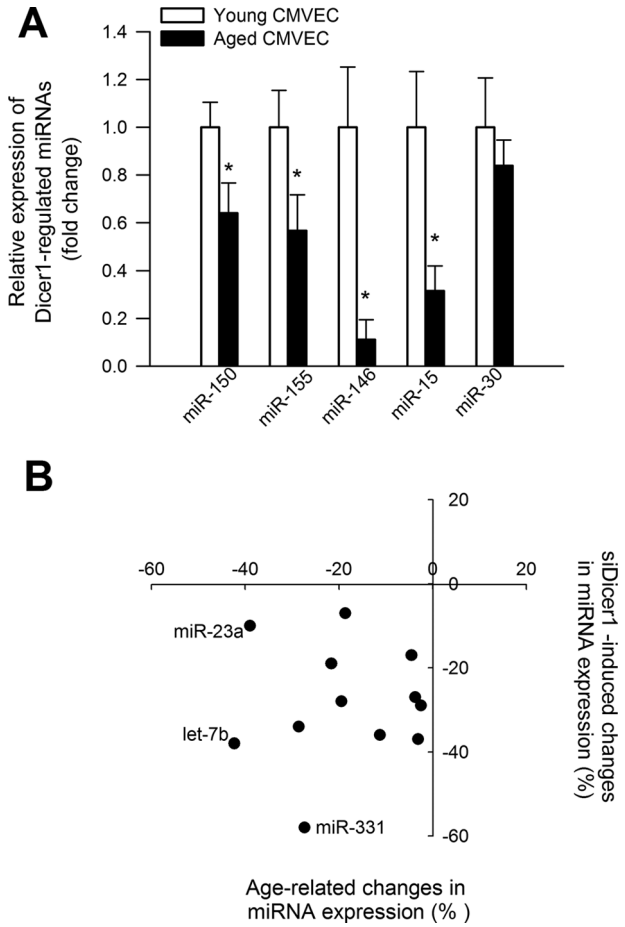

Figure 4. Expression levels of known Dicer1-dependent miRNAs in cerebromicrovascular endothelial cells (CMVECs) derived from young and aged rats. Panel A depicts expression of miRNA species, which were demonstrated in previous studies to be significantly downregulated in tissues of *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice compared with that in *Dicer*$^{fl/fl}$*Tie2cre*$^{-}$ control mice (55). *$p < .05$ vs young CMVECs. Panel B depicts expression of miRNA species, which were demonstrated in previous studies to be significantly downregulated in human umbilical vein endothelial cells after siRNA knockdown of Dicer1 (30). Note that direction of siDicer1-induced changes and age-related changes in the endothelial expression of each miRNA is similar.

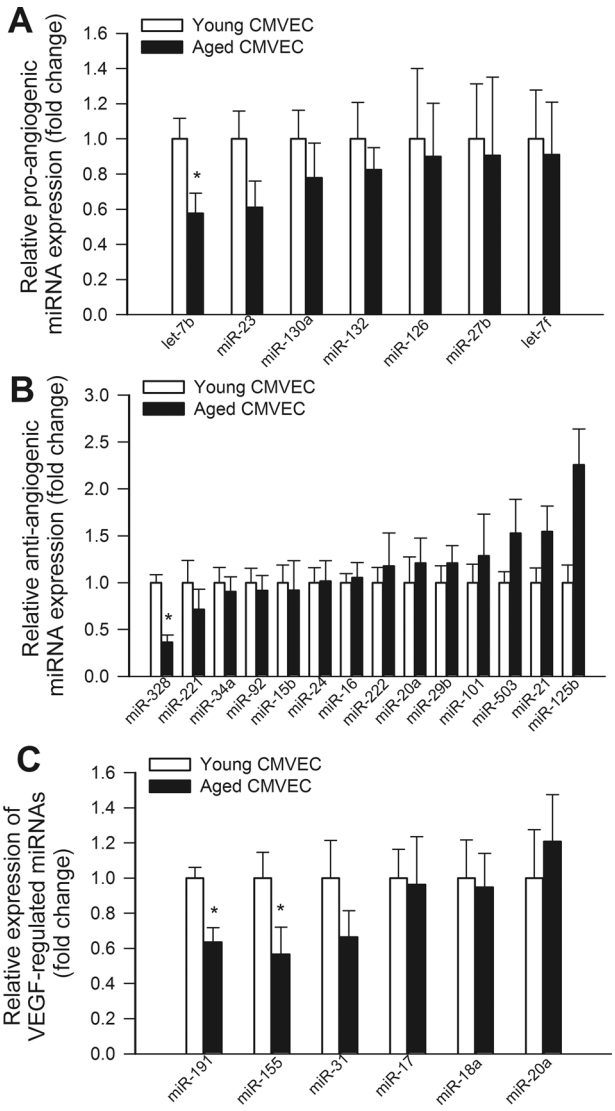

Figure 5. Panels A and B depict the expression levels of known proangiogenic and antiangiogenic miRNAs (57), respectively, in cerebromicrovascular endothelial cells (CMVECs) derived from young and aged animals. *$p < .05$ vs young CMVECs. Panel C: Expression levels of known vascular endothelial growth factor (VEGF) regulated miRNAs in CMVECs derived from young and aged animals. *$p < .05$ vs young CMVECs. List of VEGF-regulated endothelial miRNAs is taken from reference (32).

and angiogenesis. The expression level of these miRNAs was further analyzed in aged CMVECs with overexpression of Dicer1 and in young CMVECs with siRNA knockdown of Dicer1. We found that increasing Dicer1 expression in aged CMVECs significantly increased expression miR-193-b, miR-214, miR-744, miR-532, miR-672, miR-145, and miR-146 and tended to increase expression of miR-574 (Figure 3B–I). In contrast, expression of these miRNAs was decreased by siRNA knockdown of Dicer1 in young CMVECs, mimicking the aging phenotype.

We also analyzed the expression levels of known Dicer1-dependent miRNAs in CMVECs derived from young and aged rats. Figure 4A depicts expression of miRNA species, which were demonstrated in previous studies to be significantly downregulated in tissues of *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice compared with that in *Dicer*$^{fl/fl}$*Tie2cre*$^{-}$ control mice (55). We found that several Dicer1-regulated genes were underexpressed in aged CMVECs, including miR-150, a known regulator of endothelial cell migration (56) (Figure 4A). Panel B depicts expression of miRNA species, which were demonstrated in previous studies to be significantly downregulated in human endothelial cells after siRNA knockdown of Dicer1 (30). We found that expression of miRNAs, which are downregulated by siDicer1 treatment, also exhibits age-related declines (Figure 4B). We also analyzed the expression levels of known proangiogenic and antiangiogenic miRNAs (57), respectively, in CMVECs derived from young and aged

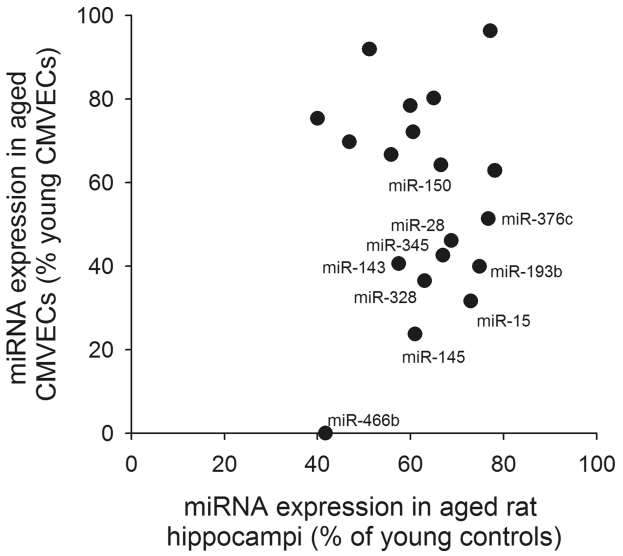

Figure 6. (A) Expression of miRNAs, which are downregulated in hippocampi of aged rats and in aged cerebromicrovascular endothelial cells.

animals (Figure 5A and B). We found that expression of let-7b significantly decreased in CMVECs derived from aged animals (Figure 5A), whereas age-related decline in expression of other proangiogenic miRNAs did not reach statistical significance. Previous studies identified VEGF-regulated endothelial miRNAs (32), which likely play a role in regulation of VEGF signaling and angiogenesis. We found that expression levels miR191 and miR155 were significantly decreased in CMVECs derived from aged animals (Figure 5C). Expression of miR31 also tended to decrease in aged CMVECs but the difference did not reach statistical significance. To further substantiate the in vivo significance of our findings, we have analyzed miRNA expression profile in the hippocampi of young and aged rats. We have identified a subset of miRNAs that were both significantly downregulated in the hippocampi of aged rats and the expression of which could be detected in cultured CMVECs. Figure 6 shows the expression of miRNAs, which are downregulated in aged hippocampi and in aged CMVECs.

Downregulation of Dicer1 Is Associated With Impaired Adhesion of CMVECs to Extracellular Matrix Proteins

Despite the rapid progress of aging research in the last few years (58–94), there are no studies to our knowledge investigating the role of Dicer1 and miRNAs in age-related endothelial alterations that contribute to the development of VCI. To determine the role of Dicer1 on angiogenic capacity, we overexpressed Dicer1 in aged CMVECs and knocked down Dicer1 using siRNA in young CMVECs. Cell adhesion experiments were performed to investigate whether downregulation of Dicer1 affects VEGF-induced adhesion of CMVECs to different components of the extracellular matrix. We found that disruption of Dicer1

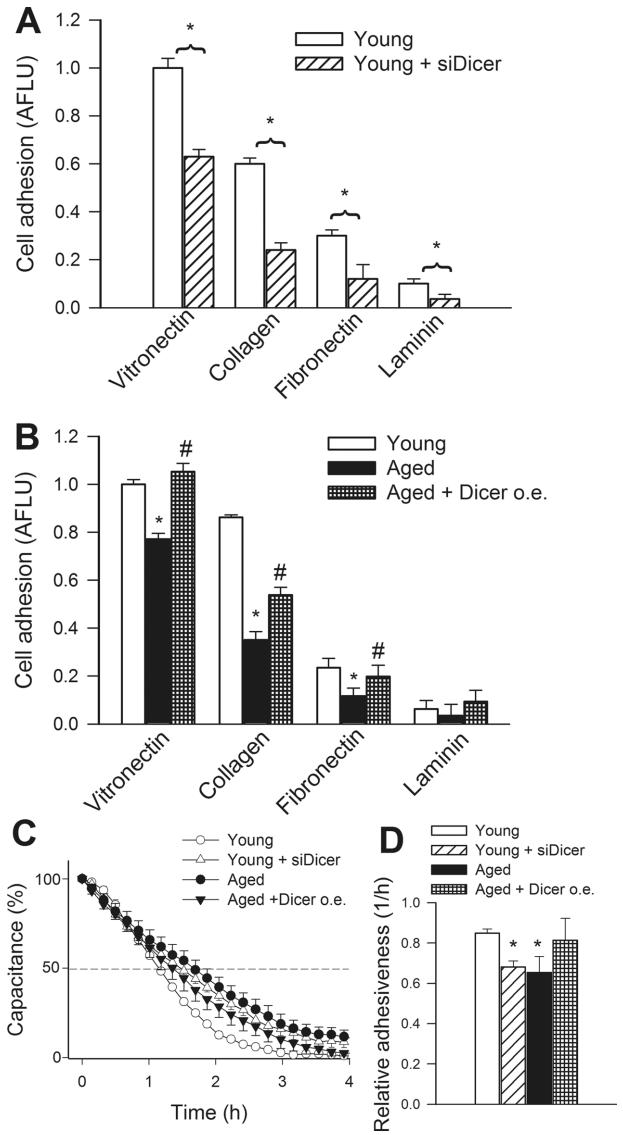

Figure 7. Disruption of Dicer-dependent pathways by siRNA knockdown of Dicer (siDicer) significantly impairs adhesion capacity of cerebromicrovascular endothelial cells (CMVECs) derived from young F344 × BN rats. CMVECs, loaded with the fluorescent dye CyQuant and stimulated with vascular endothelial growth factor (VEGF) (100 ng/mL) were seeded in vitronectin, collagen, fibronectin, or laminin-coated plates (Methods section). After 3 h of incubation, nonadherent cells were washed away, and the ratio of adhering cells was quantified by assessing the background-corrected fluorescence at 508/527 nm. Data are expressed as normalized arbitrary fluorescence light units (means ± *SEM*) ($n = 5$ in each group), $^{*}p < .05$ vs young control. (B) Adhesion capacity of CMVECs isolated from aged F344 × BN rats is impaired compared with that of young cells, and it is significantly improved by overexpression (o.e.) of Dicer. Data are means ± *SEM* ($n = 5$ in each group), $^{*}p < .05$ vs young control, $^{\#}p < .05$ vs untreated. (C) Analysis of VEGF (100 ng/mL) stimulated cell adhesion by electric cell–substrate impedance sensing technology (Methods section). Panel C: Time course of changes of capacitance (at 60 kHz) after addition of CMVECs to collagen-coated wells. 100% change corresponds to the maximum level of cell coverage reached on the active electrode. Time to reach 50% cell adhesion was obtained, and its inverse was used as an index of adhesiveness. Panel D depicts the summary data for relative cell adhesiveness in the four experimental groups. Data are means ± *SEM* ($n = 5$ in each group), $^{*}p < .05$ vs young control.

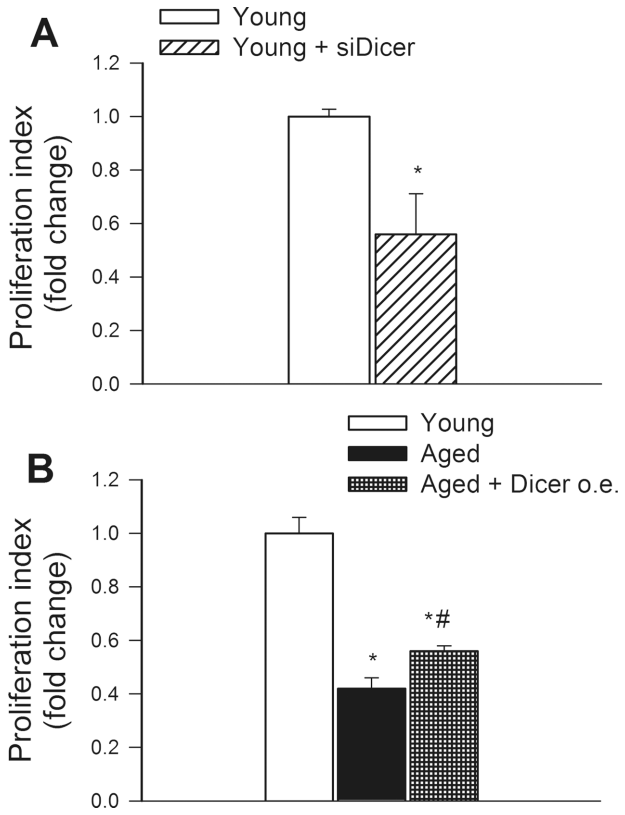

Figure 8. (A) Disruption of Dicer-dependent pathways by siRNA knockdown of Dicer (siDicer) significantly impairs proliferation capacity of cerebromicrovascular endothelial cells (CMVECs) isolated from young F344 × BN rats. Cell proliferation capacity was assessed in CMVECs stimulated with vascular endothelial growth factor (100 ng/mL) using the flow cytometry based Guava CellGrowth assay (Methods section). As index of proliferation capacity, the inverse of the mean fluorescence intensity of the indicator dye CFSE was used. Data are means ± *SEM* ($n = 5$ in each group), $^{*}p < .05$ vs control. (B): Proliferation capacity of CMVECs isolated from aged F344 × BN rats is impaired compared with that of young cells and it is significantly improved by overexpression (o.e.) of Dicer. Data are means ± *SEM* ($n = 5$ in each group), $^{*}p < .05$ vs young control, $^{\#}p < .05$ vs untreated.

signaling by Dicer1 siRNA treatment impaired the ability of CMVECs to adhere to vitronectin, collagen, fibronectin, and laminin (Figure 7A). Aged CMVECs exhibited impaired adhesion to vitronectin, collagen, and fibronectin (Figure7B). The ability of aged CMVECs to adhere to these extracellular matrix components was significantly improved by overexpression of Dicer1 (Figure 7B).

In other experiments, we used ECIS technology to monitor changes of capacitance (at 60 kHz) due to the adhesion of VEGF (100 ng/mL) stimulated cells to the collagen-coated active electrode (Figure 7C). Time to reach 50% cell adhesion was used to calculate an index of adhesiveness. We found that aged CMVECs, but not aged CMVECs overexpressing Dicer1, exhibited impaired adhesiveness to collagen (Figure 7D). Further, siRNA knockdown of Dicer1 significantly impaired the ability of VEGF-treated young CMVECs to adhere to collagen, mimicking the aging phenotype (Figure 7D).

Downregulation of Dicer1 Is Associated With Impaired Proliferative Capacity of CMVECs

Proliferation represents a key step in angiogenesis. Proliferative capacity of young and aged CMVECs was compared after incubation with VEGF for 24 hours. We found that siRNA knockdown of Dicer1 significantly increased CFSE fluorescence in CMVECs, indicating that proliferation capacity is impaired by Dicer1 dysregulation (Figure 8A). Aged CMVECs exhibited impaired proliferative capacity, which was significantly improved by overexpression of Dicer1 (Figure 8B).

Downregulation of Dicer1 Is Associated With Impaired Migratory Capability of CMVECs

The migratory capability of vascular endothelial cells has a pivotal role in the maintenance of microvascular integrity and angiogenesis. An ECIS-based wound-healing assay was used to assess the effect of downregulation of Dicer1 on migratory capability of VEGF-treated CMVECs (Figure 9A). We found that aged CMVECs exhibited impaired migratory capability compared with young CMVECs (Figure 9B). In contrast, the calculated migration rate of aged CMVECs with overexpression of Dicer1 did not differ from that of young CMVECs (Figure 9B). In young CMVECs, siRNA knockdown of Dicer1 significantly decreased migration rate, mimicking the aging phenotype (Figure 9B).

Downregulation of Dicer1 Is Associated With Impaired Formation of Capillary-Like Structures by CMVECs

When seeded onto Geltrex matrices, young CMVECs formed elaborated capillary networks in the presence of VEGF (Figure 10A). We found that siRNA knockdown of Dicer1 significantly inhibited the formation of capillary-like structures by young CMVECs (Figure 10B and E). Compared with young cells in aged CMVECs, formation of capillary-like structures was significantly impaired (Figure 10C and E). The finding that overexpression of Dicer1 significantly improved formation of capillary-like structures by aged CMVECs (Figure 10D and E) suggests that age-related downregulation of Dicer1 is causally linked to the impaired angiogenic capacity of aged endothelial cells.

Discussion

The principal new findings of this study are that (1) age-related dysregulation of Dicer1-dependent miRNA expression is associated with impaired angiogenic response in aged rat CMVECs, that (2) a functional Dicer1-dependent pathway is essential for a healthy angiogenic response of CMVECs, and that (3) upregulation of Dicer1 in aged CMVECs confers proangiogenic effects, counteracting, at least in part, the adverse effects of aging.

To our knowledge, this is the first study demonstrating that expression of Dicer1 significantly declines with age in the cerebral circulation (Figure 1). Demonstration of

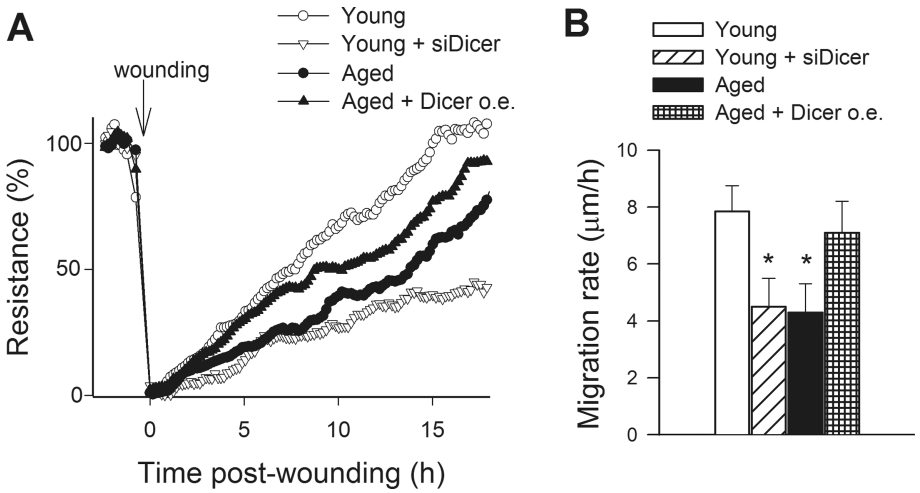

Figure 9. Migration capacity of cerebromicrovascular endothelial cells (CMVECs) isolated from aged F344 × BN rats is impaired compared with that of cells isolated from young F344 × BN rats, and it is significantly improved by overexpression (o.e.) of Dicer. In contrast, disruption of Dicer-dependent pathways by siRNA knockdown of Dicer (siDicer) significantly impairs migration capacity of young CMVECs, mimicking the aging phenotype. Vascular endothelial growth factor (100 ng/mL) stimulated cell migration was monitored by electric cell-substrate impedance sensing technology in a wound-healing assay (Methods section). Panel A: Time course of resistance recovery after wounding (electric pulse of 5 mA for 20 s at 60 kHz; 100% represents prewounding levels). Resistance (at 4,000 Hz) was monitored in every 160 s. Data are mean ± *SEM* ($n = 5$ in each group). Time to reach 50% resistance recovery (corresponding to 50% confluence on the active electrode) was determined for each group, and this parameter and the known physical dimensions of the electrode were used to calculate the migration rate (expressed as μm/h). Panel B depicts the summary data for migration rate in each group. Data are means ± *SEM* ($n = 5$ in each group), $^*p < .05$ vs young control.

age-related downregulation of Dicer1 in the CMVECs (Figure 1E) is particularly important, as Dicer1-dependent pathways have been shown to regulate, in addition to angiogenesis (30–33), multiple aspects of cellular physiology relevant for vascular aging, including replicative senescence (95,96), mechanotransduction (97), NO production (31,97), endothelial apoptosis (98), and inflammation (31). Age-related alterations in Dicer1 expression in other organs have not yet been documented, but previous studies have linked decreased Dicer1 expression to induction of a premature senescence cellular phenotype both in vivo and in vitro (99).

Previous studies demonstrate that oxidative stress contributes to age-related microvascular alterations in aging (100) and that age-related oxidative stress is associated with a decreased angiogenic capacity of endothelial cells (20,21,101,102). Our studies demonstrate that increased H_2O_2 levels promote downregulation of Dicer1 expression in aged CMVECs (Figure 2), suggesting a novel mechanism linking age-related oxidative stress to microvascular impairment. The mechanisms involved in H_2O_2-dependent transcriptional regulation of Dicer1 in endothelial cells are presently unknown and need to be elucidated in future studies. There are data suggesting that redox signaling in endothelial cells is subject to regulation by miRNAs (103); thus, it is likely that this pathway is regulated by a series of complex feedback loops. Our findings showing upregulation of Dicer1 by resveratrol (Figure 2D) are interesting, as resveratrol treatment was shown to increase capillary density in the brain of aged mice (104). Our previous studies also demonstrated that resveratrol treatment attenuates oxidative stress in vascular endothelial cells in aged mice (105).

We have identified a number of miRNAs that are downregulated in CMVECs during physiological aging (Figures 3–5). We posit that age-related oxidative stress and downregulation of Dicer1 are causally involved in dysregulation of miRNAs in aged CMVECs (Figure 3). This concept is supported by the findings that overexpression of Dicer1 in aged CMVECs increased expression of age-sensitive miRNAs (Figure 3). Furthermore, knockdown of Dicer1 resulted in downregulation of the same miRNAs, mimicking the aging phenotype (Figure 3). Previous studies using *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice (55) and cultured human umbilical vein endothelial cells with siRNA knockdown of Dicer1 (30) have identified a group of miRNAs the production of which is Dicer1-dependent. We found age-related changes in the expression levels of these known Dicer1-dependent miRNAs are consistent with the diminished expression of Dicer1 in aged CMVECs (Figure 4). It should be noted that although the majority of miRNAs that exhibited significant age-related changes in their expression level were downregulated, there were also certain miRNAs that were upregulated in aged endothelial cells. These alterations cannot be explained by age-related downregulation of Dicer1. Analysis of age-related changes in miRNA profiles and expression of factors involved in miRNA processing in other organs (106), including the heart (107), raises the possibility that age-related changes in the expression of additional factors involved in miRNA regulation (e.g., Ago1 and Ago2 proteins (107)) may also contribute to dysregulation of miRNA expression in aged CMVECs.

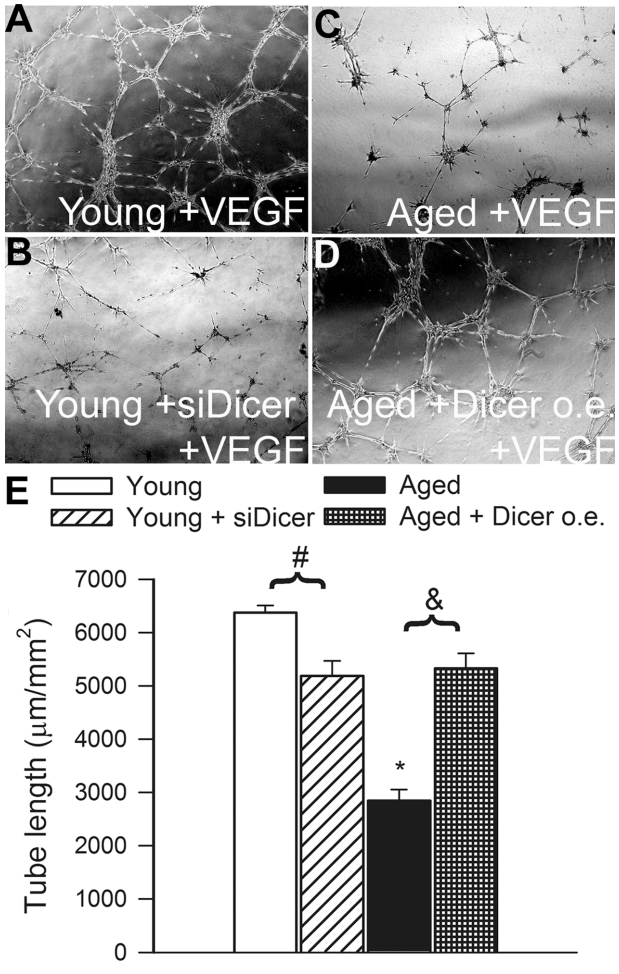

Figure 10. Tube-forming ability of cerebromicrovascular endothelial cells (CMVECs) isolated from aged F344 × BN rats is impaired compared with that of cells isolated from young F344 × BN rats, and it is significantly improved by overexpression (o.e.) of Dicer. In contrast, disruption of Dicer-dependent pathways by siRNA knockdown of Dicer (siDicer) significantly impairs the ability of young CMVECs to form capillary-like structures, mimicking the aging phenotype. CMVECs were plated on Geltrex matrix-coated wells, and tube formation was induced by treating cells with vascular endothelial growth factor (100 ng/mL, for 24 h). Representative examples of capillary-like structures are shown in Panel A. Summary data, expressed as total tube length per total area scanned (µm tube/mm^2), are shown in Panel B. Data are means ± *SEM* ($n = 5$ in each group), *$p < .05$ vs control.

Interestingly, we found that expressions of most miRNAs, which are downregulated in aged CMVECs, are also downregulated in hippocampi of aged rats (Figure 6). However, we cannot conclude the aging results in similar changes in miRNA expression profile in every tissue. A recent analysis of miRNA expression profile in aortas of 18 months old mice showed a significant age-related decline (>1.5-fold) in the expression of 14 miRNAs (108). Yet, we could detect the expression of only four of these miRNAs in CMVECs, and only the expression of miR-31 exhibited an age-related decline in these cells (by 35% vs a 41% decline in aged mouse aortas). Recent in vitro studies identified changes in expression of various miRNAs (95,96) with increased number of passages, which have been implicated in governing senescence in human umbilical vein endothelial cells. We could not demonstrate consistent changes in these miRNAs in our study. Thus, further studies are warranted to analyze the overlap between age-related and in vitro and senescence-related changes in endothelial miRNA expression profile and to elucidate the precise functions of these miRNAs in regulating microvascular aging. It will also be important to identify miRNAs, the endothelial expression of which shows similar age-related changes across different species.

There is increasing experimental evidence for the involvement of miRNAs in the regulation of the angiogenic process (31,32,57). Here, we report that age-related dysregulation of Dicer1 is associated with significant changes in various pro- and antiangiogenic miRNAs and VEGF-regulated miRNAs (Figure 5). Our studies provide strong evidence that a functional Dicer1-dependent pathway is essential for a healthy endothelial angiogenic response in the cerebromicrovasculature because all the major steps of the angiogenic process, including adhesion (Figure 7), proliferation (Figure 8), migration (Figure 9), and formation of capillary-like structures (Figure 10), are compromised by disruption of Dicer1 signaling in CMVECs, extending previous findings in different cell types (30–33). Because overexpression of Dicer1 in aged CMVECs exerts proangiogenic effects, improving cell adhesion (Figure 7), proliferation (Figure 8), migration (Figure 9), and endothelial tube formation (Figure 10), it is likely that age-related decline in Dicer1 expression contributes to the aging-induced impairment of endothelial angiogenic capacity. Other mechanisms, which likely contribute to the induction of the antiangiogenic phenotype in CMVECs include circulating and/or paracrine IGF-1 deficiency (7,24,109), age-related Nrf2 dysfunction (51), and alterations in expression of pro- and anti-inflammatory factors in the brain (110).

The pathophysiological consequences of impaired angiogenesis associated with dysregulation of Dicer1-dependent pathways are likely multiple. Dicer1 depletion may decrease capillary density in the brain and negatively affect cerebral angiogenesis and/or collateral formation induced by physiological (e.g., exercise, local ischemia) or pharmacological stimuli. Indeed, aging results in cerebromicrovascular rarefaction (4,7) and impaired cerebral angiogenesis in response to hypoxia or VEGF administration (26,111). It is thought that microvascular rarefaction and impairment of compensatory proliferation of the cerebral resistance vessels and the capillary network play a prominent role in impairment of regional cerebral blood flow and the occurrence of VCI with age. Because the role of miRNA regulation and function in the aging cardiovascular system is a new emerging area, additional investigations are needed to study the contribution of individual miRNAs or miRNA clusters in controlling gene expression that underlie microvascular aging.

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