MicroRNA Expression in the Blood and Brain of Rats Subjected to Transient Focal Ischemia by Middle Cerebral Artery Occlusion

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Background and Purpose—Several hundred small RNAs called microRNAs (miRNAs) have been identified and characterized from various organisms, including humans. In humans, some of these miRNAs have been found to regulate (patho)physiologic conditions such as tumor progression/regression, cholesterol and glucose homeostasis, etc. In this report, we present data on the miRNAs expressed under ischemic conditions in both the brain and blood of rats subjected to middle cerebral artery occlusion (MCAo).

Methods—Sprague-Dawley rats subjected to MCAo were reperfused for either 24 or 48 hours, and both blood and brain samples were harvested. miRNA expression profiling and oligonucleotide microarray were carried out, and the data were validated by quantitative real-time polymerase chain reaction and correlated with published data on protein and gene expression in MCAo rats.

Results—We report here for the first time the involvement of miRNA regulation in brain pathogenesis associated with MCAo. Comparison with the corresponding DNA microarray data revealed that the target mRNA expression is correlated with the regulation of miRNA. We have also provided evidence that some of the miRNAs that are highly expressed in the ischemic brain can be detected in blood samples.

Conclusions—Further studies are needed to evaluate the possible use of miRNAs as biomarkers in stroke and related pathologies. (Stroke. 2008;39:959-966.)

Key Words: ischemia ▪ middle cerebral artery occlusion ▪ microRNA ▪ profiling ▪ Sprague-Dawley rats

The discovery of microRNAs (miRNAs) has broadened an overall understanding of the mechanisms that regulate gene expression, with the addition of an entirely novel level of regulatory control. These small, noncoding RNA molecules of 18 to 25 nucleotides modulate protein expression by binding to complementary or partially complementary target messenger RNAs (mRNAs) and thereby target the mRNA for degradation or translational inhibition.1 It is now predicted that ~40% to 50% of mammalian mRNAs could be regulated at the translational level by miRNAs. In mammals, specific miRNAs are known to control processes such as development, neuronal cell fate, apoptosis, proliferation, adipocyte differentiation, hematopoiesis, and exocytosis,2 as well as in diseases such as cancer3,4 and possibly neuronal disorders.5

Approximately 20% to 40% of miRNAs in rat primary cortical neurons appear to be developmentally regulated, whereby the expression of miRNAs such as miR-128, -191, -323, -324-5p, -326, -329, and -344 increase dramatically in parallel with cortical development.6 A distinct miRNA profile has been found in neuronal differentiation (miR-23, -23b) and synapse formation.5,7 Involvement of miRNAs in the initiation and progression of cancer pathogenesis has also been extensively studied.8 However, very few reports are available on the importance of miRNA in brain pathogenesis or central nervous system–related pathophysiologic conditions. In our study, we report miRNA profiling (screening) performed on rat brains subjected to middle cerebral artery occlusion (MCAo) and reperfusion for 24 or 48 hours. We have also identified several miRNA target genes for some of the highly expressed miRNAs.

Materials and Methods

Transient Focal Cerebral Ischemia
Male Sprague-Dawley rats (200 to 300 g) obtained from the Laboratory Animal Centre (National University of Singapore, Singapore) were maintained on an ad libitum intake of standard laboratory chow and drinking water. They were handled according to the guidelines given by the Council for International Organization of Medical Sciences on Animal Experimentation (World Health Organization, Geneva, Switzerland) and the National University of Singapore guidelines. Transient focal cerebral ischemia was induced by intraluminal occlusion of the right middle cerebral artery (n=59). Infarct volume was measured according to Engelhorn et al.10
Extraction of miRNA From Rat Blood and Brain Samples

Total RNA (plus miRNA) was extracted from blood and brain samples with use of the Mouse Ribopure-Blood RNA isolation kit and mirVana miRNA isolation kit from Ambion (Austin, Tex), respectively, according to the manufacturer’s protocol. The concentration and integrity of RNA were determined by NanoDrop ND-1000 spectrophotometry (NanoDrop Tech, Rockland, Del) and gel electrophoresis, respectively.

mRNA and miRNA Real-Time Quantitative PCR

Real-time polymerase chain reaction (PCR; with gene-specific primers and SYBR green assay) was performed to quantify the genes encoding aquaporin-4 (AQP4), visinin-like-1 (VSNL1), transgelin, and matrix metalloproteinase-9 (MMP9). Quantification of miRNAs (miR-124a, miR-223, and miR-494) was performed with stem-loop real-time PCR chemistry as described by the manufacturer (Applied Biosystems). Both reverse transcription and PCRs were performed in triplicate in 3 separate experiments on an Applied Biosystems 7000 sequence detection system. Ribosomal RNA (18S) was used as an internal calibrator.

Oligonucleotide (DNA) Microarray Analysis

The oligonucleotide (DNA) microarray was performed according to Cher et al.11 The fold change values presented are the mean of 3 independent microarray (3 chips for each sample) experiments. The raw data were subtracted from the data for normal brains and further filtered for signal log ratio (>1 and <1) and a detection probability value of <0.01.

μParaflo miRNA Microarray Assay and Analysis

Total RNA (2 to 5 μg) from the brain and blood was size-fractionated with a YM-100 Microcon filter (Millipore). The small RNAs (<300 nucleotides) isolated were 3′-extended with a poly(A) tail with the use of poly(A) polymerase. Two different oligonucleotide tags were ligated to the poly(A) tails of the test and control RNA samples for fluorescent dye staining. Hybridization was performed overnight on a μParaflo microfluidic chip with use of a microcirculation pump (Atactic Technologies).12 On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to the target miRNA (from miRBase 9.0; http://microrna.sanger.ac.uk/sequences/) or other RNA (control sequences) and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were made in situ synthesis with photogenerated reagent chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization used 100 μL of 6× SSPE buffer (0.90 mol/L NaCl, 60 mmol/L Na2HPO4, 6 mmol/L EDTA, pH 6.8) containing 25% formamide at 34°C. Tag-specific Cy3 and Cy5 dyes were used for detection. Hybridization images were collected by a laser scanner (GenePix 4000B, Molecular Devices) and a digitizer (Media Cybernetics). Data were analyzed by first subtracting the background and then normalizing the signals with a locally weighted regression filter.13 For 2-color experiments, the ratio of the 2 sets of detected signals (log, transformed, balanced) and probability values of the t test were calculated; differentially detected signals were defined as those with <0.01 probability values. Microarray analysis involved multiple sample analyses, including normalization, data adjustment, t test/ANOVA, and clustering. The t test was performed between control and test sample groups for each group, t values were calculated for each miRNA, and probability values were computed from the theoretical t distribution. miRNAs with probability values below a critical value (typically 0.01) were selected for cluster analysis according to a hierarchical method, which was performed with average linkage and euclidean distance metrics. The clustering plot was generated with TIGR multiple experimental viewer software.14

Results

Brain and blood samples from rats subjected to MCAo were screened for a total of 236 Rattus norvegicus-miRNAs (rno-miR; 12 spots each, as listed in Sanger miRBase release 9.0; www.sanger.ac.uk/Software/Rfam/mirna/). Only 20 and 25 miRNA transcripts were detected in the blood and =106 and 82 transcripts were detected in the brain of MCAo rats reperfused for 24 and 48 hours, respectively. Hierarchical clustering of the miRNAs detected in the brain showed 7 distinct clusters, A to G (Figure 1a). Cluster A contained the very highly expressed genes, followed by cluster D in both the 24- and 48-hour reperfusion brain samples. The down-regulated genes were found in clusters B and C, whereas miRNAs in cluster F remained upregulated in the 24-hour samples only. Cluster E included genes that were upregulated at 48 hours of reperfusion only. The genes in cluster G, however, were found to be downregulated at 24 hours but upregulated at 48 hours of reperfusion. A distinct expression was observed for miR-138 and miR-145: they showed exclusive upregulation at 24 and 48 hours, respectively. The heat map and dendrogram of miRNAs detected in both brain and blood samples are shown in Figure 1b and are represented as a Venn diagram in Figure 1c. The results are also presented as supplementary data (supplemental Tables I and II, available online at http://stroke.ahajournals.org).

miRNAs in Blood Samples of Rats Subjected to MCAo

Comparison of the miRNA profile for 24- and 48-hour reperfusion showed that the miRNAs present in both samples could be divided into 3 categories on the basis of their expression pattern. The first group representing the 24-hour blood samples (supplemental Figure Ia and Table III, available online at http://stroke.ahajournals.org) showed the effects of acute ischemic injury and recovery events mediated by the MCAo within 24 hours of reperfusion. Of these, rno-miR-19b, -290, and -292-5p were found to be highly expressed, whereas rno-miR-103 and rno-miR-107 were found to be poorly expressed. Interestingly, an entirely new group of miRNAs was observed in the 48-hour-reperfusion blood samples (supplemental Figure Ia and Table III). These could possibly represent the miRNAs that were being expressed as the injury progressed and initiated secondary effects due to focal ischemia. Among the 14 miRNAs that appeared at both time points, rno-miR-150, -195, and -320 showed an opposite trend in expression at 24 and 48 hours, whereas rno-miR-103, miR-107, and miR-191 showed almost the same level of downregulation at both time points. Transcripts that were detected in the 24-hour-reperfusion blood samples only included rno-miR-16, -19b, -23a, -106b, -122a, and -292-5p (supplemental Figure Ia).

miRNAs in Brain Samples of Rats Subjected to MCAo

Although there were 56 common miRNAs present at both time points, ~8 miRNAs were found to be present exclusively in the 48-hour samples. Similarly, 32 miRNAs were found to be present in the 24-hour samples only (Figure 1c). The miRNAs that were present in the 48-hour brain samples
only included rno-miR-99a, -181 (a, b, and c), -195, -328, -379, and -539. The miRNAs that were expressed in the 24-hour brain samples only included rno-miR-16, -17, -20a, -21, -24, -25, -30a-3p, -34a, -92, -124a, -130a, -132, -134, -151*, -210,-215, -324-3p, -322, -329, -342, -361, -374, -382, -383, -422b, -433, -451, -497, -505, -664, let-7d, and let-7f. The let-7 (a, b, c, and e) miRNAs were downregulated in the 24-hour-reperfused MCAo rat brains but were subsequently upregulated during the 48-hour reperfusion. The miRNAs detected in the brain comprised the reported brain-specific and enriched transcripts as well as 46 new transcripts (supplemental Tables III and IV). In addition to these, 4 other miRNAs that have been reported in heart and skeletal muscle (miR-206 and -143), lung and kidney (miR-24), and spleen (miR-150) were also detected in the ischemic brain samples. The miRNAs that were highly upregulated during the ischemia/reperfusion periods (24 and 48 hours) comprised rno-miR-206, -214, -223, -290, -292-5p, -298, -327, and -494 (supplemental Table II). Of these, miR-290, -292-5p, -327, and -494 were identified as the highly expressed miRNAs in

Figure 1. Hierarchical clustering analysis of 236 miRNAs expressed in the MCAo rat brain and blood with average linkage and euclidean distance as the similarity measure. Green indicates downregulation and red, upregulation. Reperfusion time points (24 and 48 hour) at which the brain and blood samples were collected are also noted. For both times, MCAo was maintained for 60 minutes. Each miRNA listed was detected as significantly and differentially expressed between the normal and ischemic brain. Heat map and dendrogram for miRNA profiles of (a) brain samples only and (b) transcripts detected in both brain and blood samples are indicated. c, Venn diagram illustrating the miRNAs detected in the brain and blood.
the ischemic brain. To date, no other reports are available on these miRNAs as being either brain specific or brain enriched.

miRNAs in Blood and Brain Samples
Among the differentially regulated miRNA transcripts, only 10 miRNAs (Figures 1c and supplemental lb) were found to be present in both the blood and brain at both reperfusion times (24 and 48 hours). miR-290 was observed to be highly upregulated, whereas let-7i was downregulated. Transcripts that were common to both the blood and brain at 24-hour reperfusion (supplemental Figure 1c) included rno-miR-16, -23a, -103, -107, -150, -185, -191, -292-5p, -320, -451, -494, and let-7 (a, d, f, and i). miRNAs found at 48-hour reperfusion (supplemental Figure Id) in both the blood and brain were miR-26a, -26b, -103, -107, -140*, -150, -185, -195, -191, -214, -320, -328, -352, -494, and let-7 (a, c, and i). The 22 miRNAs that were expressed in 24-hour blood as well as 48-hour blood sample are shown in supplemental Figure 1e. miRNAs that were present in 1 sample (either blood or brain) at any 1 reperfusion time (either 24 or 48 hours) are shown in Figure 1c.

miRNA Targets, DNA Microarray, and Real-Time PCR
An independent DNA microarray study was performed on brain samples from MCAo rats that were subjected to 24-hour reperfusion. We selected 659 genes (signal log ratio >1 and <-1 and a detection probability value of <0.01) to be significant to our study (data were similar to those of Lu et al15). miRNAs that target these genes were identified from 5 different databases (PicTar, TargetScan3.1, mirGen, mirBase, and TargetScans). The genes (mRNAs) with the highest-ranking scores for each database were selected as targets for the miRNA identified in our profiling analysis. The miRNA targets that were found in our DNA microarray analysis are listed in the Table. The expression of selected miRNAs was further validated16 by stem-loop real-time PCR (supplemental Table V). AQP4, VSNL1, transgelin, and MMP9 gene expression in brain samples was also quantified (supplemental Table V). AQP4, VSNL1, transgelin, and MMP9 gene expression in brain samples was also quantified (supplemental Table V). AQP4, VSNL1, transgelin, and MMP9 gene expression in brain samples was also quantified (supplemental Table V). AQP4, VSNL1, transgelin, and MMP9 gene expression in brain samples was also quantified (supplemental Table V).

miRNA Profiling in Ischemic Brain
Cerebral ischemia triggers a multifaceted cascade of physiologic and biochemical events. It is believed that these events are mediated in part by alterations in molecular processes such as transcription and translation. An extensive DNA microarray analysis by Lu et al15 identified 12 functional categories that contribute to the temporal changes in gene expression after transient MCAo in rats. To date, no reports are available on the miRNA microarray profiling of the ischemic brain. Nevertheless, several reports have demonstrated the roles of specific miRNAs in neuronal differentiation, neurogenesis, neural cell specification, and neurodevelopmental function.6,7,17 We identified the expression of 114 miRNAs in ischemic brain samples. Among them, 106 and 82 transcripts were detected in the 24-hour and 48-hour-reperfusion brain samples, respectively (Figures 1a and 1c). These transcripts included some of the previously reported miRNAs in the brain,7,17-20 as well some new miRNAs (supplemental Table IV). Our data also show that miRNAs are actively regulated and that their expression pattern changed with reperfusion time, thus indicating temporal expression during ischemic injury. Many reports have demonstrated that neuronal damage increases with prolonged reperfusion time, and this damage could be more severe than the ischemic injury itself.21 It is noteworthy that the let-7 family, which has been implicated in neural cell specification of miRNAs (let-7a, b, c, and e20), was upregulated in the 48-hour-reperfused MCAo brain samples along with miR-150 and -125a. Distinct upregulation was also observed for miR-138 and -145 at 24 and 48 hours of reperfusion, respectively. miR-138 has been reported to be developmentally regulated, and its expression is restricted to distinct neuronal populations in the cerebrum, cerebellum, and midbrain of adult mice,22 whereas miR-145 has been identified as a specific miRNA that is downregulated in cancers.23

The 24-hour brain samples showed 32 unique transcripts, whereas the 48-hour brain samples had only 8 unique detectable miRNAs (Figure 1c). miR-7, -27a, -29 (b and c), -30e, -98, -101a, -137, -148b, -204, -218, -301, -338, -335, -369-5p, -376 (b and b*), and -424 were found to be downregulated at both reperfusion times (24 and 48 hours). Downregulation of these transcripts after MCAo (eg, miR-338), however, was correlated with the upregulation of its target gene, such as transgelin (8.15±0.099, Figure 2), an early marker of smooth muscle cell gene expression during vascular/cardiac development.24

Among the highly expressed miRNAs, miR-210, -215, -324-3p, -422b, -451, -497, and the brain-specific miR-134 were upregulated >2-fold and were detected only at the 24-hour time point. miR-290 showed high expression at 24 hours (2405.5±0.3) and increased by 5 times at the 48-hour reperfusion time. The miR-292-5p transcript showed the highest expression after ischemic injury in the brain, 3150.90±1.34 at 24-hour reperfusion, and increased even more, by 1.5 times, at 48-hour reperfusion. Our DNA microarray data also showed that many genes that were targets of miR-290 were downregulated (the Table).

miR-214 was highly expressed in brain samples at 24-hour reperfusion (114±0.33) but was decreased (70.27±0.201) in the 48-hour-reperfused brain. miR-214 has been reported to be required for the specification of muscle cell fate during somitogenesis, and its inhibition results in a reduction or loss of slow-muscle cell types.25 Among the brain-specific/enriched transcripts identified, miR-7, -9, -92, and -125a were found to be downregulated in MCAo rat brains at both reperfusion times.

Target Genes for miRNA Were Found to Be Regulated in Parallel
Repression of endogenous laminin-1γ1 and integrin-β1genes during neural differentiation has been attributed to the function of the brain-enriched miR-124.26 These 2 genes were found to be downregulated in our DNA microarray study (the Table). Cao et al26 proposed that miR-124 may not act as a
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<th>Affymetrix Probe Set ID</th>
<th>Representative Public ID</th>
<th>Gene Title</th>
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<th>MCAo Brain vs Sham, Signal Log Ratio</th>
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(Continued)
primary determinant of neuronal differentiation but could play a role in ensuring that progenitor genes are posttranscriptionally inhibited in neurons. On the other hand, Schratt et al17 showed that miR-134 regulates local dendritic translation, and its expression increases as the brain matures. Upregulation of both of these transcripts (miR-124 and -134; supplemental Tables II and IV) indicates that injured brain cells could be actively involved in regeneration during the first 24 hours of reperfusion. Screening for miRNA targets identified 1 novel target for miR-124a, -290, and -494, the gene encoding VSNL1, a neuronal calcium sensor protein that is expressed in granule cells of the cerebellum. The VSNL1 protein was identified as a specific and promising biomarker in the plasma of stroke patients and in the cerebrospinal fluid of poststroke rats.27 Repression of VSNL1 gene expression was reflected by decreased expression of the VSNL1 gene in our DNA microarray data as well. Expression of the gene encoding VSNL1 further decreased in the 48-hour-reperfused brain (supplemental Figure II). This was correlated with the high expression of miR-290 at 24 hours and a further increase at 48 hours (5 times in the brain), which was detected in blood samples. Similarly, the VSNL1 gene is also a target for miR-124a, which was upregulated at 24-hour reperfusion and an additional 5-fold increase in the 48-hour brain samples (the Table, supplemental Figure II). The upregulated expression of miR-290 and of brain-specific miR-124a is correlated with repression of the VSNL1 gene. However, the very high expression of miR-290 (2405.491/110060.31 at 24 hours and 12520.60/110060.52 at 48 hours; supplemental Table II) indicates that the VSNL1 gene is most probably the typical target of miR-290.

We also observed that highly expressed (brain) miRNAs (miR-223, -290, -292-5p, -327, and -494) target the genes encoding aquaporins 1, 4, 5, 6, and 11. AQP4, which is

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<td>-0.96</td>
<td>miR-290</td>
</tr>
<tr>
<td>1370543_at</td>
<td>U76557</td>
<td>O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)</td>
<td>Ogt</td>
<td>-2.43</td>
<td>miR-290</td>
</tr>
<tr>
<td>1368015_at</td>
<td>AF280967</td>
<td>Prostaglandin E synthase</td>
<td>Ptges</td>
<td>-1.08</td>
<td>miR-290</td>
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<tr>
<td>1371508_at</td>
<td>AI412098</td>
<td>Protein tyrosine phosphatase 4a2</td>
<td>Ptpra2</td>
<td>1.17</td>
<td>miR-290</td>
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<tr>
<td>136991_at</td>
<td>NM_013119</td>
<td>Sodium channel, voltage gated, type III, α-polypeptide</td>
<td>Scn3a</td>
<td>1.36</td>
<td>miR-214, -223, -494</td>
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<tr>
<td>1370116_at</td>
<td>NM_019375</td>
<td>Septin 3</td>
<td>Sep3</td>
<td>-2.28</td>
<td>miR-23a, -23b, -181b, -290, 324-5p, -328, -382, let-7d*</td>
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<tr>
<td>1368986_at</td>
<td>BF567766</td>
<td>Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7</td>
<td>Stcl7a7</td>
<td>-3.2</td>
<td>miR-7, -327</td>
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<tr>
<td>1369718_at</td>
<td>NM_031120</td>
<td>Signal sequence receptor, γ</td>
<td>Sar3</td>
<td>-2.28</td>
<td>miR-290</td>
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<td>1387359_at</td>
<td>NM_053788</td>
<td>Syntaxin 1A (brain)</td>
<td>Stx1a</td>
<td>-2.46</td>
<td>miR-24, -29a, -29b, -29c, -298, -497</td>
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<tr>
<td>1387662_at</td>
<td>L38247</td>
<td>Synaptotagmin IV</td>
<td>Syt4</td>
<td>-0.81</td>
<td>miR-30a-35p, 30e, -292-5p</td>
</tr>
<tr>
<td>1367570_at</td>
<td>NM_031549</td>
<td>Transgelin</td>
<td>Tagln</td>
<td>3.14</td>
<td>miR-34a, -98, -139, -204, -290, -320, -338, -382, -485, let-7a, let-7b, let-7e</td>
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<td>1371241_x_at</td>
<td>AF370889</td>
<td>Tropomyosin 1, α</td>
<td>Tpm1</td>
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<td>miR-21, -29c, -329, -338</td>
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<td>Vim</td>
<td>2.74</td>
<td>miR-92, -129, -138, -185, -223, -320, -382, let-7a, let-7b, let-7c, let-7d*, let-7i</td>
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<td>AU227991</td>
<td>Visinin-like 1</td>
<td>Vsnl1</td>
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abundantly expressed in the brain, forms a target for miR-30a-3p, -99 (a and b), -100, -223, and -383. Expression of the AQP4 gene has been shown to be downregulated at the onset of ischemia but is upregulated during recovery, with a concomitant decrease of the protein in cerebral edema.28 AQP4-null mice are protected from cytotoxic brain edema; however, deletion of AQP4 aggravates vasogenic brain edema.29 We also observed that the AQP4 gene was downregulated in the brain at 24 hours of reperfusion but was increased at 48 hours of reperfusion. The expression profile of miR-30a-3p and of miR-383 (supplemental Table II) corresponds to the increased expression of AQP4 mRNA (supplemental Figure II).

Increased expression of MMP9 has been demonstrated in both rodent and nonhuman primate stroke models as an early marker of injury (within 3 hours of MCAo) and is associated with alterations in the blood-brain barrier and early vasogenic edema after transient focal cerebral ischemia.30 MMP9 is involved in degradation of the basal lamina and extracellular matrix components. MMP9 levels in the peripheral blood of stroke patients have also been shown to be the highest 24 hours after admission.31 We also observed that the mRNA encoding MMP9 was upregulated in the first 24 hours of MCAo and increased further at 48 hours. A concomitant increase in laminin breakdown products, a decrease in intact laminin molecules, and increased MMP9 protein levels were observed by Horstmann et al.32 Upregulation of the MMP9 gene (supplemental Figure II) and downregulation of the corresponding miR-132 and -664 (supplemental Table II) at both 24 and 48 hours of reperfusion, as well as downregulation of laminin and integrin genes and the concomitant upregulation of brain-specific miR-124a, reflect the tight relationship between miRNA and mRNA regulation.

**Summary**

We have presented the profiling of miRNA during ischemia and reperfusion in a rat MCAo model and have identified distinct regulation patterns for 7 clusters of miRNA. We have also correlated the results with relevant DNA microarray data and have identified some of the miRNAs that control the expression of 4 genes known to be important in the progression of cerebral ischemia. These results may be useful in pursuing further studies on the possible use of microRNAs as biomarkers in stroke.

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**Disclosures**

None.
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MicroRNA Expression in the Blood and Brain of Rats Subjected to Transient Focal Ischemia by Middle Cerebral Artery Occlusion
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