surgery. In total, 18 gerbils were randomized into three groups. When the animals began to regain consciousness after anesthesia, both common carotid arteries (CCAs) of the first and second groups (n=8 each) were occluded with surgical micro clips (RS-5424; Roboz Surgical Instrument Co., Gaithersburg, MD, USA) for 2 min of tCCAO. For the second group (n=8), secondary occlusion of both CCAs for 2 min was induced 2 days later, and then in the same way for tertiary occlusion, with a total of three rounds of tCCAO at 2-d intervals. Gerbils of the sham-control (SC) group were treated only once with a neck incision without any artery occlusion. The animals were allowed to recover for 1 d, 7 d, 1 month (M), or 6 M at ambient temperature (23-25°C), then were deeply anesthetized with pentobarbital (i.p., 40 mg/kg; Kyoritsu Seiyaku Corporation, Saitama, Japan) and decapitated. SC animals were sacrificed in the same way just after the sham operation.

tCCAO and reperfusion

Transient global forebrain ischemia was induced into gerbils by occluding bilateral CCA, as per our previous method (3). Briefly, gerbils were lightly anesthetized after inhaling a nitrous oxide/oxygen/isoflurane (69%:30%:1%; Air Water Inc., Okayama, Japan) mixture. Body temperature was monitored in all animals, and was maintained at 37 ± 0.3°C by using a heating pad (model BMT-100; Bio Research Center, Nagoya, Japan) during surgery. A midline neck incision was made and both CCAs were exposed. Animals began to regain consciousness after the end of anesthesia, and both CCAs were occluded with surgical micro clips. After 2 min of

tCCAO, cerebral blood flow was restored by removing the micro clips. At 1 d, 7 d, 1 M and 6 M after reperfusion, the animals were euthanized as described above (n=2) using a previously defined method (28). Then, all brains were dissected, quickly frozen in powdered dry ice, and stored at -80°C until subsequent preparation.

All animal experiments were approved by the Animal Committee of the Graduate School of Medicine and Dentistry, Okayama University. All institutional and national guidelines for the care and use of laboratory animals were followed.

Total RNA (including microRNA) isolation from gerbil brain samples

The bilateral hippocampal CA1 region of the frozen brain was dissected and combined in a cryostat (HM 500M; Microm Inc., Mound, MN, USA) at -18°C. Total RNA containing miRNA was isolated from about 5 μg of the hippocampal CA1 region suspended in RNA-Later ICE (Ambion Inc., Austin, TX, USA) using a *mirVana*™ miRNA Isolation Kit (Ambion). The concentration, quality and integrity of total RNA were determined using a NanoDrop Spectrophotometer (ND-1000; NanoDrop Tech, Rockland, DE, USA). The ratio of OD_{260}/OD_{280}, which was >1.8 for all RNA samples, was used to ascertain the quality of RNA. RNA samples were stored at -80°C before miRNA array studies.

Gerbil microRNA sequencing

For each SC or ischemic brain sample, an equal amount of total RNA was pooled from three biological replicates to generate enough RNA (approximately 1,000 ng) for

deep sequencing (Table 1). Briefly, the isolated total RNAs of each sample were resolved on a denaturing 15% polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA) for size selection and these small RNAs (≤30 bases) were ligated to a pair of Solexa adaptors at the 5′ and 3′ ends using T4 RNA ligase. After ligation and purification, adapter-ligated small RNAs were reverse transcribed and amplified for 15 cycles (cDNA RT-PCR) with a pair of adapter complementary primers in order to produce sequencing libraries. PCR products were then purified and directly sequenced using an Illumina HiSeq2000 instrument (Illumina Inc., San Diego, USA).

MicroRNA microarray analysis

Groups of gerbils subjected to tCCAO were sacrificed at various reperfusion periods (1 d, 7 d, 1 M and 6 M; n=2 at each time point). To perform the miRNA array experiment, total RNA (2-5 μg) was size fractionated on a Microcon centrifugal filter (YM-100; Millipore, Billercia, MA, USA). To the small RNAs (<300 nucleotides), poly-A tails were added at the 3′ end mediated by poly(A) polymerase, and nucleotide tags were ligated to the poly-A tails. Each sample was hybridized to a microarray (LC Sciences, Houston, TX, USA) that contained gerbil miRNA probes. Thereafter, an oligonucleotide was ligated to the poly-A tail for later staining with fluorescent dye Cy3. Hybridization was performed overnight using μParaFlo® mcirofluidic technology. Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Device, Sunnyvale, CA, USA) and digitized using Array-Pro image analysis software (Media Cybernetics, Bethesda, MD, USA). Correlations between

fluorescence signal intensities from the laser scanner and expression levels of different miRNA sequences were generally proportional. Since fluorescence intensity was the read out of the laser scanner, fluorescence intensity data was expressed with no absolute units in the figures of this study.

Statistical analysis

All data in this study are presented as means ± SD. Data from different groups were normalized and statistically analyzed using one-way ANOVA, followed by Tukey's post hoc comparison. The normalized hybridization data of the microarrays were subjected to hierarchical cluster analysis using the Euclidian distance function. To increase the validity of the data, cross-comparison matrices were generated. For example, data from the two sham chips were cross-compared with the two tCCAO chips (at each time point) to generate a matrix of four comparisons. Statistical analyses were performed using SPSS (ver. 13.0; IBM, Armonk, NY, USA). All tests were considered statistically significant at a p-value < 0.05, as per our previous report (12).

RESULTS

miRNA expression profile in gerbil hippocampus

A total of 1067 miRNAs (LC Sciences, Houston, TX; USA) were screened in samples of the CA1 region from gerbils subjected to tCCAO. A total of 251 miRNAs were detected in samples of one or three doses of tCCAO. Since no miRNA data for

gerbils is available, the sequences of mouse and some other animal species miRNAs were used for this study (Fig. 1 A; Table 1). Each column in Fig. 1 B-E represents a single sample, and each row represents a single miRNA. Black squares represent the median level of miRNA, and green or red squares represent lower or higher levels than the median level of miRNA expression, respectively.

Hierarchical clustering of the miRNAs detected in the brain showed four distinct clusters for neurogenesis and protein expression (Fig. 1 B-E). Cluster B contained *Mus musculus* (mouse)-let-7b (mmu-let-7b), *Macaca mulatta* (rhesus monkey; mml)-miR-9, mmu-miR-10b, mmu-miR-15a, mmu-miR-15b, *Equus caballus* (horse; eca)-miR-16, mmu-miR-16, mmu-miR-20 (a, b), mmu-miR-28 (a, b), mmu-miR-29 (a, b, c), mmu-miR-34 (a, b, c), mmu-miR-124, mmu-miR-128, oan-miR-128, mmu-miR-125b, *Ornithorhynchus anatinus* (platypus; oan)-miR-125, mmu-miR-133 (a, b), mmu-miR-146b, *Gorilla gorilla gorilla* (gorilla; ggo)-miR-146b and rno-miR-146b, related to neurogenesis (18,27,29); cluster C contained mmu-let-7 (b, d, i), oan-let-7b, *Canis familiaris* (dog; cfa)-mir-24, mmu-miR-24, *Sarcophilus harrisii* (Tasmanian devil; sha)-miR-24, mmu-miR-33, mmu-miR-132, mmu-miR-143, mmu-miR-330 and mmu-miR-574, related to 43kDa transactivation response DNA-binding protein (TDP43) (9,10,24); cluster D contained mmu-miR-9, oan-miR-9, mmu-miR-12, mmu-miR-132, mmu-miR-192, mmu-miR-199 (a, b) and sha-miR-199a, related to fused in sarcoma/translocated in liposarcoma (FUS/TLS) (38); cluster E contained mmu-miR-181 (c, d), mmu-miR-200 (a, b, c) and mmu-miR-378 (a, b, c, d), related to heat shock protein 70 (HSP70) (41,49).

Target analysis of differently expressed miRNAs

Among the above four clusters, miRNA expression profiles for mmu-miR-15a-5p, sha-miR-24_L+3R+1, oan-let-7b-3p_R+2, mmu-miR-125b-5p, mmu-miR-132-5p, mmu-miR-181c-5p, and mmu-miR-378a-5p were compared in the CA1 subfield of gerbils subjected to one or three doses of 2 min-tCCAO. Heat maps showed the pattern of miRNA expression among the different experimental groups. These expressed miRNAs were used for further analyses (Fig. 2, 3, 4).

Mmu-miR-15a-5p expression for neurogenesis

The signal strength of mmu-miR-15a-5p for the hippocampal CA1 region is shown in Fig. 2. Signal strength was 820 ± 22 in the SC brains (Fig. 2, gray circle). After one dose of 2 min-tCCAO, the signal strength was slowly, but progressively induced in the CA1 region. From the baseline of the SC level, the strength of miRNA increased to 943 ± 153, 1,125 ± 196, 1,163 ± 37, and 1,373 ± 244 at 1 d, 7 d, 1 M and 6 M, respectively (Fig. 2, open diamonds). In the group of three doses of 2 min-tCCAO, the signal strength showed a similar pattern to that of the one dose, but the strength was much higher: 1,301 ± 186, 1,438 ± 311, 1,525 ± 356 and 1,386 ± 166 at 1 d, 7 d, 1 M and 6 M, respectively (*p<0.05 vs SC, **p<0.01 vs SC, Fig. 2, filled squares).

Sha-miR-24 and oan-let-7b-3p expression for TDP43

The signal strength of sha-miR-24_L+3R+1 was 7,098 ± 920 in the

hippocampal CA1 region of the SC brains (Fig. 3 B, gray circle). From the baseline level of the SC brain, the strength of miRNA increased to 9,327 ± 2,889 and 12,134 ± 240 at 1 d and 7 d, peaked at 1 M (13,790 ± 2,885) then slightly returned at 6 M (11,543 ± 1,776) after one dose of 2 min-tCCAO (Fig. 3 B, open diamonds). In the group of three doses of 2 min-tCCAO, the peak time accelerated to 1 d (13,122 ± 781), then gradually returned until 6 M. The strength of miRNA was 12,421 ± 2,319, 12,452 ± 1,159, 10,586 ± 167 at 7 d, 1 M and 6 M, respectively (*p<0.05 vs SC, **p<0.01 vs SC, Fig. 3 B, filled squares).

The signal strength of oan-let-7b-3p_R+2 was 18 ± 2 in the hippocampal CA1 region of the SC brains (Fig. 3 D, gray circle). From the baseline level of the SC brain, the strength of miRNA increased to 17 ± 1, 25 ± 16, 30 ± 18 and 46 ± 11 at 1 d, 7 d, 1 M and 6 M, respectively after one dose of 2 min-tCCAO (Fig. 3 D, open diamonds). In the group of three doses of 2 min-tCCAO, the signal strength peaked at 1 d (63 ± 18), then returned from 7 d to 6 M. The strength of miRNA was 46 ± 6, 35 ± 1 and 32 ± 6 at 7 d, 1 M and 6 M, respectively (*p<0.05 vs SC, **p<0.01 vs SC, Fig. 3 D, filled squares).

Mmu-miR-125b-5p and mmu-miR-132-5p expression for FUS/TLS

The signal strength of mmu-miR-125b-5p and mmu-miR-132-5p for the hippocampal CA1 region is shown in Fig. 3A and 3C. The signal strength of mmu-miR-125b-5p (Fig. 3A) showed a similar increasing pattern in both groups of 2 min-tCCAO at both doses. From the baseline level of the SC brain (17,508 ± 2,915, Fig.

3A, gray circle), the strength of mmu-miR-125b-5p was 18,392 ± 2,680, 25,851 ± 715, 27,273 ± 166, 22,717 ± 3,889 at 1 d, 7 d, 1 M and 6 M after one dose of 2 min-tCCAO (Fig. 3A, open diamonds), and 24,879 ± 456, 26,344 ± 152, 24,574 ± 1,301, 26,728 ± 1,236 at 1 d, 7 d, 1 M and 6 M after three doses (Fig. 3A, filled squares, $*p<0.05$ vs SC).

The signal of mmu-miR-132-5p increased and peaked at 1 d then was down-regulated from 7 d to 6 M in both groups (Fig. 3 C). From the baseline level of the SC brain (705 ± 30, Fig. 3C, gray circle), the strength of mmu-miR-132-5p was 769 ± 209, 710 ± 57, 601 ± 37, 541 ± 46 at 1 d, 7 d, 1 M and 6 M after one dose of 2 min-tCCAO (Fig. 3C, open diamonds), and 1,009 ± 152, 637 ± 64, 574 ± 71, 678 ± 176 at 1 d, 7 d, 1 M and 6 M after three doses (Fig. 3C, filled squares, $*p<0.05$ vs SC).

Mmu-miR-181c-5p and mmu-miR-378a-5p expression for HSP70

The signal strength of mmu-miR-181c-5p was 530 ± 45 in the hippocampal CA1 region of the SC brains (Fig. 4 A, gray circle). From the baseline level of the SC brain, the signal strength of miRNA increased to 2,056 ± 2,091, 2,587 ± 1,144 and 3,094 ± 144 at 1 d, 7 d and 1 M, peak at 1 M, then returned slightly at 6 M (2,479 ± 892) after one dose of 2 min-tCCAO (Fig. 4 A, open diamonds). In the group of three doses of 2 min-tCCAO, the peak time accelerated to 1 d (3,330 ± 224), then gradually returned to 3,278 ± 772, 2,675 ± 226, 1,870 ± 264 at 7 d, 1 M and 6 M, respectively ($*p<0.05$ vs SC, $**p<0.01$ vs SC, Fig. 4 A, filled squares).

The signal strength of mmu-miR-378a-5p was 56 ± 8 in the hippocampal CA1

region of the SC brains (Fig. 4 B, gray circle). From the baseline of the SC level, the strength of miRNA decreased to 56 ± 2, 48 ± 7, 42 ± 1 and 52 ± 8 at 1 d, 7 d, 1 M and 6 M, respectively after one dose of 2 min-tCCAO (Fig. 4 B, open diamonds). In the group of three doses of 2 min-tCCAO, signal strength was induced in the CA1 region with a peak time at 7 d (75 ± 23), then returned from 1 M to 6 M. The strength of miRNA was 58 ± 1, 68 ± 3 and 55 ± 0 at 1 d, 1 M and 6 M, respectively (*p<0.05 vs SC, **p<0.01 vs SC, Fig. 4 B, filled squares).

DISCUSSION

In the present study, miRNA microarray technology detected 251 miRNAs from the hippocampal CA1 region of gerbils after tCCAO. Among these miRNAs, seven miRNA expression profiles were compared in terms of ischemic tolerance in gerbils subjected to one or three doses of 2 min-tCCAO (Table, Fig. 1). Mmu-miR-15a-5p for neurogenesis showed increased expression after one dose of 2 min-tCCAO, which was much higher in the three-dose group (Fig. 2). Time-dependent increases of sha-miR-24 and oan-let-7b-3p for TDP43 were observed after one dose of 2 min-tCCAO, but the peak time accelerated to an earlier period of reperfusion after three doses (Fig. 3B, D). In contrast, mmu-miR-125b-5p and mmu-miR-132-5p for FUS/TLS showed a similar increase in both groups after one or three doses (Fig. 3A, C). Mmu-miR-181c-5p and mmu-miR-378a-5p for HSP70 also showed accelerated expression after three doses (Fig. 4).

Mongolian gerbils are particularly susceptible to bilateral carotid occlusion,

which results in transient global brain ischemia, due to an incomplete circle of Willis (15). We already reported typical cresyl violet staining of the hippocampal CA1 region in our previous paper (48). Moreover, hippocampal CA1 cells are the most vulnerable region in cerebral ischemia (17). CA1 pyramidal neurons both in experimental animals and in humans undergo selective cell death after transient ischemia (8). No change in cell density of the CA1 pyramidal neurons was observed between the SC group and the gerbil group subjected to a single 2-min tCCAO from 1 day to 6 months later. However, in the gerbil group subjected to 2-min tCCAO three times, neuronal loss was observed in the CA1 region. Although most hippocampal CA1 neurons survived at 1 day after 3×2-min tCCAO, large numbers of CA1 neurons were gradually lost from 3 to 7 days. After the significant loss at 7 days, CA1 cell density gradually recovered from 1 to 6 months (48). Although single 2 min-ischemia depletes high-energy phosphate compounds and perturbs protein synthesis, it has not been reported to cause neuronal death. Therefore, 2 min-ischemia was selected as mild ischemic treatment for subsequent neuroprotection (36). In the gerbils' hippocampal CA1 region, 2 min-ischemia for ischemic preconditioning is extremely effective in providing protection against cell death.

Our previous study showed that ischemic preconditioning offers a way to induce endogenous neuroprotection and neurogenesis in gerbils (48). Changes in TDP43, FUS/TLS and HSP70 proteins were involved in this function. Significant changes to TDP43 and FUS/TLS were observed in the ischemic brains. The colocalization of TDP43 with markers for RNA transport and stress granules suggests that TDP43 may

play a role in sequestering and regulating the stability of mRNAs necessary for the neuronal response to stress or injury (37). TDP43 and FUS/TLS are both DNA/RNA-binding proteins involved in transcriptional regulation, mRNA splicing, transport, and translation (39). Previously, we showed that ischemic preconditioning accelerated HSP70 gene expression at the transcriptional level, ameliorated the translational disturbance of mRNA to protein, and saved CA1 cells from subsequent lethal ischemia (2). Molecular mechanisms indicate how ischemic preconditioning alters the balance between cell-survival and cell-detrimental signaling pathways.

Transient cerebral ischemia reprograms the response of nerve cells for subsequent ischemia. A previous report suggested that neuroprotection by a short period of transient ischemia may depend on altered microRNA expression (46). Ischemic tolerance can be obtained by a large change in gene expression, the functions of which influence protein turnover, cell-cycle regulation, and ion-channel abundance. For this tolerance, an endogenous genomic program may be present, serving as an adaptation to improve survival against oxygen limitation (14). The microarray findings in this study suggest that miRNAs are effectors of ischemic tolerance (Fig. 1).

The present study shows that transient forebrain ischemia induces a gradual increase of miR-15a neurogenesis in the hippocampus (Fig. 2). We previously found that three repetitions of 2-min ischemia caused neuronal damage in selectively vulnerable hippocampal CA1 neurons, but exhibited a significant recovery after 7 d to 6 M (48). In the present study, miR-15a showed an increase up to 6 M after one dose

of 2 min-tCCAO, but the increase was much greater in the three-dose group (Fig. 2, filled squares). As was reported in cancer cells (11,35), miRNA-15 may promote neuronal survival by regulating neurogenesis and the apoptosis pathway in this tCCAO model.

Related to the miRNA pathway, TDP43 and FUS/TLS are associated with frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS) (33,38). Let-7b miRNA expression was down-regulated following TDP43 knockdown in culture cells (10), and the expression of some miRNAs decreased or increased in TDP43 mutant flies (18). MiR-24-1* was lower in a PGRN (+, [pro]granulin) FTLD TDP patient than in a PGRN (-) FTLD TDP patient (24). FUS affects the biogenesis of miRNAs for neuronal function, differentiation and synaptogenesis with miR-125b and miR-132 (16,26,42). The present study showed that the expression of miR-24 and let-7b for TDP43 accelerated to an earlier period of reperfusion after three doses (Fig. 3B, D), but the expression of mmu-miR-125b-5p and mmu-miR-132-5p for FUS/TLS did not show any evident acceleration (Fig. 3A, C). We previously found that the redistribution of both TDP43 and FUS/TLS immunoreactivity to cytoplasm was accelerated to an earlier period of reperfusion after three doses of 2 min-tCCAO from 3 M to 7 d or from 7 d to 1 d, respectively (48). Thus, the remarkable acceleration of TDP43 may be well correlated to the present expression data of miR-24 and let-7b for TDP43 (Fig. 3B, D).

HSP70 is a major stress protein which is strongly induced under stresses including cerebral ischemia, heat, and metal toxicity (1,3,5). MiR-181 was identified

as a regulator of several HSP70 family members, and an increased level of miR-181a is associated with a decrease in endoplasmic reticulum GRP78 protein related to cerebral ischemic injury (41). Cardiac ischemic preconditioning of mice decreased HSP70 related to miR-378* microRNAs (49). Our previous immunohistochemical finding that HSP70 was induced only at 7 d after three doses of 2 min-tCCAO is well correlated to our present data of mmu-miR-181c-5p and mmu-miR-378-5p, which accelerated at 1-7 d (Fig. 4) (48).

In summary, the present study shows the profiling of miRNAs for ischemic preconditioning after tCCAO in gerbil, and identifies distinct regulation patterns for seven miRNA clusters. This data could provide new perspectives of miRNA expression proteins for neuroprotection, TDP43, FUS/TLS and HSP70, not only in transient cerebral ischemia, but also in ischemic preconditioning. These results may be useful for pursuing further studies on the possible use of miRNAs as biomarkers in cerebral ischemia and neuroregeneration.

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