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BASIC SCIENCE ARTICLE

Temporal brain microRNA expression changes in a mouse model of neonatal hypoxic–ischemic injury

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BACKGROUND: Neonatal hypoxic–ischemic brain injury (HIBI) results in significant morbidity and mortality despite current standard therapies. MicroRNAs (miRNAs) are a promising therapeutic target; however, there is a paucity of data on endogenous miRNA expression of the brain after HIBI during the primary therapeutic window (6–72 h after injury).
METHODS: Postnatal day 9 mouse pups underwent unilateral carotid ligation+hypoxia (HIBI), sham surgery+hypoxia, or sham surgery+normoxia (controls). miRNA sequencing was performed on the ipsilateral brain of each of the three groups plus the contralateral HIBI brain at 24 and 72 h after injury. Findings were validated in eight key miRNAs by quantitative polymerase chain reaction.
RESULTS: Hypoxia resulted in significant differential expression of 38 miRNAs at both time points. Mir-2137, -335, -137, and -376c were significantly altered by neonatal HIBI at 24 and 72 h, with 3 of the 4 demonstrating multiphasic expression (different direction of differential expression at 24 versus 72 h).
CONCLUSIONS: Our global assessment of subacute changes in brain miRNA expression after hypoxia or HIBI will advance research into targeted miRNA-based interventions. It will be important to consider the multiphasic miRNA expression patterns after HIBI to identify optimal timing for individual interventions.

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IMPACT:

- This study is the first to comprehensively define endogenous brain microRNA expression changes outside of the first hours after neonatal hypoxic–ischemic brain injury (HIBI).
- Mir-2137, -335, -137, and -376c were significantly altered by neonatal HIBI and therefore deserve further investigation as possible therapeutic targets.
- The expression profiles described will support the design of future studies attempting to develop miRNA-based interventions for infants with HIBI.
- At 24 h after injury, contralateral HIBI miRNA expression patterns were more similar to ipsilateral HIBI than to controls, suggesting that the contralateral brain is not an appropriate "internal control" for miRNA studies in this model.

INTRODUCTION

Neonatal hypoxic–ischemic encephalopathy—the clinical phenotype resulting from perinatal hypoxic–ischemic brain injury (HIBI)—is estimated to affect on average 8 out of every 1000 live births worldwide, with some regions demonstrating rates as high as 15 per 1000 live births.[1] Therapeutic hypothermia for the first 3 days after injury decreases mortality and neurodevelopmental impairment in survivors but has not been shown to be beneficial in low-resource settings and is only partially effective in higher-resource settings.[2,3] Despite widespread utilization of therapeutic hypothermia in many countries, more than half of infants with moderate-to-severe injury die or develop severe neurodevelopmental disability.[3] Because of this, there is an urgent need for the development of targeted therapies to supplement therapeutic hypothermia in these high-risk infants.

MicroRNAs (miRNAs) are small, non-coding RNAs that are thought to play a significant role in modulating the neuroinflammation after HIBI and therefore may act as effective targets for therapeutic intervention.[4] Much of the literature to date regarding miRNAs in neonatal HIBI has focused on a class of miRNAs known as hypoxamiRs. HypoxamiRs, including not only the commonly studied miR-21 and -210 but also miR-335, -137, and -376c, are miRNAs that are not only regulated by hypoxia but also in turn regulate cell response to decreased oxygen.[5] These hypoxamiRs have been shown to play a significant role in several pathological states, from cardiac injury to cancer,[6,7] and altering miRNA levels in the brain may

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provide neuroprotection after HIBI.[8–10] Due to their small size (21–25 nucleotides in length), miRNAs are more likely to cross the blood–brain barrier than other larger molecules currently being tested as therapeutics.[11] As such, miRNA-based interventions may be able to provide targeted therapy to supplement hypothermia in neonates suffering after HIBI, but the question of which miRNA(s) to target remains a significant hurdle in the path toward developing successful miRNA-based therapies.

As most of the previous studies assessing endogenous miRNA expression after HIBI were seeking to use miRNA expression as an early biomarker of injury, rather than assessing for therapeutic targets, they have primarily used peripheral blood for miRNA measurements.[12–15] The circulating miRNA levels, however, are unlikely to adequately reflect the brain microenvironment given that the peripheral blood contains miRNAs from every organ, not just the brain, and those results should therefore be used with caution when attempting to identify investigating therapeutic targets for neuroprotection. Additionally, despite a recent study showing altered gene expression in school age children who suffered neonatal HIBI,[16] previous miRNA studies in neonatal HIBI have mainly focused on changes in the seconds (cord blood) or first few hours after injury.[12–15] The expression pattern of miRNAs changes rapidly in the first minutes to hours after neonatal HIBI,[12,13] and clinically neonatal encephalopathy is often not diagnosed within the first hour after injury. As such, miRNA expression will likely have shifted significantly from birth by the time that therapy is initiated. The miRNA expression patterns in cord blood or immediately after injury in animal models, therefore, has limited utility in the development of miRNA-targeted interventions that would most likely be administered over the course of hypothermia (approximately 2–72 h after injury). Although some studies have profiled the expression of a few selected brain miRNAs outside of the first few hours after neonatal HIBI, the global profile of miRNA expression changes 24–72 h after neonatal HIBI has not yet been established.

Designing interventions to effectively target miRNAs after neonatal HIBI will require a greater knowledge of the subacute miRNA expression changes that occur after injury. To that aim, the goal of the current project was to assess changes in brain-specific miRNA expression at 24 and 72 h after injury in a mouse model of neonatal HIBI. In order to provide a comprehensive miRNA profile, we used next-generation miRNA sequencing (miRNA-Seq), validated by quantitative polymerase chain reaction (qPCR). The results of this study will provide important differential expression information necessary to design future studies targeting miRNA expression in the first 72 h after neonatal hypoxic–ischemic encephalopathy.

METHODS

This study was approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

HIBI model

Timed pregnant CD1 mouse dams were obtained from Charles River Laboratory (Wilmington, MA). After delivery, pups were maintained in a 12-h light and 12-h dark environment with the dam and littermates. At postnatal day 9, pups of both sexes were randomized to three groups of at least 12 pups each. The HIBI group was anesthetized with 2.5% isoflurane and underwent right carotid artery dissection and ligation[17] followed by 2 h of recovery and then 30 min at 8% oxygen in a hypoxia chamber (BioSpherix, Parish, NY). The control group underwent the same anesthesia but a sham surgery of carotid artery dissection without vessel ligation. After recovery, the control group was separated from the dam in a warm normoxic environment for 30 min to maintain consistency with the HIBI group procedures. Lastly, the hypoxia only group underwent sham surgery followed by 2 h of recovery and then 30 min at 8% oxygen. Surgeries took no longer than 5 min, and the animals were maintained at normothermia throughout the surgery, recovery, and hypoxia/normoxia using warming pads.

Half of the pups in each group were sacrificed at 24 h and the other half at 72 h after injury. The whole brain was extracted as quickly as possible, and the hemispheres separated. Each hemisphere was cut into 1 mm coronal sections and then submerged in RNAlater Stabilizing Reagent (Invitrogen, Carlsbad, CA) until RNA extraction. For the hypoxia and control groups, the right hemisphere was used for analyses. For HIBI pups, ipsilateral (right) and contralateral (left) hemispheres were processed separately.

RNA extraction

Between 15 and 20 mg of tissue at a time was transferred into a dounce homogenizer with Qiazol Lysis Reagent (Qiagen, Hilden, Germany). The tissue was fully homogenized, and the RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen) per the manufacturer instructions. RNA concentration was determined through spectrophotometry (DeNovix DS-11, Wilmington, DE). Only samples with ratios of 260/280 nm absorbance >1.9 were used for analysis.

RNA sequencing and analysis

Three samples from each group underwent quality confirmation through parallel capillary electrophoresis on a Fragment Analyzer (Agilent, Santa Clara, CA) and then were processed for miRNA-Seq. MiRNA-Seq libraries were prepared from 200 ng RNA per sample with the NEXTFLEX Small RNA Kit (PerkinElmer, Waltham, MA) per the manufacturer's instructions. The libraries were then sequenced on the Illumina NextSeq 550 platform (San Diego, CA).

Sequencing results were aligned to mature mouse miRNA from miRBase using bowtie2 alignment, and differential expression analysis was performed using EdgeR. Two samples with alignment <25% were excluded and replaced with new samples with alignment >25%. A false discovery rate <0.05 or p value <0.05 for this exploratory study were considered significant. Conservation between mammalian species for each of the miRNAs was assessed using microRNAviewer.[18] MiRNAs that demonstrated >90% sequence homology between species were considered to be "highly conserved".

xFor validation of miRNA-Seq findings, we performed qPCR on eight of the miRNAs that were highly differentially expressed and/or were previously reported to have biological relevance in neonatal HIBI (mmu-miR-1195, mmu-miR-137-3p, mmu-miR-155-5p, mmu-miR-2137, mmu-miR-335-5p, mmu-miR-376c-3p, mmu-miR-6240, and mmu-miR-665-3p). We performed qPCR on six biological replicates per group. miRCURY LNA Reverse Transcription Kit (Qiagen) was used to generate cDNA from 200 ng of RNA. We performed qPCR using the miRCURY LNA SYBR Green qPCR Kit (Qiagen) with manufacturer-generated primers for each of the target mature miRNAs (specific sequences listed in Supplemental Table 1). Values were normalized to U6 as an endogenous control. All qPCR samples were run in triplicate. Cycle threshold (Ct) values were recorded, and expression levels were calculated by the ΔΔCt method.[19] Expression fold change in the HIBI group compared to controls was reported after $\log_2$ transformation.

Clustering analyses

To assess expression pattern relationships between controls, ipsilateral HIBI, and contralateral HIBI, we used a stepwise approach combining significance and magnitude of change approach. First, we filtered out miRNA species with an average raw count of <10, as these low expressors tend to give rise to more unreliable data. Then we calculated counts per million (CPM) for each sample and $\log_2$ transformed the CPM to establish differential expression for ipsilateral–control, ipsilateral–contralateral, and contralateral–control comparisons. For this analysis, differentially expressed miRNA species were defined by an uncorrected p value of 0.05 and a magnitude change of 30% in at least one of the three comparisons. The data used for clustering can be found in Supplemental Tables 2 and 3. These data underwent unsupervised two-way (gene versus sample) hierarchical clustering based on Euclidian distance using the Morpheus program (Broad Institute, Cambridge, MA). This procedure was separately performed for samples obtained at 24 or 72 h.

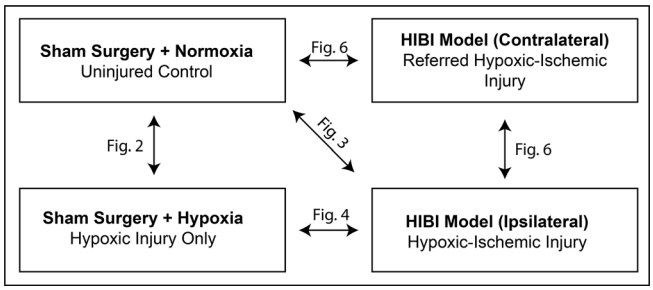

Fig. 1 Group comparisons made throughout this study, with references to the relevant figures that contain each of the comparisons. Hypoxic–ischemic brain injury (HIBI) model consists of unilateral carotid artery ligation with 30 min of hypoxia at 8% oxygen. Sham surgery includes dissection and visualization of carotid artery but no ligation. The hypoxia only group received sham surgery followed by 8% oxygen for 30 min.

RESULTS

Analyses were performed between four different groups (Fig. 1): normoxia controls, hypoxia only, ipsilateral HIBI, and contralateral HIBI. Figure 2 demonstrates the results of the first comparison: those miRNAs with significant differential expression in hypoxia only compared to normoxia controls. They include 51 miRNAs at 24 h (Fig. 2b), 129 at 72 h (Fig. 2c), and 38 with significant differential expression at both time points (Fig. 2d); 33 of which are highly conserved between mammalian species. Notably, all miRNAs that were differentially expressed at both time points after hypoxia only were expressed in the same direction (positive or negative) at both time points.

A similar analysis was performed between the ipsilateral (lesioned) HIBI samples and the normoxia controls (Fig. 3). Figure 3a demonstrates the number of miRNAs with a significant p value, with associated $\log_2$ fold change. The analyses demonstrated 16 miRNAs with significant differential expression at 24 h after HIBI (Fig. 3b) and 26 at 72 h after HIBI (Fig. 3c). Five of these miRNAs had significant altered expression at both 24 and 72 h: miR-137-3p, -2137, -335-5p, -376c-3p, and -5126 (Fig. 3d). The conservation of miR-5126 between mammalian species has not been well described; however, the other four miRNAs are highly conserved. Of note, miR-2137 and -5126 maintained similar relative expression directionality at both 24 and 72 h after injury (both were upregulated at both time points after HIBI). MiR-335-5p, -137-3p, and 376c-3p demonstrated multiphasic expression, with all three being upregulated after ipsilateral HIBI at 24 h after injury but downregulated at 72 h.

To attempt to differentiate the effects of hypoxia versus ischemia, Fig. 4 demonstrates the miRNAs with significant differential expression at 24 and 72 h after HIBI compared to the differential expression in hypoxia only at each of the time points. The five miRNAs that were differentially expressed after HIBI at both time points are listed at the top of the figure (within the dashed box) for ease of comparison. With only a few exceptions, the direction of expression after hypoxia only and HIBI were similar between the two comparisons at 24 h (Fig. 4a) but were more often inverse to each other at 72 h (Fig. 4b). Figure 4c is a Venn diagram demonstrating the overlap miRNAs with significant differential expression for each of the hypoxia only and HIBI groups at each time point.

Each of the three miRNA-Seq samples also had qPCR performed. Figure 5 demonstrates strong correlation ($R^2 = 0.634$) between qPCR $\log_2(\Delta\Delta Ct)$ fold change and miRNA-Seq fold change for each of those samples. MiR-665 was an outlier for many of the correlated values and post hoc exclusion of miR-665 values improved the R^2 to 0.743. In addition to running qPCR on the miRNA-Seq samples, three additional brains per group were used for qPCR, for a total of 6 samples per group. Supplemental Fig. 1 summarizes the total ΔΔCt fold change qPCR results for all six biological replicates in each of the analyzed miRNAs, compared to the miRNA-Seq fold change values.

To aid in understanding the pathways affected by the upregulated miRNAs in this study, we performed a literature search to identify the most commonly identified mRNAs involved in neonatal HIBI. We identified 63 such mRNAs (listed in Supplemental Table 4).[20–25] A list of miRNAs known to be associated with each of the mRNAs was obtained from miRTarBase and then compared to the list of miRNAs with significant differential expression from our study. Table 1 lists those differentially expressed miRNAs from our study that were associated with one or more of the identified mRNAs, as well as the experimental group and time point at which that miRNA demonstrated significant altered expression.

Lastly, as contralateral brain tissue has often been proposed as a valid internal control for unilateral HIBI models, we investigated the pattern of differential miRNA expression in the normoxia controls, ipsilateral HIBI, and contralateral HIBI groups using two-way, unsupervised hierarchical clustering. Figure 6 demonstrates the clustering results. These analyses revealed that the pattern of 107 miRNAs differentially expressed at 24 h strongly separated the three experimental groups (vertical dendrogram in Fig. 6a), with the normoxia control group showing the most distinct separation. It is notable that the miRNA expression in the contralateral hemisphere was closer to the patterns seen in ipsilateral HIBI than to the normoxia controls at 24 h. In contrast, at 72 h (Fig. 6b), the contralateral HIBI samples appeared to demonstrate recovery, showing an expression pattern with greater similarity to the miRNA profile in the normoxia controls. At 72 h, a strong miRNA lesion signature started to emerge in the ipsilateral tissue, providing greater separation from the other two groups. Comparisons between the miRNA expression in the contralateral HIBI samples versus controls at 24 h after injury are also shown in Supplemental Fig. 2. Between the contralateral HIBI and control groups, there were no miRNAs with significant differential expression at 72 h after injury.

DISCUSSION

The results from this study provide a comprehensive assessment of the subacute brain miRNA expression changes at 24 and 72 h after hypoxia or HIBI, resulting in several novel conclusions: (1) hypoxia alone results in significantly altered expression at both 24 and 72 h of several miRNAs, many of which are hypoxamiRs; (2) HIBI resulted in much more phase-specific expression, with few miRNAs demonstrating significant differential expression at both time points, and most of those demonstrating inverse differential expression at 24 h compared to 72 h; and (3) at 24 h, most miRNAs with significant differential expression had differential expression in HIBI similar in direction (positive or negative) to hypoxia, but at 72 h HIBI miRNAs tended to have inverse directionality to hypoxia. Lastly, we investigated the relationships between the miRNA expression in ipsilateral HIBI versus contralateral HIBI versus controls and found that the ipsilateral-injured brain was more similar to controls than was the contralateral brain at 24 h, though the contralateral brain became more similar to controls than ipsilateral brain by 72 h.

Given the significant role of hypoxamiRs in regulating the cell response to hypoxia, it is not surprising that many of the known hypoxamiRs (including miR-135a, -34a, -21a, -369, -128, and -92)[26,27] demonstrated significant differential expression in the hypoxia only group versus controls in our study. The direction of differential expression was consistent between 24 and 72 h after hypoxic injury (as compared to HIBI where many of the miRNAs showed inverse differential expression at 24 versus 72 h). Although there are very few time-series studies assessing

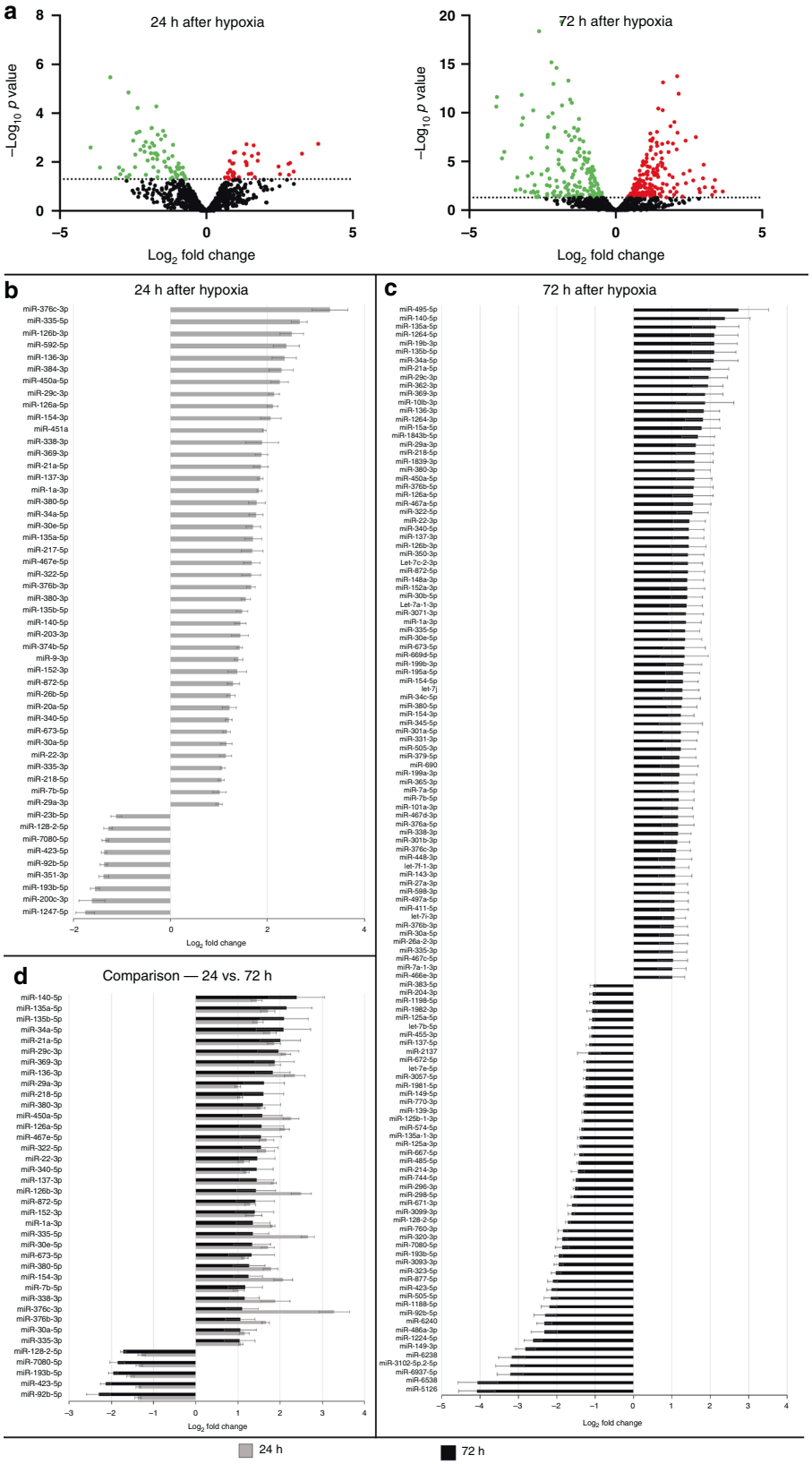

Fig. 2 MicroRNAs with significant differential expression in hypoxia only injury compared to uninjured controls. a Volcano plots demonstrate total number of microRNAs with significant *p* values (above dashed line) and corresponding $\log_2$ fold change. The microRNAs with significant differential expression (defined by $\log_2$ fold change >1, *p* value and/or false discovery rate <0.05, and average count per million reads >10) are shown, stratified by their altered expression at **b** 24 h, **c** 72 h, and **d** both.

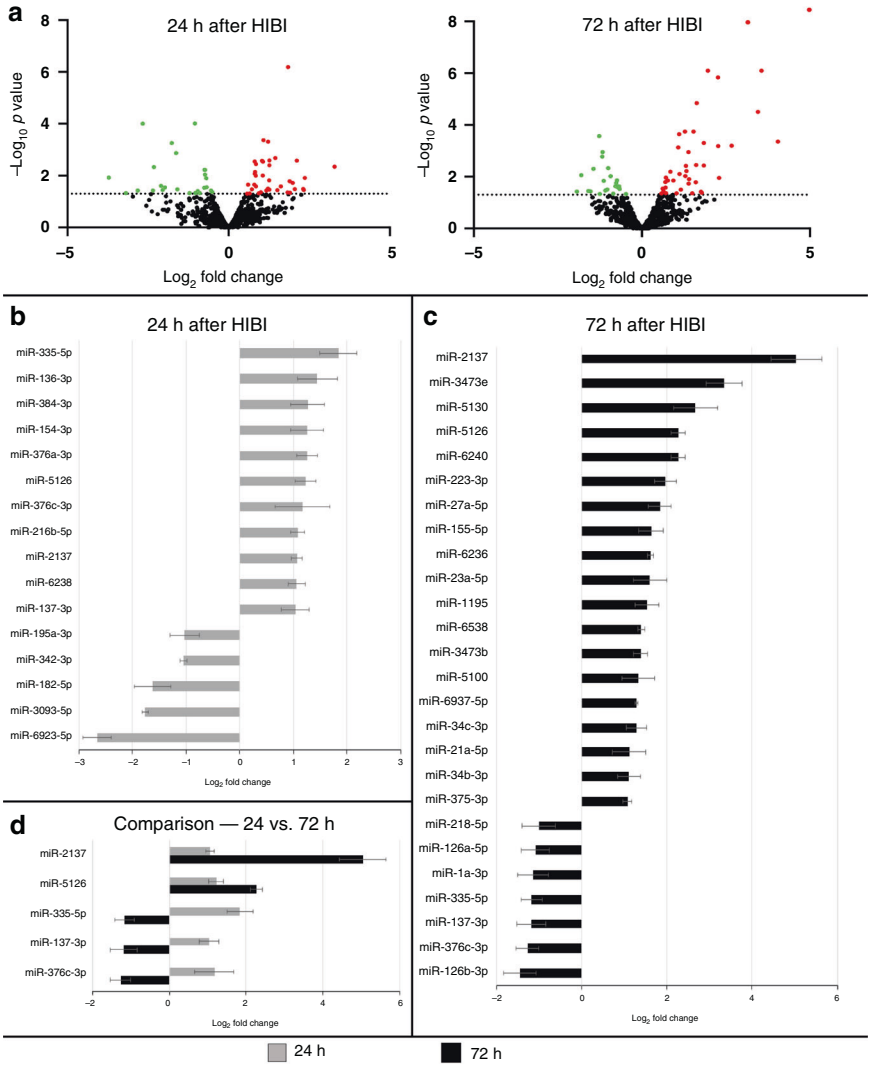

Fig. 3 MicroRNAs with significant differential expression in ipsilateral hypoxic–ischemic brain injury (HIBI) compared to uninjured controls. a Volcano plots demonstrate total number of microRNAs with significant *p* values (above dashed line) and corresponding $\log_2$ fold change. The microRNAs with significant differential expression (defined by $\log_2$ fold change >1, *p* value and/or false discovery rate < 0.05, and average count per million reads >10) are shown, stratified by their altered expression at **b** 24 h, **c** 72 h, and **d** both.

temporal changes in miRNA expression after hypoxia, those that have been published demonstrate similarly persistent expression of hypoxamiRs up to 48 h after hypoxia.[28,29] In our study, however, the primary value of assessing miRNA expression after hypoxia is in comparison with the miRNA expression after HIBI in order to better understand the contribution of hypoxia versus ischemia in driving miRNA expression.

In the HIBI tissue, four highly conserved miRNAs demonstrated significant differential expression at both 24 and 72 h after injury: miR-2137, -335, -137, and -376c. Of these, miR-2137 was the only HIBI-specific miRNA that was upregulated at both 24 and 72 h after injury. MiR-2137 is involved in inflammation, has been associated with elevated levels of tumor necrosis factor-α (TNF-α), and its inhibition has been shown to increase the anti-inflammatory interleukin (IL)-10 in oral inflammation.[30] Increased miR-2137 expression has also been shown in the penumbral tissue after traumatic brain injury,[31] and a previous study demonstrated upregulation of miR-2137 up to 7 days after injury in an adult ischemic stroke model.[32] Contrary to our findings, however, the post-stroke data showed a decrease in miR-2137 differential expression between 1 and 3 days after injury while ours demonstrated an increase. It is notable that miR-2137 is the only of the four key miRNAs identified in HIBI in our study that has not yet been described as a hypoxamiR, and its differential expression after HIBI was inverse to that of the hypoxia only group at both 24 and 72 h, suggesting a unique association with ischemia rather than hypoxia.

Neonatal HIBI is a triphasic injury.[33] The secondary phase occurs between 12 h and a few days after injury and is characterized by deteriorating mitochondrial function and an acute inflammatory response. After the secondary phase is the tertiary phase that brings partial recovery from the injury but is characterized by continued inflammation and gliosis. Similarly, miRNA expression also varies over time after ischemic injury. Thought to be related to the multiphasic nature of hypoxia-inducible factor (HIF)-1α expression after ischemia, several hypoxamiRs have been shown to have multiple phases of expression throughout the first 72 h after injury. For instance, miR-335 was shown to be down-regulated in the first hours after middle cerebral artery occlusion, followed by significant upregulation around 24 h and then downregulation again after 24 h; an expression pattern inverse to that of HIF-1α.[10]

Consistent with the triphasic pattern seen in the pathophysiology of neonatal hypoxic–ischemic encephalopathy, we

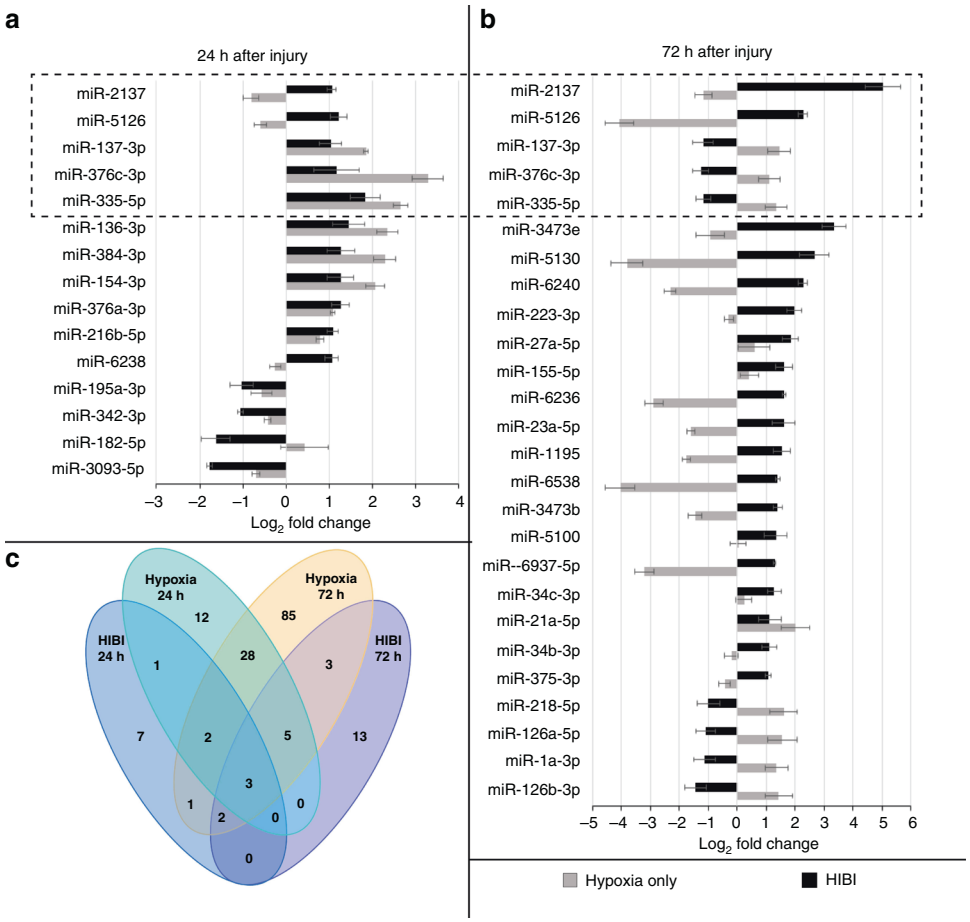

Fig. 4 Comparing MicroRNA Differential Expression After Hypoxia Only Verus Hypoxic-Ischemic Brain Injury. MicroRNAs with significant differential expression in ipsilateral hypoxic–ischemic brain injury (HIBI) compared to controls (black bars), with associated differential expression of the same miRNAs in hypoxia only compared to controls (gray bars) at **a** 24 h or **b** 72 h. The five microRNAs with significant differential expression at both time points are enclosed in the dashed box. Significance defined by HIBI versus control comparison with $\log_2$ fold change >1, *p* value and/or false discovery rate <0.05, and average count per million reads >10. A Venn diagram in **c** shows the number of microRNAs with significant differential expression under each condition and time point.

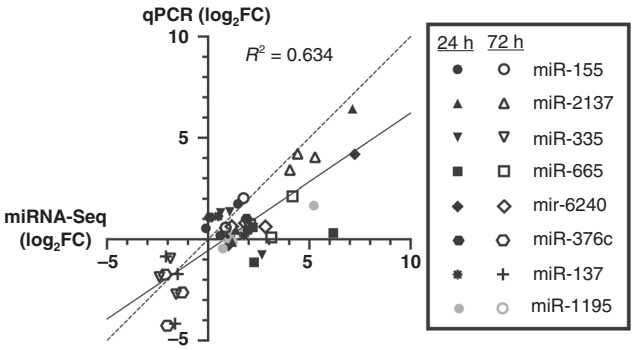

Fig. 5 Correlation between next-generation microRNA sequencing (miRNA-Seq) and quantitative polymerase chain reaction (qPCR) validation of eight highly expressed and/or physiologically relevant microRNAs. The *x*-axis values are miRNA-Seq $\log_2$ fold change ($\log_2$FC, ipsilateral hypoxic–ischemic brain injury versus control) values and *y*-axis are qPCR ΔΔCt $\log_2$FC values. Filled shapes are from the samples obtained at 24 h and the open shapes are from the samples obtained at 72 h. The solid line represents the best-fit regression of the data points with strong correlation (R^2 = 0.634) and the dashed line represents the ideal correlation line ($x = y$). The top right and lower left quadrants contain the majority of samples, which all demonstrated concordant $\log_2$FC directionality between miRNA-Seq and qPCR, while the top left and lower right quadrants show the few samples with discordant directionality.

demonstrated multiphasic differential expression in several of the key HIBI-related miRNAs, including miR-335. MiR-335 is a hypoxamiR, regulates cholesterol metabolism,[34] and acts as a direct regulator of HIF-1α, as well as being predicted to regulate several other mRNAs known to be affected by neonatal HIBI (Table 1). MiR-335 has been shown to be downregulated in the cord blood of infants who suffered moderate-to-severe HIBI.[13] In the adult stroke model, miR-335 has been shown to have a triphasic expression pattern: downregulated immediately after middle cerebral artery occlusion, peaked upregulation around 24 h, and then downregulation again after 24 h. Reflecting this pattern, miR-335 mimic has been shown to be neuroprotective when administered immediately after injury, when endogenous miR-355 expression is lowest, but miR-335 antagonist was neuroprotective when administered at 24 h after injury, corresponding to increased endogenous expression.[10] Our data showed similar trends, with upregulation at 24 h after HIBI but downregulation by 72 h.

MiR-137 is another hypoxamiR that has also been associated with the inflammatory response after cerebral ischemia.[35] miR-137 may also play a significant role in brain development and neural stem cell differentiation,[36] with decreased levels of miR-137 during brain development being associated with increased anxiety-like behaviors later in life.[37] Similar to the pattern that we observed in miR-335, our results showed upregulation of miR-137 at 24 h after hypoxia–ischemia (HI) but downregulation at 72 h. Although the

Table 1. Differentially expressed microRNAs (miRNA) associated with one or more hypoxic–ischemic brain injury (HIBI)-associated messenger RNAs (mRNA) identified by literature search.

miRNA	Differential expression				Predicted mRNA targets
	Hypoxia		HIBI		
	24 h	72 h	24 h	72 h	
miR-1a-3p	↑	↑		↓	Nfat5
miR-101a-3p		↑			Dusp1
miR-1195				↑	Eno2, Nfat5
miR-135b-5p	↑	↑			Il1r1
miR-140-5p	↑	↑			Adam9
miR-149-3p		↓			Nfat5
miR-15a-5p		↑			Hspa12a
miR-155-5p				↑	Anxa2, Il13ra1, Junb, Nes, Nfat5, Tnf
miR-19b-3p		↑			Npy2r
miR-193b-5p	↓	↓			Hspb8
miR-200c-3p	↓				Dusp1
miR-218-5p	↑	↑		↓	Anxa2, Clcf1, Ddx58, Eno2, Nrp1, Uchl1
miR-223-3p				↑	F3, Tnf
miR-23a-5p		↓		↑	Casp7
miR-29a-3p	↑	↑			Casp8, S100b
miR-29c-3p	↑	↑			Casp8, Fzd4
miR-296-3p		↓			Tnf
miR-298-5p		↓			Ddx58, Tnf
miR-30e-5p	↑	↑			Adam9, Adrb2, Csf1, Dap, Eno2, Nfat5
miR-335-5p	↑	↑	↑	↓	Csf1, Dap, Dusp5, F3, Gfap, Grik4, Nfat5, Nrp1, Spp1, Tlr1
miR-338-3p	↑	↑			Nrp1
miR-34a-5p	↑	↑			Anxa4, Bag3, Casp6, Casp7, Casp8, Cybb, Tnf
miR-340-5p	↑	↑		↓	Cyp1b1, Spp1
miR-362-3p		↑			Adam9, Atg10, Ctsl, Grin2a, Nfat5, Scg2, Tmbim1
miR-375-3p				↑	Ctsc, F3, Fzd4, Procr
miR-376b-5p	↑	↑			Casp8
miR-669d-5p		↑			Eno2
miR-744-5p		↓			Nfat5
miR-877-5p		↓			Nfat5, Uchl1
miR-93-3p	↑				Eno2

The direction of differential expression for each group, relative to controls, at each time point (24 or 72 h after injury) are designated by up (upregulation in HIBI versus controls) or down (downregulation in HIBI versus controls) arrows.

multiphasic expression is not as well described as that of miR-335, it is likely that miR-137 also has a triphasic pattern, since overexpression of miR-137 at 30 min after cerebral ischemia attenuated brain levels of Janus-activated kinase 1, signal transducer and activator of transcription factor 1, TNF-α, IL-1β, and IL-6 and resulted in decreased infarct size and neurological function score, suggesting that levels were low or downregulated early after ischemia.[35]

In addition to miR-335, miR-376c was also seen in previous studies of cord blood after perinatal asphyxia or neonatal hypoxic–ischemic encephalopathy.[14,15] The relative downregulation of miR-376c demonstrated in the cord blood studies, in conjunction with relative upregulation at 24 h and downregulation at 72 h after HI injury in our study, suggests that miR-376c also may have a triphasic response. This is further supported by an in vitro study demonstrating that overexpression of miR-376c-3p at the time of oxygen–glucose deprivation increased cell viability and decreased apoptosis by inhibiting ING5 in two neuroblast cell lines.[38]

Lastly, our clustering analyses revealed distinct miRNA signatures between the sample groups, which were quite different at 24 and 72 h. They suggested that the early subacute phase of the injury (at 24 h) continues to evolve and change, giving rise to a less extensive but more robust miRNA profile in HIBI animals at 72 h. In addition, our data suggest that using the contralateral hemisphere of the lesioned animals as a baseline control in the first 24 h after HIBI is perhaps less than ideal: the unilateral lesion appears to have a strong effect on the miRNA expression level of the whole brain, and not only on the lesioned side.

This study's limitations included a modest sample size. Due to the exploratory and descriptive nature of the study, we used the minimal number of samples for miRNA-Seq that would provide for valid statistical analyses. In order to help overcome the lower sample size for miRNA-Seq, however, we performed qPCR as a validation for several of the key miRNAs. Due to the modest sample size, we were unable to assess for differences in expression between sexes, which may affect miRNA expression.[39] Additionally, given our descriptive methods, we are not able to assess whether the alterations in miRNA expression that were demonstrated were harmful and part of the pathophysiology of the HIBI

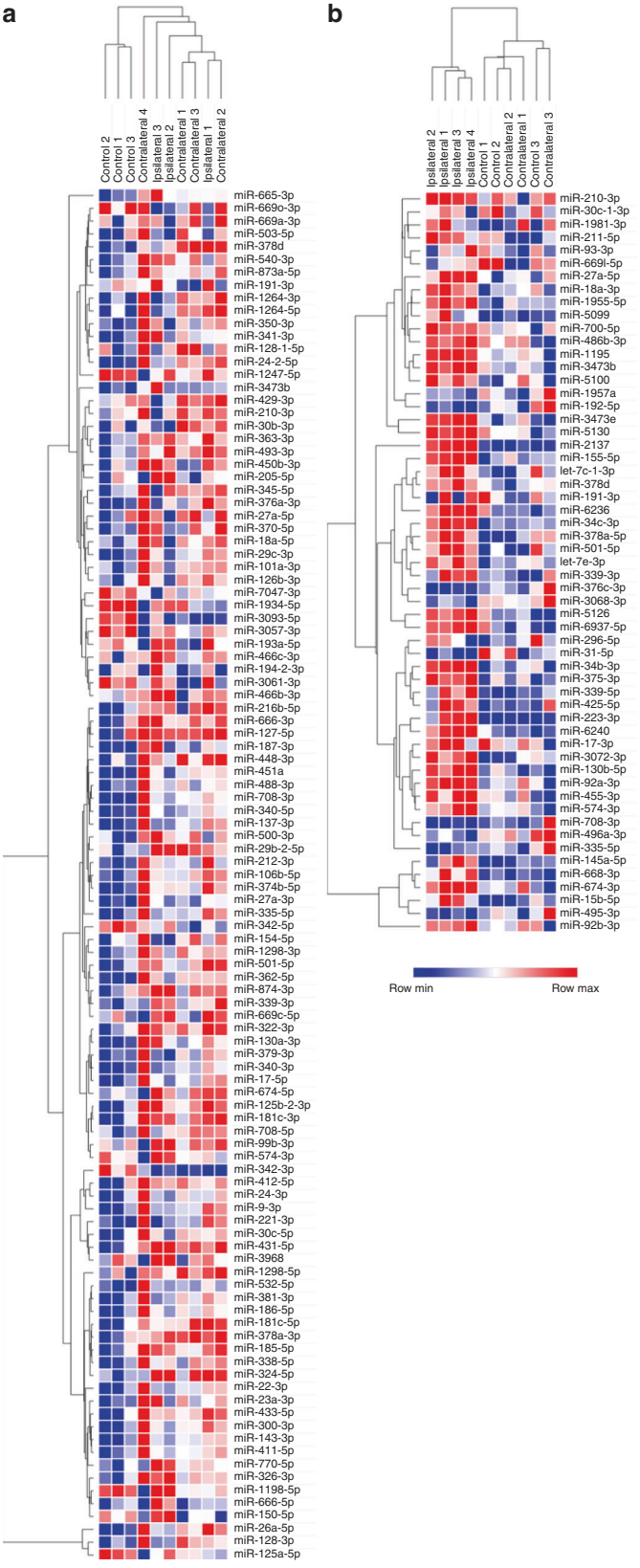

Fig. 6 Two-way unsupervised clustering of differentially expressed microRNAs at 24 and 72 h. Rows represent microRNA species and columns represent samples. Each square represents an expression of a single microRNA species in a single sample, color-coded for magnitude of change relative to other samples in the comparison. The unsupervised clustering **a** fully separated the control samples from the ipsilateral and contralateral hypoxic–ischemic brain injury (HIBI) samples at 24 h and **b** clearly separated out the ipsilateral HIBI samples from the control and contralateral HIBI samples at 72 h after injury.

or were part of the endogenous cellular repair mechanisms. Lastly, this study used whole-brain samples, so we cannot delineate possible region-specific miRNA expression changes within the brain.

In conclusion, this study provides a global assessment of the subacute changes in brain miRNA expression after hypoxia or HIBI in mouse models of injury. To our knowledge, this is the first study to include brain-specific miRNA-Seq as late as 72 h after injury. As investigators continue to advance research into targeted miRNA-based interventions for neonatal HIBI, it will be very important to take into account the multiphasic expression patterns that we observed in this study to identify optimal timing for the individual interventions. Future studies should consider assessing region-specific miRNA changes, utilizing miRNA mimics and antagonists to aid in differentiating pathologic versus protective miRNA expression changes, and evaluating the effects that hypothermia may have on miRNA expression.

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AUTHOR CONTRIBUTIONS

E.S.P. participated in the conception and design, acquisition of data, interpretation of data, and drafting and critical revision of the manuscript. N.S. and W.S. participated in the acquisition of data, analysis of data, and critical revision of the manuscript. K.M. participated in the interpretation of data and critical revision of the manuscript. All authors provided final approval of the version to be published.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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