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REVIEW

MicroRNA Changes in Preconditioning-Induced Neuroprotection

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Abstract Preconditioning is a paradigm in which sublethal stress–prior to a more injurious insult–induces protection against injury. In the central nervous system (CNS), preconditioning against ischemic stroke is induced by short durations of ischemia, brief seizures, exposure to anesthetics, and other stresses. Increasing evidence supports the contribution of microRNAs (miRNAs) to the pathogenesis of cerebral ischemia and ischemic tolerance induced by preconditioning. Studies investigating miRNA changes induced by preconditioning have to date identified 562 miRNAs that change expression levels after preconditioning, and 15% of these changes were reproduced in at least one additional study. Of miRNAs assessed as changed by preconditioning in more than one study, about 40% changed in the same direction in more than one study. Most of the studies to assess the role of specific miRNAs in the neuroprotective mechanism of preconditioning were performed in vitro, with fewer studies manipulating individual miRNAs in vivo. Thus, while many miRNAs change in response to preconditioning stimuli, the mechanisms underlying their effects are not well understood. The data does suggest that miRNAs may play significant roles in preconditioning-induced neuroprotection. This review focuses on the current state of knowledge of the possible role of miRNAs in preconditioning-induced cerebral protection.

Keywords Preconditioning · MicroRNA · Neuroprotection · Stroke

Introduction

Cerebral ischemia is a leading cause of death and disability worldwide, and a serious complication during the perioperative period. Preconditioning (PC) is an endogenous neuroprotective response induced by a mild stress or subthreshold stimulus in the brain, including a sublethal duration of ischemia, brief episodes of seizures, and exposure to bioactive pharmaceutical drugs (including anesthetics) given before a subsequent injurious ischemia [1–4].

It is known that PC induces neuroprotection with two possible time courses. Rapid tolerance happens within minutes after PC and is associated with posttranscriptional modification of proteins and activation of signaling pathways that converge on the mitochondria to reduce cell death [2, 5, 6]. Delayed tolerance develops more slowly with neuroprotection against subsequent injury only evident after about 24 h, peaking at 3 days and diminishing over 1 week [7–9]. Delayed tolerance is characterized by a dependence on de novo protein synthesis of heat shock and other proteins [10, 11] and suppression of the transcriptional response to ischemic injury [12]. PC causes a complex reprogramming of the genetic response to ischemic injury, modifying metabolic, cell-cycle regulatory, ion channel, and immunologic pathways [12–14].

MicroRNAs (miRNAs) are small non-coding RNAs (~20–22 nucleotides) which regulate gene expression largely at the posttranscriptional level [15–17]. MiRNAs modulate mRNA

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function mainly by binding to complementary sites in the 3′-untranslated region (3′-UTR) of target mRNAs and inhibiting translation or inducing mRNA degradation [18]. However, additional mechanisms have also been demonstrated including miRNA binding to gene promoters in some genes to induce their expression [19] or binding protein-coding regions to alter expression [20]. MiRNAs are abundant in the nervous system where they serve important roles in brain development and neuronal function including synaptic plasticity and neurogenesis as well as in neurodegeneration and neuroinflammation [21–25].

Emerging evidence demonstrates that miRNAs contribute to the pathogenesis of cerebral ischemia [16, 26–32], representing both endogenous attempts at repair, as well as contributing to pro cell-death pathways. As part of the overall epigenetic control of gene expression, miRNAs also play prominent roles in ischemic tolerance induced by PC [33, 34]. MiRNA profiling has been performed for ischemic PC [26, 35, 36] and ischemic tolerance–the protected state after ischemic injury preceded by brief ischemic or other noxious stimulation [35]–and has demonstrated miRNA-dependent regulation of a number of neuroprotective pathways.

Recent studies in cerebral ischemia models have documented miRNA changes, and modulating an array of miRNAs is neuroprotective experimentally [28, 37–45]. The clinical application of ischemic preconditioning in the cardiovascular and central nervous system (CNS) is readily apparent, given its ease of implementation if non-invasive (such as application of a blood pressure cuff to induce remote ischemia). A few preconditioning treatments have been tested clinically [46–49]. Slagsvold et al. [47] showed that remote ischemic PC preserved mitochondrial function and activated pro-survival kinase Akt in the left ventricle during cardiac surgery. In patients with symptomatic intracranial arterial stenosis, repetitive bilateral arm ischemic PC safely reduced stroke recurrence, protected against brain ischemia, and improved inflammation and coagulation [46]. Tian et al. [48] showed that isoflurane PC in combination with angiotensin-converting enzyme inhibitors (ACEi) additively attenuated myocardial ischemia-reperfusion injury by reducing oxidative stress and inflammation. We and the others are also currently involved in clinical trials for remote ischemic preconditioning in hemorrhagic shock.

While clinical use of PC is in its infancy, preclinical data on its efficacy is extensive. One reason why clinical data is sparse is that many of the models are not practical clinically (i.e., subthreshold seizure induction, brief cerebral ischemia). If the mechanisms of neuroprotection by PC can be parsed out, the eventual goal would be to mimic the effects of PC by directly manipulating the pathways involved pharmacologically. In this review, we sought to clarify the role of miRNAs in PC-induced cerebral protection in preclinical models and evaluate the current state of the field. The paper is organized according to the method of preconditioning used: ischemia, 3-nitropropionic acid (3-NPA), exposure to anesthetic, brief seizures, and hibernation. The potential clinical use of miRNAs to mimic PC for cerebral protection is highlighted.

Ischemic Preconditioning

Several preclinical studies have investigated miRNA changes in ischemic PC (IPC) (Table 1), and while these are summarized in the accompanying tables, we will discuss the salient findings here. Dharap et al. [36] used hypertensive rats subjected to brief middle cerebral artery occlusion (MCAO) to identify miRNAs that change in response to IPC. Of the 265 miRNAs analyzed, 28 were uniquely changed only at 6 h, while 13 miRNAs showed increased expression and 10 miRNAs decreased expression up to 3 days after PC. Of these 23, 3 miRNAs were no longer significantly changed at the 72-h timepoint. Overall miRNA expression changed with time following IPC. Bioinformatic investigation of possible mRNA targets identified Fragile X mental retardation 1, disheveled-axin domain containing 1 (DIXDC1), a protein controlling Wnt signaling and synaptic structure, and ubiquitin carboxyl-terminal hydrolase 1(UCHL1), an emerging neuroinjury biomarker responsible for protein degradation targeted by several miRNAs upregulated by PC [36]. These likely play a role in IPC-induced ischemic tolerance and possibly neurogenesis. MiRNAs downregulated after IPC targeted methyl CpG-binding protein 2 (MeCP2) and Fas ligand (Faslg) 1L [36]. These changes might also play a role in PC-induced neuroprotection [50, 51]. Notably, Wnt signaling plays a critical role in cell survival during neuronal development, making epigenetic modulation of this pathway by PC intriguing [52, 53]. MiRNA-21, one of the upregulated miRNAs identified by Dharap and colleagues, was also shown to be upregulated in rat MCAO by another group [38]. Buller et al. found that overexpression of miR-21 protects against ischemic neuronal death through downregulation of Faslg, an important cell death-inducing ligand targeted by miR-21 in vitro [38].

Sun et al. [54] identified several miRNAs that increased with a preconditioning stimulus of sublethal global ischemia in gerbils. Their work identified 43 kDa transactivation response DNA-binding protein (TDP43), fused in sarcoma/translocated in liposarcoma (FUS/TLS), and heat shock protein 70 kDa (HSP70) as potential mRNA targets. This group demonstrated the neuroprotective role of these proteins in a previous investigation [55]. Other corroborative work has shown that miR-132, which targets FUS/TLS, increased and peaked at post-injury day 1, but was subsequently downregulated from 7 days to 6 months after ischemia [54]. Of interest, this investigation found that three repeated episodes of

Table 1 Preconditioning-induced miRNA changes in vivo

Preconditioning	MiRNA changes with PC	Species, sex, time assessed	Reference
MCAO	miRNAs altered 6 through 72 h after PC, 11↑, 9↓ miRNAs altered only 6 and 24 h after PC 2 ↑, 1↓ miRNAs altered only 6 h after PC 13↑, 15↓	Rats, hypertensive, male; 6, 24 and 72 h	[36]
MCAO	miR-200 family ↑ (miR-200a, miR-200b, miR-200c, miR-141, miR-429) miR-182 family ↑ (miR-182, miR-183, miR-96)	Mice, male; 3 and 24 h	[26]
MCAO	180 miRNAs ↑, 93 miRNAs ↓	Mice, male; 24 h	[35]
HPC	4 miRNAs ↑, 13 miRNAs ↓	Mice, male; 6 h	[56]
tCCAO	mmu-miR-15a-5p ↑, sha-miR-24 ↑, oan-let-7b-3p ↑, mmu-miR-125b-5p ↑, mmu-miR-132-5p ↑, mmu-miR-181c-5p ↑	Gerbil, male; 1 and 7 days	[54]
3-NPA	miR-199a ↓	Rats, male; 2 and 4 days	[71]
3-NPA	miR-33a ↓	Rats, male; 4 days	[72]
Isoflurane	miR-350 ↑, miR-647-3p ↑	Rats, male; 6 h	[77]
Sevoflurane	miR-15b ↓	Rats, male; 72 h	[78]
Seizure	25 miRNAs ↑	Mice, male; 8 h	[87]
Hibernation	miR-200 family ↓ (200a, 200b, 200c, 141, 496) miR-182 family ↓ (182, 183, 96); overall more ↑ than ↓, members of 33 miR families altered	Ground squirrels, adult male and female	[59]

MCAO middle cerebral artery occlusion, *HPC* hypoxic preconditioning, *tCCAO* transient common carotid artery occlusion, *3-NPA* 3-nitropropionic acid

preconditioning more rapidly induced miRNA expression; however, this three-dose protocol also led to neuronal loss in CA1 of the hippocampus, in contrast to a single exposure. This complicates interpretation of the data, as their findings with the three-dose protocol may be more related to miRNAs involved in neuronal death, rather than protection.

In a study investigating the miRNA profile in mouse cortex following IPC, Lee et al. found that the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) and the miR-182 family (miR-182, miR-183, and miR-96) were upregulated early (i.e., 3 h) after IPC [26] with protection linked to downregulation of prolyl hydroxylase 2 (PHD2). As the prolyl hydroxylases play a critical role in the degradation of hypoxia inducible factors (HIFs), HIF stabilization and transcription of its target proteins might have played a critical role in this investigation. In contrast, our lab demonstrated that post-stroke increases in miR-200c increased neuronal cell death through inhibiting reelin expression (an extracellular matrix glycoprotein modulating synaptogenesis and neuronal migration), while downregulation of miR-200c protected against neuronal injury by enhancing reelin expression in vivo and in vitro [28].

Liu et al. [56] demonstrated significantly altered expression of 17 miRNAs, with 4 upregulated and 13 downregulated in the cerebral cortex of hypoxic PC (HPC) mice. HPC with subsequent ischemia (HPC+MCAO) reversed the upregulation of miR-30a seen following HPC or ischemia alone. Others have suggested that downregulation of miRNA-30a alleviates neuronal injury by enhancing beclin1-mediated autophagy in N-2a cells after oxygen glucose deprivation (OGD) [41]. Downregulation of miR-30a by antagomir to miR-30a also prevented neural ischemic injury through upregulating glucose-regulated protein of 78 kDa/binding immunoglobulin protein GRP78/BIP expression in vivo and in vitro [42]. There is also a potential role for protein kinases. Bioinformatic analysis suggested 19 miRNAs were differentially expressed following HPC and MCAO including ones regulating protein kinase C (PKC) βII, PKCϒ, and nPKCε-interacting protein [56].

Another study, by Lusardi et al., investigating miRNA changes with ischemic preconditioning in mice identified transcriptional and translational regulators as prominent targets of miRNAs altered by IPC, with the largest number of miRNAs targeting methyl CpG binding protein 2 (MeCP2) [35]. These investigators reported that IPC neuroprotection is associated with downregulation of miR-132 and increased MeCP2 protein in the mouse cortex 24 h after ischemic PC without altering MeCP2 mRNA levels. The investigators suggested that IPC leads to depression of miR-132 resulting in increased synthesis of MeCP2 protein [35] which participates in down regulation of some of the transcriptional responses to ischemia. They focused on miR-132 of the 287 profiled miRNAs altered after PC, because of its effects on MeCP2 expression in neurons [57]. However, their results are not sufficient to define how important miRNA-132 is for IPC, as they did not manipulate any individual miRNAs to investigate their impact on IPC or ischemia. In contrast, Hwang et al. reported that overexpression of miR-132 affords neuroprotection

against global ischemia-induced neuronal death in in vivo and in vitro models of ischemia [58].

The miR181 family of miRNAs has also garnered a lot of interest in ischemia and preconditioning. While Lusardi et al. [35] identified miR-181a, b, and c as downregulated with IPC, Lee et al. reported that miR-181a and c were upregulated [59], and Dharap et al. reported that miR-181b and d were upregulated [36]. Peng et al. [60] demonstrated neuroprotection against ischemic injury by downregulating miR-181b which in turn upregulated GRP78/BIP and UCHL1 in mice after PC. Indeed, GRP78/BIP antagonizes neuronal apoptosis induced by ischemic brain injuries [61, 62]. UCHL1 is a potential biomarker of traumatic brain injury, involved in a cellular pathway responsible for the ubiquitination and degradation of damaged proteins [63–65]. Intracerebroventricular (ICV) injection of miRNA-181b antagomir increased GRP78/BIP and UCHL1, decreased neuronal cell loss, and reduced neurological deficits 24 h after MCAO [60]. Further, we [40] investigated the effect of increased miR-181a and found that increased miR-181a targeted GRP78/BIP and increased injury. GRP78/BIP is a central participant in ER-dependent protein folding, being one of the three arms of the unfolded protein response, as well as having a role in autophagy and inhibition of apoptosis [66]. Consistent with this, inhibition of miR-181a provided neuroprotection from ischemia, increasing the levels of GRP78/BIP in a mouse MCAO model [40]. In rat forebrain ischemia, untreated rats showed increased miR-181a levels and decreased Bcl-2 protein in hippocampal CA1 [39]. Notably, miR-181a antagomir enhanced CA1 neuron survival, increased pro-survival Bcl-2 protein, and prevented the decrease of glutamate transporter 1 (GLT-1) characteristic of selective loss of CA1 neurons [39]. Lastly, we found [67] that post-stroke treatment with miR-181a antagomir was neuroprotective, increasing levels of the apoptotic inhibitory proteins Bcl2 and XIAP in mice. Collectively, the miR181 family appears to play an integral role in the neuronal apoptotic response to ischemia and is influenced by preconditioning stimuli.

In vitro ischemic PC is typically performed using OGD (Table 2) and is known to change a number of different cell survival pathways. Similar to PC effects of brief ischemia in whole animal preparations, sublethal in vitro OGD as a form of PC using neuroblastoma (N-2a) cells resulted in upregulation of the miR-200 family, resulting in increased cell survival and downregulated PHD2 levels, again highlighting the involvement of HIF regulatory pathways in IPC [26]. In accordance with the aforementioned animal data, another N-2a cell OGD model demonstrated that overexpression of miR-181b promoted OGD-induced cell death with decreased GRP78/BIP and UCHL1 [60]. This is also consistent with other in vitro studies of miR-181a, another member of miR-181 family, that suggest a regulatory role in cerebral ischemia. Inhibition of miR-181a was shown to provide neuroprotection by targeting GRP78/BIP [40] or Bcl-2 in vitro [39].

The migration of neural progenitor cells is also thought to play an important role in neuronal repair from ischemia. Relevant to this, Shin et al. [68] demonstrated that a decrease in miR27b after stimulation with ischemic brain extract (IBE) increased the expression of its target mRNA and protein, stromal cell-derived factor 1 (SDF-1), critical for progenitor migration. This effect was mediated, interestingly, via miR-223, which itself downregulated miR-27b. This is an interesting example of epi-epi-genetic modulation. Other investigators have highlighted SDF-1 as conferring neuroprotection and synaptic modulation after ischemic injury [69, 70] using primary rat astrocytes. Lastly, knockdown and inhibition of CXCR2, a chemokine receptor, inhibited the IBE-induced miR-223 expression and abolished IBE-mediated SDF-1 expression, suggesting that CXCR2 is required for the increased expression of miR-223 and subsequent SDF-1 induction [68].

Chemical Preconditioning with 3-Nitropropionic Acid

Chemical preconditioning using 3-NPA, an inhibitor of succinate dehydrogenase, was used by Xu et al. [71] to investigate the role of miR-199a. This form of PC was associated with downregulation of miR-199a and protection from subsequent MCAO [71]. The expression of miR-199a during ischemic PC showed region specific timecourses. In cortex and striatum, miR-199a was downregulated on the second and fourth day after PC, while in the hippocampus, it was only downregulated the second day after PC [71]. Another study investigating altered miRNA expression following PC induced by 3-NPA showed that decreased miR-33a significantly reduced infarct volume in rats [72].

Volatile Anesthetic Preconditioning

Cerebral ischemia is a serious complication that can occur in the perioperative period. Volatile anesthetics such as sevoflurane have been found to have preconditioning neuroprotective effects against ischemic injury in experimental settings [73–76]. To date, few studies have investigated a possible role for miRNAs in anesthetic PC. Cao et al. [77] showed that 2% isoflurane exposure for 1 h is neuroprotective and significantly increased miR-350 and miR-647-3p in rats. A study investigating the neuroprotective effect of sevoflurane PC with 2.4% sevoflurane for 30 min per day on four consecutive days, showed that sevoflurane PC was associated with decreased miR-15b expression and increased Bcl-2 protein in rats [78]. ICV injection of Bcl-2 inhibitor following

Table 2 Effects of altering miRNAs in vitro

Preconditioning	MiRNA	Target gene	Effect	Manipulation	Cells	Reference
OGD	miR-200 family↑	PHD2 ↓ HIF-1α ↑	Increased survival	miR-200a,b,c, miR-429 mimics	Mouse neuroblastoma cells (N-2a)	[26]
	miR-182 ↑	HIF-1α ↑	Increased survival	miR-182 mimic		
	miR-181b ↑	GRP78/BIP ↓ UCHL1 ↓	Decreased survival	Pre-miR-181b	N-2a	[60]
	miR-181b↓	GRP78/BIP ↑ UCHL1 ↑	Increased survival	Anti-miR-181b		
	miR-615-3p ↑	Unknown	Decreased survival	Pre-miR-615-3p	N-2a	[56]
	miR-615-3p ↓	Unknown	Increased survival	Anti-miR-615-3p		
Ischemic brain extract	miR-27b ↑	SDF-1 ↓	Reduced viability	Pre-miR-27b	C6 rat astrocytoma cells	[68]
	miR-27b ↓	SDF-1 ↑	Increased survival	Anti-miR-27b		
	miR-223 ↑	IKKα mRNA ↓ SDF-1↑	Increased survival miR-27b↓	Pre-miR-223		
	miR-223 ↓	IKKα mRNA↑ SDF-1 ↓	Decreased survival miR-27b ↑	Anti-miR-223		
	miR-27b	miR-27b	Increased survival	IKKα siRNA		
	miR-223 ↓ miR-27b ↑	Restoration of IKKα decrease SDF-1 ↓	Decreased survival	siCXCR2		
3-Nitropropionic acid (3-NPA)	miR-199a ↑	Sirt1 ↓	Decreased survival	Pre-miR-199a	Rat hippocampal neurons	[71]
	miR-199a ↓	Sirt1 ↑	Increased survival	Anti-miR-199a		
Isoflurane	miR-203 ↑	Unknown	Increased survival, P-Akt ↑	miR-203 overexpression	Rat B35 cells	[77]
Sevoflurane	miR-15b ↑	Bcl-2 ↓	Decreased sevo PC-protection	Lenti-miR-15b overexpression	Rat cortical neurons	[78]
	miR-15b ↓	Bcl-2 ↑	Increased survival	miR-15b inhibitor		
Sevoflurane	miR-101a ↑	Unknown	Decreased survival	miR-101a mimic	Rat PC-12 cells	[80]
	miR-34b ↑	Unknown	Increased survival	miR-34b mimic		
	miR-34b ↓	Unknown	Decreased survival	miR-34b inhibitor		
Hibernation	miR-200c ↑	NEDD8, Ubc9, SUMO3	no change in survival	miR-200c mimic	Human neuroblastoma SHSY5Y cells	[59]
	miR-200c ↓	NEDD8, Ubc9, SUMO3	Increased survival	miR-200c inhibitor		
	miR-182 ↑	NEDD8, Ubc9	Decreased survival	miR-182 mimic		
	miR-182 ↓	NEDD8, Ubc9	Increased survival	MiR-182 inhibitor		
	miR-183 ↑	Ubc9, UFM1	Decreased survival	MiR-183 mimic		
	miR-183 ↓	Ubc9, UFM1	Increased survival	MiR-183 inhibitor		
	miR-141 ↑	UFM1, SUMO1	No change	miR-141 mimic		
	miR-141 ↓	UFM1, SUMO1	Increased survival	miR-141 inhibitor		

sevoflurane PC and MCAO significantly increased infarct volume, although there was surprisingly no effect on ischemic infarct in the absence of sevoflurane PC, suggesting a role for Bcl-2 in sevoflurane PC.

Anesthetic preconditioning and its associated modulation of miRNAs have also been demonstrated in vitro. In B-35 cells, a neuroblastoma cell line, isoflurane PC significantly increased miR-203 expression, significantly reducing OGD-induced cell injury. This suggests miR-203 may be a target for isoflurane in the brain [77]. Overexpression of miRNA-203 increased phospho-Akt, a kinase important in survival signaling in response to growth factors; this mechanism might similarly mediate isoflurane-induced neuroprotection [79]. The increased activation/phosphorylation of Akt induced by miR-203 overexpression may also be a mechanism by which miR-203 increases the tolerance of cells to OGD.

Shi et al. [78] demonstrated a critical role for miR-15b in sevoflurane-dependent PC (Table 2). Ischemic injury to cortical neurons in their model upregulated miR-15b, suppressing the expression of the pro-survival protein Bcl-2; this was reversed with sevoflurane preconditioning. Sun et al. [80] further identified miRNAs altered after 30 min exposure to 2% sevoflurane using pheochromocytoma-12 (PC-12) cells, which have a characteristically neuronal phenotype [81, 82]. In this study, sevoflurane PC induced neuroprotection by downregulation of miR-101a and upregulation of miR-34b. Overexpression of miR-101a or administration of miR-34b

inhibitor significantly enhanced apoptosis of hypoxic PC-12 cells [80].

Epileptic Preconditioning

Brain injury secondary to status epilepticus can also be reduced by preceding brief seizures [83]. One study profiled miRNAs in a model of epileptic tolerance, that is, mice were given intraperitoneal kainic acid to induce brief seizures for PC followed 24 h later by status epilepticus induced with intra-amygdalar kainic acid [84] and compared to mice only subjected to status epilepticus. Changes in miRNA levels were assessed 24 h after status epilepticus, and 23% of miRNA expression was increased while 77% decreased in the tolerant state compared to control [84]. This contrasted to the status epilepticus alone which compared with control had many more upregulated compared to downregulated miRNAs. With preconditioning and subsequent severe seizures (tolerance), one miRNA was uniquely upregulated (15 in the non-preconditioned mice) and eight miRNAs were uniquely downregulated. Expression of miR-27a, 200a, and 326 were increased in the injured, non-preconditioned mice, but decreased in the preconditioned animals, suggesting a specific role for these miRNAs in epileptic preconditioning-induced tolerance. MiR-132, which plays a role in regulating neuronal structure [85, 86], was upregulated after status epilepticus in the hippocampal subfield CA3, but this was prevented in preconditioned mice. When miR-132 antagomir was injected ICV, it was found to increase neuronal survival after status epilepticus [84].

McKiernan et al. profiled changes in miRNAs following preconditioning seizures alone [87]. Seizure PC increased 39 miRNAs and the most upregulated miRNA was miR-184. Upregulation of miR-184 was shown to contribute to neuroprotection by epileptic PC, as ICV injection of mir-184 antagomir increased neuronal cell death despite epileptic PC. MiR-9, another upregulated miRNA in epileptic PC, was found to be neuroprotective in mouse MCAO when upregulated and associated with decreased Bcl2-l1 protein and reduced neuronal apoptosis in OGD-treated hippocampal neurons [43].

Hibernation

Hibernation is a state of natural tolerance to ischemia. Hibernating mammals lower their energy consumption, breathing, blood flow, and body temperature, but have no cerebral ischemia despite what would under normal conditions be a lethal level of cerebral blood flow [88]. It has been reported that massive global SUMOylation, a form of posttranscriptional protein modification with the small ubiquitin-related modifier (SUMO), occurs during hibernation in the brains of ground squirrels [89]. SUMOylation is involved in ischemic tolerance in human neuroblastoma cells and primary cortical neuronal cultures in rats and mice [90, 91]. However, the ischemic tolerance level induced by SUMOylation did not reach the degree of tolerance to brain hypoperfusion seen during hibernation [89], so there are likely additional factors participating in protection in combination with SUMOylation. Lee et al. [59] showed that two miRNA families (the miR-200 and 182 families) were downregulated and miR-34 and miR-206 were upregulated in ground squirrels during hibernation torpor. They demonstrated that inhibition of the miR-200 family and/or miR-182 family in human neuroblastoma SHSY5Y cells increases protein conjugation by several ubiquitin-like protein modifiers (ULMs), which are increased in the brains of ground squirrels during hibernation torpor, and reduces OGD-induced cell death in this neuronal cell line [59].

Discussion

The above evidence suggests ischemic preconditioning has a highly heterogeneous and complex effect on miRNA expression. We have attempted to summarize the relatively large body of data, focusing on parsing out the consistencies. In total, 562 miRNAs were reported to change expression after PC in the nine studies listed in Table 3. Of these, 79 miRs were reported in two studies, 13 in three (Table 4). The single miR reported changed in four studies, miR-34b, was increased in all four, suggesting it may play an important role. Mir-34b is interesting as it plays a role in brain development [92] and in Parkinson's disease [93] as well as regulating apoptosis of astrocytes in the setting of seizures [94]. Of 79 miRNAs reported changed in two studies, 44% reported the same direction of change (increased or decreased) in both. In the case of three reports (13 miRNAs), 38% reported change in the same direction. Thus, while there are clues from this work on possible candidate miRNAs, it remains difficult to identify a small target group of miRNAs given that bi-directionality of change was frequently observed.

The studies reviewed here used different experimental designs, including different PC stimuli, in vivo or in vitro models, different species, cell types, timing, and number of miRNAs assessed. These differences likely underlie some of these differences in outcomes. Of the different preconditioning paradigms, MCAO was used in three studies. Useful data comes from the work of Lusardi et al. [35], who used microarray probes that contained 489 miRNAs in IPC by brief MCAO in vivo. They found that 180 miRNAs were increased and 93 miRNAs were

Table 3 Preconditioning stimuli and species for references in Table 4

Reference	Reference number	Species	Preconditioning stimulus
McKiernan et al. 2012	[87]	Mouse	Seizure/epilepsy
Sun et al. 2015	[54]	Gerbil	tCCAO
Liu et al. 2012	[56]	Mouse	Hypoxia
Dharap et al. 2010	[36]	Rat	MCAO
Lee et al. 2010	[26]	Mouse	MCAO
Lee et al. 2012	[59]	Ground squirrel	Hibernation
Lusardi et al. 2010	[35]	Mouse	MCAO
Sun et al. 2015	[80]	Rat	Sevoflurane
Cao et al. 2012	[77]	Rat	Isoflurane

decreased, 24 h after IPC in three male mice. Another useful array study reported by Dharap et al. [36] measured 265 miRNAs at three time points (6, 24, and 72 h) after IPC (also brief MCAO) in six hypertensive male rats. Many of these miRNAs differed in the direction of change from the work done by Lusardi's group, making firm conclusions difficult.

In addition to different designs and models, challenges have been identified in performing miRNA research. Proper validation of hybridization, amplification, and interpretation is required [95]. While a full discussion of the unique challenges of miRNA research is beyond the scope of this paper, we direct the reader to the excellent article by Witwer and Halushka for further discussion [95].

The effect of manipulation of individual miRNAs is also inconsistent across studies. Lusardi et al. [35] suggested that decreased miR-132 expression correlated with increased MeCP2 and induced neuroprotection following ischemic PC in mice. Jimenez-Mateos et al. [83] also investigated miRNA-132 and reported that it was increased after status epilepticus but not increased after status epilepticus following seizure PC. They did not test the effect of PC alone. Decreased miR-132 by the use of miR-132 antagomir ICV injection induced neuroprotection from seizure [83]. However, Hwang et al. [58] showed that miR-132 overexpression by injection of lentiviral miR-132 increased neuron survival following global ischemia in rats. They also suggested that miR-132 overexpression decreased cell death following OGD in vitro [58]. Notably, there are nuances to transfecting or introducing miRNAs or their inhibitors into cells. The supraphysiologic overexpression of miRNAs obtained by transfection may have different biologic effects [95]. Mayya et al. have shown that miRNAs work in a variable dose-dependent fashion with their mRNA binding sites [96]. If the miRNA is at a low concentration, it may modulate only a subset of genes, whereas at high concentrations, it may find additional mRNA binding sites. The effects of levels of expressions likely impact biologic pathways in different ways [95].

Reasonably consistent results supporting mechanisms for the role of three families have been identified. Both in vitro and in vivo studies have consistently demonstrated that miRNA-181 (a and b) targets the stress protein GRP78/BIP, which plays a critical role in the unfolded protein response and inhibition of apoptosis. Ischemic preconditioning from various labs, using diverse models, have shown changes in levels of miR-181-a, b, c, and d; however, the direction of changes and specific family members is not always consistent between reports (see Tables 3 and 4). Stabilization or increased levels of GRP78/BIP provide neuroprotection from subsequent lethal stimuli when exogenous miR-181 antagomirs are transfected into cells or administered ICV [39, 40, 60]. Two other compelling miRNA families affected by ischemic preconditioning that appears promising for translational therapy are the miR-200 and miR-182 families, reported changed by both hibernation and ischemic PC (see Tables 1 and 4). Both of these families are implicated in protein conjugation with ubiquitin-like protein modifiers, such as SUMO, and increased protein conjugation by these modifiers is associated with protection from ischemia, and with the resistance to ischemia seen in hibernation [26, 59]. In addition, the miR-200 family was shown to target PDH2 leading to increased levels of HIF-1α [26], an additional established protective mechanism.

Off-target effects remain an issue for therapeutic applications of miRNAs, and empiric tests for miRNAs with assessment of off-target effects are needed [97]. MiRNAs can target multiple mRNAs, but most current miRNA studies focus on one miRNA and one selected target, for which interaction and silencing is demonstrated [98]. In fact, often multiple miRNAs target a single mRNA, just as miRNAs have many different mRNA targets. Therefore, the binding of a miRNA to a target mRNA is dependent on the cell type and physiologic state [98]. Assessment of multiple pathways regulated by a particular miRNA should be performed. This is

Table 4 MiRNAs that change in more than one PC study

MicroRNA	Increased	Decreased
let-7a	[36]	[35]
let-7b		[35] [80]
let-7e		[35] [56]
let-7f	[87]	[35]
miR-7	[35] [87]	
miR-9	[87]	[35]
miR-10a	[35] [87]	
miR-15a		[35] [56]
miR-15b	[36] [59]	[35]
miR-16	[80]	[35]
miR-23a		[35] [80]
miR-24	[54]	[35]
miR-26b	[35] [36]	
miR-27a	[36] [80]	[35]
miR-27b	[36]	[35]
miR-28	[35] [87]	
miR-29a	[87]	[35]
miR-30a	[36]	[56]
miR-30c-2*	[56]	[36]
miR-30d		[35] [36]
miR-31	[87]	[35]
miR-34b	[35] [59] [80] [87]	
miR-34b-3p	[59] [80]	
miR-34c	[59] [87]	
miR-92	[59]	[35]
miR-92a	[59]	[36]
miR-92b	[59]	[36]
miR-96	[26] [35]	[59]
miR-99b		[35] [36]
miR-100	[87]	[35]
miR-125a	[59]	[35] [80]
miR-129	[87]	[35]
miR-130b	[87]	[59]
miR-132	[87]	[35]
miR-134	[26]	[35]
miR-137	[36]	[35]
miR-139		[35] [59]
miR-140	[35] [87]	
miR-141	[26] [35]	[59]
miR-144	[26] [35]	
miR-145		[35] [36]
miR-146a	[36]	[59]
miR-148b	[35] [59] [87]	
miR-153	[35] [36]	
miR-155	[35] [59] [87]	
miR-181a	[59]	[35]
miR-181b	[36]	[35]
miR-181c	[59]	[35]
miR-182	[26]	[59]
miR-183	[26] [35]	[59]
miR-184	[87]	[59]
miR-185	[87]	[35]
miR-195	[80]	[35]
miR-196a	[35] [87]	
miR-200a	[26]	[59]
miR-200b	[26]	[59]
miR-200c	[26] [35]	[59]
miR-204	[36] [87]	
miR-206	[35] [59]	
miR-216	[35] [87]	
miR-223	[26] [35]	
miR-292-5p	[35]	[36]
miR-294	[35]	[80]
miR-299-5p	[59] [87]	
miR-300	[80]	[35]
miR-320		[35] [36]
miR-324-3p		[35] [36]
miR-328	[59]	[35] [36]
miR-331		[35] [36] [77]
miR-335	[36] [35] [87]	
miR-339	[59]	[35]
miR-344	[56]	[35]
miR-346	[59]	[35]
miR-350	[77]	[35]
miR-369-3p	[35] [87]	
miR-374	[35] [36]	
miR-375	[35] [87]	
miR-376a	[35] [87]	
miR-378	[35]	[59]
miR-382	[36]	[35]
miR-411	[59]	[35]
miR-425		[36]
miR-429	[26] [35]	[59]
miR-448	[35] [87]	
miR-450	[35] [87]	
miR-452	[26] [35]	
miR-493	[35] [87]	
miR-494	[87]	[36]
miR-504	[35] [59]	
miR-674-3p	[77]	[36]
miR-690	[26]	[59]

For this table, species differences were ignored

important in the development of successful miRNA-based therapeutics.

Conclusions

Several laboratories have performed miRNA profiling studies using different models of PC. However, there are still few reports demonstrating the therapeutic effects of miRNAs by artificially induced overexpression and/or inhibition of miRNAs during PC. Most of the validation studies suggesting miRNAs contribute to PC-induced cell resistance were performed in vitro. Further, many investigations identified upregulated miRNAs during preconditioning and subsequently tested those miRNAs in ischemic models. However, there is a paucity of data manipulating individual miRNAs during the preconditioning phase. Although in vitro studies provide useful information, more in vivo observations are needed to make advances towards the development of clinical therapies. MiRNA changes likely play important roles in PC-induced neuroprotection, but the limited consistent evidence is not sufficient to understand the extent of their contribution. To clarify the significance of miRNAs in PC-induced neuroprotection, further in vivo investigations for specific miRNAs are required. Once a clearer picture is obtained manipulating miRNAs in vivo, PC might eventually be mimicked via exogenous miRNA mimic or inhibitor delivery. The use of miRNAs in clinical practice is already emerging in clinical trials, so the identification of candidate miRNAs involved in PC is likely to have a high chance of clinical translation.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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