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microRNAs: innovative targets for cerebral ischemia and stroke

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Abstract

Stroke is one of the leading causes of death and disability worldwide. Because stroke is a multifactorial disease with a short therapeutic window many clinical stroke trials have failed and the only currently approved therapy is thrombolysis. MicroRNAs (miRNA) are endogenously expressed noncoding short single-stranded RNAs that play a role in the regulation of gene expression at the post-transcriptional level, via degradation or translational inhibition of their target mRNAs. The study of miRNAs is rapidly growing and recent studies have revealed a significant role of miRNAs in ischemic disease. miRNAs are especially important candidates for stroke therapeutics because of their ability to simultaneously regulate many target genes and since to date targeting single genes for therapeutic intervention has not yet succeeded in the clinic. Although there are already quite a few review articles about miRNA in ischemic heart disease, much less is currently known about miRNAs in cerebral ischemia. This review summarizes current knowledge about miRNAs and cerebral ischemia, focusing on the role of miRNAs in ischemia, both changes in expression and identification of potential targets, as well as the potential of miRNAs as biomarkers and therapeutic targets in cerebral ischemia.

Keywords

BCL2 family; cerebral ischemia; heat shock protein; microRNA; mitochondria

Introduction

Cerebral hypoxia/ischemia occurs in several disease states; of these, stroke, cardiac arrest and resuscitation, and head trauma are major ones. Stroke (rodent models of middle cerebral artery occlusion) is one of the leading causes of death worldwide and a major cause of long-term disability [1]. Although many clinical stroke trials have been completed, the only efficacious treatment to date is thrombolysis [2]. Similarly, the cerebral injury resulting from cardiac arrest and resuscitation (rodent models of global/forebrain ischemia) leads to death and neurological impairment, and has only been effectively treated in the clinical setting with hypothermia [3,4]. Suggested reasons for the failures include the complex interplay among signaling pathways, the potentially short therapeutic window for acute neuroprotection, and defining the treatment window for specific targets. In animal models, focal ischemia/stroke and global ischemia have similar underlying injury mechanisms, including excitotoxicity, mitochondrial dysfunction, and oxidative stress [5,6]. Molecular

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chaperones or stress proteins, and some antiapopototic members of the BCL2 family of apoptosis regulatory proteins, can protect mitochondrial function, reduce oxidative stress, and protect from cerebral ischemia [7–14]. They have been suggested as targets for neuroprotection, and here we discuss them as miRNA targets.

MicroRNAs (miRNAs) are important post-transcriptional regulators that interact with multiple target messenger RNAs (mRNA) coordinately regulating target genes. There are so far a handful of reports on miRNA in focal ischemia [15–17] and a single report in forebrain ischemia [18]. We recently reported that a highly brain-enriched miRNA, miR-181, targets both of these protective mechanisms – the chaperone and anti-apoptotic BCL2 family members BCL2 [14,19]. Studying miRNAs as part of the brain's response to ischemia, and defining targets and mechanisms underlying their effects, will both add critical knowledge to our understanding of miRNA function and identify promising new targets for the development of novel treatments for ischemic brain injury and neurodegenerative diseases. In this review we will summarize the current state of understanding of the role of miRNAs in cerebral ischemia, with a focus on potential effects on genes known to play a role in ischemic outcome: heat shock protein 70 (HSP70) family members and BCL2 family members, and discuss their potential use as biomarkers and potential therapeutic targets.

A. miRNA Overview

The discovery of posttranscriptional gene silencing by miRNAs has led to an explosion of new hypotheses in human disease. As many as 30% of human protein coding genes are likely regulated by miRNAs. Of the ~1000 human miRNAs known thus far, each has the potential to bind tens to hundreds of mRNA targets [20]. miRNAs are now implicated in most biological processes including embryonic development, cellular differentiation, metabolism, and many pathological processes. In some research areas including cancer, liver and heart disease, miRNA research has quickly moved from screening into therapeutic development programs.

miRNA biogenesis—Mature miRNAs are 21 to 23 nucleotides (nt) long and modulate protein expression by binding to complementary or partially complementary mRNAs, thereby targeting the mRNA for degradation or translational inhibition [21] (Fig. 1). Sequential endonucleolytic maturation steps are required to generate the mature miRNA from miRNA genes, starting with long primary miRNA transcripts (pri-miRNA), which are processed to ~70-nt precursor miRNA (pre-miRNA), and then to mature miRNA (Fig. 1). A short (5–7 nt long) sequence, referred to as the seed sequence, in the miRNA determines the specificity of binding to the mRNA, so miRNAs can bind multiple mRNAs and mRNAs can be bound by multiple miRNAs, creating a new and complex regulatory layer to post-transcriptional control of the proteome. While most authors observed a repressive role of miRNAs, a few examples of induction by miRNA have been described [22,23]. Furthermore, emerging evidence suggests that pri-mRNA and pre-miRNA can also potentially interact with target mRNAs and repress gene expression [24,25]. While most miRNA target binding sites studied to date are in the 3′ UTR of mRNAs, functional binding sites have also been identified in other parts of the mRNA [23].

miRNA mimics and inhibitors—There are two major approaches to developing miRNA-based therapeutics: mimics to increase effective levels of a miRNA, and inhibitors or antagomirs to reduce them. miRNA mimics are small, chemically modified, double-stranded RNA molecules that load the active strand into the RNA induced silencing complex (RISC) which then binds the target mRNA to induce translational silencing (Fig. 1). miRNA mimics can be used to restore a loss of function of beneficial miRNAs. miRNA inhibitors and antagomirs (which differ in their chemical modifications and intended use *in vivo*) are

modified single stranded antisense oligonucleotides harboring the full or partial complementary sequence to the mature miRNA, to reduce endogenous levels of the miRNA and increase expression of its mRNA targets. miRNA inhibitors/antagomirs can inhibit endogenous miRNAs and could be applied to reduce miRNAs with pathogenic function in stressed cells or diseased tissues. Currently locked nucleic acids which contain modified RNA nucleotides are in clinical trials to manipulate miRNA in specific disease settings (see section H for details).

Technologies for miRNA research—miRNA microarray analysis has been used to profile miRNA expression in many settings. Another widely used tool to study the expression of miRNAs is real-time PCR, which has high sensitivity and specificity. In this case individual miRNAs are evaluated.

Two approaches have been used so far to identify candidate genes targeted by miRNAs-computational target prediction algorithms and experimental target identification strategies. Many computational miRNA/mRNA target programs are available online for prediction of miRNA recognition sites in the 3′UTR of target mRNAs. Those include TargetScan (http://www.targetscan.org), MicroCosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm), Pictar (http://pictar.org), ElMMo (http://www.mirz.unibas.ch) and DIANA-microT (http://microrna.gr/microT). Since miRNA functionality usually requires seed sequence complementarity [26] the main prediction feature used in most of these programs is the sequence alignment of the miRNA seed to the 3′UTR of candidate target genes. The major limitation of this approach is that not all binding sites are in the 3′UTR. Additionally, many current algorithms utilize conservation of miRNA/mRNA target sites across species as a parameter to help rank the likelihood that the target is real. Since binding sites can occur outside the 3′UTR, and since some mammalian miRNAs bind to an mRNA with imperfect complementarity, the bioinformatically predicted targets might be either false-positive or false-negative predictions. Therefore, experimental validation of targets is an important step in defining the functions of individual miRNAs.

Experimental approaches for target validation can be subdivided into four different strategies. The first strategy is at the mRNA level. mRNA is purified after treatment with miRNA mimic [27] or inhibitor/antagomir [28,29], and analyzed by microarray hybridisation or sequencing. mRNA can also be detected by Northern blot after treatment [19]. The second strategy assesses protein levels, since the main regulatory action of miRNAs is to inhibit translation and reduce the amount of protein made from the target mRNA. Changes in protein levels can be detected using stable isotope labelling of amino acids [30,31] or by Western blot [14,19] after altering levels of the miRNA. The main problem with both these strategies is that they identify candidate genes that could be either direct or indirect targets of the tested miRNA. The third strategy is immunoprecipitation. To experimentally validate candidate targets, the RNA-induced silencing complex (RISC) loaded with a specific miRNA is purified by immunoprecipitation along with its bound mRNA targets. This technique has been used in target identification of hypoxia-induced miR-210 [32]. The forth strategy is the only currently used method to validate a sequence as a direct target of a miRNA [33]. It is based on the differential activity of a reporter gene, usually luciferase, attached to a candidate 3′UTR sequence in the presence or absence of the targeting miRNA. Several miRNA targets have been validated using such a Luciferase Reporter System after cerebral ischemia [14,19,34].

B. Cerebral ischemia and miRNAs

In the brain, the complexity and plasticity of the neuronal network and the functional specialization of neurons and glia depend on highly organized and coordinated gene

expression. It has been estimated that at least 30% of protein-coding genes are regulated by miRNAs [35], so it is not surprising that initial studies characterizing miRNAs in the brain revealed a role in neuronal cell differentiation [36–40] and embryonic development of the nervous system [41]. Moreover, our laboratory and others have demonstrated that miRNAs play a role in modulating dendritic plasticity and neuronal outgrowth [42–44]. Finally, alterations in the miRNA expression profile have been observed in diseases of the brain, including Alzheimer's disease [45] and schizophrenia [46], and in acquired pathologies such as tumors [47], suggesting that miRNAs contribute to the progression of chronic neurological diseases. It is also the case that miRNAs play critical roles in acute brain pathologies. In the setting of stroke the significance of miRNA-based therapeutic intervention may lie in the role miRNAs play in the response to an acute cell stress, and how they can be manipulated to improve outcome.

Cerebral ischemic models—Animal models of ischemic stroke are used to study the basic pathophysiological processes and potential therapeutic interventions for this disease. The extension of knowledge gained from these animal models will lead to the development of both novel and improved medical treatment of human stroke in the future.

Focal cerebral ischemia induced by middle cerebral artery occlusion (MCAO) in rats or mice is the rodent model most immediately relevant to human stroke. Using this method, transient ischemia is achieved by inserting a suture into the carotid artery and advancing it to block the middle cerebral artery. After temporarily blocking blood flow to the middle cerebral artery territory for a predetermined duration of minutes to hours, depending on the specific study, the suture is removed to allow reperfusion [19,48,49]. In some cases permanent occlusion is used.

Global cerebral ischemia or transient forebrain ischemia, which induces a histopathological picture similar to that seen with cardiac arrest and resuscitation, is often induced by occluding both carotid arteries and inducing hypotension in rats for short durations of about 10 minutes [5,6]. Ten minutes of transient forebrain ischemia causes delayed neuronal death selectively of hippocampal CA1 pyramidal neurons after 2–4 days of reperfusion, whereas other regions within the hippocampus, dentate gyrus and CA3, as well as most cortical neurons remain essentially intact.

Glucose deprivation (GD) and combined oxygen-glucose deprivation (OGD) are common *in vitro* models of brain ischemia. Primary brain cell cultures or acute or cultured slices generally from the hippocampus, are subjected to medium lacking glucose, and in the case of OGD, also placed in a chamber with very low oxygen levels for a fixed period of time [5,8–10,19,50,51] followed by restoration of oxygen and glucose to the medium to imitate reperfusion. The advantage of cell culture is that individual cell types can be studied, while in the case of hippocampal slice a brain slice containing some intact circuitry and relatively intact anatomical connections is studied.

Mechanism of cerebral ischemic injury—Complex interplay among multiple pathways including excitotoxicity, mitochondrial dysfunction, ionic imbalance, oxidative stress, and inflammation are involved in the mechanism of cerebral ischemic injury. These processes lead to both necrotic and apoptotic modes of cell death. One cell death pathway known to participate in injury after cerebral ischemia is mitochondrial permeability transition (MPT) pore opening (Fig. 2A). Ischemia leads to energy deprivation and loss of ion homeostasis. As the cells are unable to maintain a negative membrane potential, they depolarize, leading to opening of voltage-gated calcium channels and release of excitatory amino acids into the extracellular space [52]. This cascade of events leads to massive entry of calcium into the cell and this increase in free cytosolic calcium is transmitted to the

matrix of mitochondria by Ca^{2+} channels and exchangers located on the inner mitochondrial membrane.

Recently ER stress was found to be one of the effects of excitotoxicity, or exposure to toxic levels of excitatory neurotransmitters. ER stress leads to release of Ca^{2+} from the ER via both ryanodine receptors and inositol trisphosphate receptors (IP3R) leading to mitochondrial Ca^{2+} overload and activation of apoptosis [53]. Excessive increases in matrix Ca^{2+} alter the permeability of mitochondria and finally open the MPT pore [48], causing release of cytochrome c [54] and other pro-apoptotic factors into the cytoplasm. The released cytochrome c activates the caspase cascade including caspase-3, one of the executioner caspases, to initiate cell death. Excessive accumulation of calcium in mitochondria is a key factor in the final outcome of the cascade leading to neural cell death (Fig. 2A, B) [55].

Mitochondria can accumulate large amounts of calcium through a Ca^{2+}-selective channel known as the mitochondrial Ca^{2+} uniporter (MCU) [56,57]. However MCU has a relatively low Ca^{2+} affinity [58]. It is interesting that in response to cytosolic Ca^{2+} transients not exceeding concentrations of 1–3 μM, mitochondrial Ca^{2+} concentrations rise almost simultaneously to values above 10 μM [59]. The existence of close contact points between the ER and mitochondria (the mitochondria-associated ER membrane, MAM) is thought to provide a selective direct pathway for calcium from the ER to mitochondria. Upon cell stimulation, the release of high concentrations of Ca^{2+} at MAM leads to the formation of microdomains of high Ca^{2+} concentration that is crucial for efficient Ca^{2+} uptake by mitochondria [60,61]. It has been demonstrated recently that MAM coexists with many molecular chaperones [62] and the anti-apoptotic protein BCL2 (Fig. 2B, C).

miRNA in cerebral ischemia—Cerebral ischemia/reperfusion injury induces several genes [63], which activate molecular cascades leading to both necrotic cell death in the anoxic core, and delayed apoptosis-mediated cell death in the surrounding penumbra [64,65]. While the fate of brain cells in the anoxic core is likely fixed relatively soon after the initial insult, cells in the peri-ischemic penumbra represent targets for rescue from delayed cell death. There is thought to be a temporal window in which reversal or prevention of induction of the apoptotic cascade can occur, and such prevention of cell death would improve functional outcome [66]. Transient translational arrest is a component of the acute response to ischemia/reperfusion, which, together with the induction of the heat shock family of proteins, serves to limit the production of unfolded/misfolded proteins, thereby inhibiting the induction of pro-death pathways [67,68].

Translational arrest also permits rapid modulation of gene expression patterns prior to restarting *de novo* protein synthesis, and may therefore provide a target in the prevention of the delayed phase of neuronal cell death following stroke. Several studies have demonstrated alterations in the cerebral “miRNA-ome” following ischemia/reperfusion [16,17,69] suggesting that miRNA mediated translational arrest may be an important factor in modulating the gene expression cascade that occurs in response to ischemia/reperfusion.

Studies of miRNAs in cerebral ischemia are relatively recent, and most have focused on profiling changes in miRNAs with ischemia. Changes in miRNAs with ischemic brain injury have been identified using miRNA profiling techniques in a rat MCAO model [15–17] and in forebrain ischemia [18] as well as in stroke patients [69]. An acute alteration of the miRNA profile following cerebral ischemia would suggest that miRNAs play a role in the early stress response to ischemia in the brain, as either a negative or positive regulator of cell survival. Dharap *et* al. [15] demonstrated in a rat model of middle cerebral artery occlusion that while the expression of several miRNAs was altered up to 3 days post-ischemia/

reperfusion, a progressive increase in miR-140, miR-145 and miR-331 was observed as early as 3 hours following reperfusion (the first time point measured).

In a subsequent study investigating changes in miRNA levels following ischemic *preconditioning*, the phenomenon whereby an initial sub-lethal "priming" period of ischemia induces cytoprotection to a subsequent period of ischemia, Lee *et* al. [70] observed 8 genes (miR-200a, miR-200b, miR-200c, miR-141, miR-429, miR-182, miR-183, and miR-96) which were elevated following 3 hours of reperfusion. The authors subsequently demonstrated in neuronal cell culture that several miRNAs (miR-200b, miR-200c, and miR-429) were themselves inherently cytoprotective. These findings demonstrate the complexities of post-transcriptional regulation whereby alterations in miRNA expression can contribute to both pro-survival and pro-death outcomes. Adding to this complexity, Siegel *et* al. [71] recently demonstrated in rats subjected to MCAO a differential response between sexes in miR-23a levels, adding an additional potential mechanism of the sex-based dimorphism in response to cerebral ischemia [71].

While the kinetics of miRNA expression patterns in response to cerebral ischemia may be complex, an interesting observation made by Dharap *et* al. [72] was that miRNAs altered by ischemia/reperfusion demonstrated sequence homology with several mRNAs associated with the post-ischemic brain, including cytokines, transcription factors, heat shock proteins, ion channels and neurotransmitter receptors [72]. Whether the specific miRNA-mRNA homology observed in that study corresponds to *in vivo* functionality remains to be determined, however this finding raises the possibility that individual miRNAs may act to coordinate multiple genes in parallel to produce a concerted outcome. Indeed, it has been estimated that an individual miRNA may regulate up to hundreds of target genes [73]. The notion of coordinated gene regulation is supported by the observation that the genomic loci of several miRNAs which share a common functional outcome appear to occur in spatially localized gene "clusters" (see [74], which demonstrate similar temporal patterns of expression. Gene clustering was also demonstrated by Lee *et al.* [70], who noted that miRNAs upregulated in brains subjected to ischemic preconditioning belonged to two families of miRNAs, located on two chromosomes [70].

Recently a few studies have evaluated the significance of individual miRNAs in ischemic brain damage [14,19,34,75]. miR-15a has been shown to contribute to the pathogenesis of ischemic vascular injury. Gain or loss of miR-15a significantly reduced or increased oxygen-glucose deprivation induced cerebral vascular endothelial cell death, respectively [75]. *In vivo* repression of miR-497 using antagomirs was found to effectively lower miR-497 levels, reduce MCAO induced infarct, and improve neurological deficits [34]. Transfection of miR-200 b and c into Neuro-2a cells increased neural cell survival when subjected to oxygen glucose deprivation [70]. Additionally, miR-210 is positively correlated with better prognosis in stroke patients [76].

A key finding demonstrated by Buller *et al.* was regional expression of miR-121, primarily in the ischemic penumbra [77]. Such regional specificity of expression adds another layer of complexity to miRNA expression profiles, and may explain seemingly conflicting results in the literature. For example, in a rat model of global cerebral ischemia, Yuan *et* al. [18] reported that hippocampal miR-181a was upregulated following 30 min of reperfusion, however no change in brain miR-181 was observed either following permanent focal ischemia [17] or following transient focal ischemia [15]. Our laboratory recently demonstrated [19] in a mouse model of MCAO that regional expression of miR-181 differed according to the distribution of blood flow and that anti-miR-181 can protect the brain from ischemia (see details in sections C and H).

C. miRNAs and chaperones

Molecular chaperones and cerebral ischemia—Molecular chaperones are a functionally related group of proteins that assist in the folding or unfolding of proteins, the sequestration of denatured proteins, and the assembly or disassembly of multiprotein complexes. One major function of chaperones is to prevent newly synthesised polypeptide chains and assembled subunits from aggregating into nonfunctional structures. This function indicates that many chaperones, though not all, are also heat shock proteins (HSPs), so named because they are induced by heat stress, because the tendency for proteins to aggregate increases when they are denatured by stress such as heat. HSPs are classified by size. The human 70 kDa molecular weight family (HSP70) consists of at least 12 members [78]. The best known members are the strongly heat inducible cytosolic form, HSP70/HSP72; the constitutively expressed HSC70/HSP73/HSC73; the endoplasmic reticulum form, glucose regulated protein 78(GRP78)/BIP; and the mitochondrial form, GRP75/mortalin/mtHSP70. They are all highly evolutionarily conserved and have been extensively studied.

HSP72 has been shown to provide neuroprotection from cerebral ischemia in animal and cell-culture models of stroke. While the mechanism of this protection was initially attributed to chaperone functions (i.e., maintaining correct protein folding and blocking aggregation), recent work has shown that HSP72 may also directly interfere with cell death pathways such as apoptosis and necrosis and modulates inflammation (for review, see [79]).

GRP75/mortalin is a vital mitochondrial chaperone that is important to normal brain functioning (for recent review, see [80]). Several studies have shown that overexpression of GRP75 reduces damage in both *in vitro* and *in vivo* models of ischemic stroke [12,81]. This neuroprotection is likely mediated by GRP75's ability to preserve mitochondrial function.

GRP78, also referred to as immunoglobulin heavy chain binding protein (BiP/HSPA5), is an important ER chaperone that like other chaperones, binds hydrophobic stretches in newly synthesized polypeptides, while also playing a central role in signaling the unfolded protein response. Several studies suggest that GRP78 plays a role in the regulation of cell death, including both apoptotic Purkinje cell death in the cerebellum [82] and autophagy [83], both relevant for brain cell loss following ischemia. Two reports show that prior induction of increased levels of GRP78 with a pharmacological inducer reduces neuronal loss in both forebrain [84] and focal cerebral ischemia [85]. We recently showed that GRP78 overexpression protects primary cultured astrocytes against ischemic injury *in vitro* [9].

Recently a more complex, integrating role of these chaperones has been recognized, that of stabilizing intracellular morphological and functional networks through protein-protein interactions with numerous client proteins. This chaperoning network concept is increasingly accepted as a basic regulatory mechanism in diverse cellular functions. One example of chaperones as parts of an organelle network of particular relevance to ischemia is the cooperation between the ER and mitochondria in the regulation of intracellular calcium through MAM (Fig. 2B). Several chaperones are involved in ER-mitochondrial Ca^{2+} transfer (Fig. 2B). Both the IP3R and the ryanodine receptor Ca^{2+} release channels in the ER, and the voltage dependent anion channel (VDAC) in the mitochondrial outer membrane, are important nodes of this protein interaction network [86], defining the main calcium transfer route between these organelles.

HSP70 family members have been shown to modulate the conductance of these calcium channels. GRP75 directly interacts with both VDAC and IP3R, playing a central role in scaffolding this ER-mitochondrial complex [87]. Translocation of GRP78 from ER to mitochondria observed following ER stress [88] or ischemia-like stress [86] may also play a

key role in the ER-mitochondria crosstalk during cerebral ischemia [86]. Chaperone complexes at both the ER and mitochondrion orchestrate the regulation of Ca^{2+} signaling between these two organelles and control bioenergetics, cell survival, and cell death decisions. In the brain, ER calcium release has been found to directly contribute to excitotoxicity, a neuronal death mechanism important both in acute and chronic neurodegenerative diseases. It is now appreciated that these chaperones are also regulated by miRNAs.

miRNA regulation of molecular chaperones—There are to date a few studies of the regulation of molecular chaperones by miRNA. Recent publications have shown that the level of muscle-specific miR-1 changes in ischemic myocardium [89–91] and two of miR-1's targets are HSP60 and HSP70 [92]. miR-320 has been shown to be involved in the regulation of heart ischemia/reperfusion injury via targeting HSP20 [93]. The target was validated experimentally by utilizing a luciferase/green fluorescent protein reporter activity assay and examining the expression of HSP20 with miR-320 overexpression and knockdown in cardiomyocytes. Injection of miRNA extracted from hearts of ICR mice following ischemic preconditioning protected hearts against ischemia/reperfusion injury, possibly through upregulating HSP72, and the HSP70 transcription factor HSF-1 [94]. miRNA-1, miRNA-21, miRNA-24 and some additional miRNAs may be linked to the increased expression of the cytoprotective proteins in this study. Recently we demonstrated that a brain-enriched miRNA, miR-181, regulates GRP78 expression and outcome from cerebral ischemia (Fig. 3; Fig. 4) [19]. A reciprocal expression of miR-181a and GRP78 protein was found in both core and penumbra. Although *Grp78* mRNA was induced in both ischemic core and penumbra, GRP78 protein declined in the core following MCAO. The increased levels of miR-181a in the ischemic core could contribute to translational block and reduced levels of GRP78 protein. *In vitro* experiments shows that miR-181a mimic decreases and its inhibitor increases GRP78 protein expression [19].

D. miRNAs and the BCL2 family

BCL2 family and cerebral ischemia—The BCL2 protein family [95] is a principal regulator of apoptosis through regulating mitochondrial membrane integrity, function, and apoptotic signaling. The BCL2 protein family consists of 3 subgroups: the pro-survival proteins (BCL2, BCLxL, BCLw, MCL1 and A1), the multi-domain pro-apoptotic proteins BAX and BAK and the BH3 domain-only pro-apoptotic proteins (BIM, PUMA, BID, BAD, BIK, BMF, HRK, NOXA)(for reviews see [96–99]). In response to stress the decision to undergo apoptosis is determined by interactions between these 3 groups. BH3-only proteins are upregulated in response to apoptotic stimuli and transduce the damage signal. BH3-only proteins inhibit antiapoptotic proteins and activate pro-apoptotic proteins causing mitochondrial outer membrane permeabilization [96], cytochrome c release and activation of caspases to initiate apoptosis [100–102].

We and others have reported that overexpressing pro-survival BCL2 family members protects against cerebral ischemia *in vivo* [13,103] and *in vitro* [11]. Neuroprotection involved maintaining mitochondrial function (for review see [104]). Decreased BCL2 and increased BAX and BH3-only proteins were reported in CA1 neurons after global ischemia [105]. After global ischemia PUMA (p53-upregulated modulator of apoptosis), is upregulated in CA1 neurons, localizes to mitochondria, and binds BCLxL and BAX [106]. Selective CA1 injury induced by proteasomal inhibition was strongly reduced in PUMA knockout mice [107,108]. Interestingly anti-apoptotic protein BCL2 also exists in MAM (Fig. 2B) and affects ER and mitochondrial calcium homeostasis [109].

miRNA regulation of the BCL2 family—Compared to the number of studies on miRNA regulation of chaperones, there are more papers about the miRNA regulation of the BCL2 family, especially in cancer research [110–114]. miR-181b, miR-497, and miR-200 modulate multidrug resistance of human cancer cell lines by targeting BCL2 [114–116]. Experimental data indicate that miR-1 regulates cardiomyocyte apoptosis in cardiac ischemic injury through the posttranscriptional repression of BCL2 [90]. Abnormal expression of miR-34a may contribute to the pathogenesis of Alzheimer's disease by affecting the expression of BCL2 [117].

To date, only a few miRNAs have undergone extensive investigation to biologically validate a physiological effect through BCL2 family members following ischemic stroke. miR-15a has been shown to contribute to the pathogenesis of ischemic vascular injury through direct inhibition of the antiapoptotic gene *Bcl-2* [75]. miR-497 was demonstrated to directly hybridize to the predicted 3′UTR target sites of *Bcl-2* and regulates stress-induced neuronal death through inhibiting BCL2 translation [75,118]. *In vivo* repression of miR-497 using antagomirs was found to effectively lower miR-497 levels, reduce MCAO induced infarct, and improve neurological deficits with a corresponding increase in BCL2 protein [34]. We recently found that miR-181a reduction, which was associated with increased BCL2 and MCL1 protein levels, reduced glucose deprivation induced apoptosis, mitochondrial dysfunction, and loss of mitochondrial membrane potential in astrocytes [14]. Using the luciferase reporter assay we confirmed that BCL2 and MCL1 are the direct targets of mouse miR-181a. A prior report demonstrated a correlation between human miR-181a levels and BCL2 levels, but did not use a luciferase assay to validate the putative target sequence [119]. Positive luciferase results for human miR-181b against MCL1 have been found in studies on leukemia [120].

E. miRNAs which can target both chaperone and BCL2 families

Since targeting single genes for therapeutic intervention has not yet succeeded in the clinic, treatment using miRNAs, which coordinately regulate groups of coding genes, and which are likely to be easier to translate to clinical use, may represent part of the future of gene therapy in disease settings such as stroke. We recently reported that a highly brain-enriched miRNA, miR-181, targets both of these protective mechanisms – chaperone GRP78 (an HSP70 family member) and anti-apoptotic BCL2 family members BCL2 and MCL1. Inhibition of miR-181 was also found to protect from ischemia *in vitro* and *in vivo* [14,19].

In our experience, an mRNA with predicted conserved sites for miRNA families broadly conserved among vertebrates has a high probability to be validated as a real target by, for example, luciferase assay. According to this principle and using computational miRNA target prediction algorithms TargetScan (http://targetscan.org, Release 6.2), we found that only 4 out of hundreds of miRNAs have the potential to target both HSP70 family members and BCL2 family members in a highly conserved way (Table 1). miR-181 and miR-30 could potentially target the 3′UTRs of Hspa5 (GRP78), Bcl2 (BCL2) and Bcl2l11 (BIM). miR-181 can also target Mcl1 (MCL1) [14]. miR-200 could potentially target Hspa9 (GRP75) and Bcl2 (BCL2), and miR-17 target Hspa8 (HSP73) and Bcl2l2 (BCLw). Interestingly, from TargetSan, HSP72 has no conserved miRNA binding sites. We have validated both targets (GRP78 and BCL2) of miR-181 in our recent papers [14,19]. Experiments are ongoing in our laboratory to validate the other miRNAs which are predicted to target both chaperone HSP70 members and BCL2 family members. By targeting both GRP78 and anti-apoptotic BCL2 family members at the same time one miRNA such as miR-181 [14,19] can more effectively regulate cell fate after cerebral ischemia by targeting both chaperones and apoptosis regulatory proteins (Fig. 2C).

F. miRNA and the hypoxic response

Induction of miR-210 by hypoxia has been studied in both cancer cells and normal cells, and it has been shown to be a prominent component in the hypoxic response orchestrated by hypoxia-inducible factor (HIF) [121]. It has many identified targets including ones involved in angiogenesis, cell cycle, DNA repair, apoptosis and mitochondrial function [121]. Acute miR-210 overexpression protects from myocardial ischemia [122], and many observations demonstrate its survival benefit in cancer cells subjected to hypoxia. There are however also reports that miR-210 can reduce survival, increase apoptosis, and increase caspase activity [123–125] especially in non-hypoxic settings. Many reports demonstrate that miR-210 overexpression causes mitochondrial dysfunction, with validated targets in complexes I – IV plus a Krebs cycle protein [126]. Morphological alterations of mitochondria and reduced membrane potential occur with miR-210 overexpression [123,126]. Thus both cell type and context dependent components determine the overall effect of miR-210 increase, but it is likely to play an important role both in acute response to hypoxia/ischemia as well as the later modulation of gene expression and mitochondrial function during reperfusion.

G. miRNAs as biomarkers

Recent evidence suggests that circulating miRNAs may constitute excellent biomarkers for different human diseases [127]. Profiles composed of a few hundred miRNAs are more effective in cancer classification than profiles composed of thousands of mRNAs and may be particularly useful when the histopathological analysis is not informative [128,129]. miRNAs also have a prognostic significance, identifying differential chemotherapy response and survival [128,130]. Indeed, miRNA-based diagnostic assays have already been developed and approved for certain neoplastic diseases (rosettagenomics.com; asuragen.com). Another reason miRNAs may serve as potentially useful diagnostic biomarkers is the less invasive nature of sample procurement. Endogenous circulating miRNAs have been found to be stable because of their packaging and secretion into the blood within exosomes [131]. miRNAs have been consistently detected in serum, plasma and other bodily fluids and specific miRNA patterns have been associated with pregnancy and some diseases [132–134]. The potential diagnostic/prognostic value of miRNAs is unlikely to be limited to cancer. In the setting of cardiac disease plasma levels of miRNAs-208b and -499 have been shown to increase by more than 1000-fold after myocardial infarction [135].

In addition to the physical exam, clinical methods available for the diagnosis and prognosis of cerebral ischemia are largely limited to radiological imaging, which is associated with significant cost and sometimes limited availability. In addition obtaining scans can add to the time until a diagnosis is made. Given the limited recommended therapeutic window for thrombolysis (even the recent increase to 4.5 hours after the onset of symptoms [136]), biomarkers for stroke have the potential to expedite diagnosis and institution of treatment. Several studies have demonstrated increases in circulating miRNAs following stroke, in both animal and human models. Jeyaseelan *et al.* [16] demonstrated a large elevation in circulating miR-290 at 24 hours after reperfusion in rats subjected to middle cerebral artery occlusion. In similar rat models of stroke, Liu *et al.* [17] demonstrated increases in miR-10a, miR-182, miR-200b and miR-298 in both blood and brain 24 hours following ischemia/reperfusion [17], while Weng *et al.* [137] measured an increase in plasma levels of miR-124 as early as 6 hours following reperfusion. Unfortunately these studies did not demonstrate changes in the miRNA profile in the period immediately following ischemia, prior to reperfusion, which would be clinically relevant in determining a diagnosis and instituting treatment in people suffering acutely from stroke.

In human subjects, Tan *et al*. [69] performed miRNA profiling from whole blood in young stroke patients 6–18 months following stroke, and noted an increase in 138 miRNAs. Subsequently Gan *et* al. [138] demonstrated an approximately 2-fold elevation in miR-145 in whole blood of stroke patients relative to controls, however the time course of measurement was not described. Zeng *et* al. [76] measured serum miR-210 levels in patients within 3, 7, and 14 days of suffering a stroke, and observed that a decrease in miR-210 was associated with poorer clinical outcome. These studies collectively suggest that stroke induces changes in the miRNA profile of both brain and peripheral blood, however further work is needed to clarify the time course of expression, and to correlate miRNA changes with the severity of disease and outcome in order for them to become a clinically useful biomarker.

H. miRNAs as therapeutic targets

The identification of miRNAs as key regulators of gene expression has opened a new field of research for not only biomarkers and but also therapeutics of ischemic disease. Despite being only about 22nt long, miRNAs are thought not to readily cross the blood brain barrier due to their strongly hydrophilic nature. Several factors including blood brain barrier penetration and stability affect the potential therapeutic use of miRNAs in the brain. Locked nucleic acids (LNA) and other methods to chemically modify miRNA can alter their stability and ability to cross the blood brain barrier. Since both increased and decreased miRNA levels may be needed either as prevention or treatment of stroke, both ways to increase and decrease miRNA in the brain are currently being developed. Following a stroke the blood brain barrier is damaged so there is easy access to the affected area, but novel approaches are being developed to get miRNA related therapeutics into the brain across an intact blood brain barrier, including chemical modification, use of targeting molecules such as rabies virus glycoprotein and methods to disrupt the blood brain barrier such as mannitol [139], and encapsulation with pegylated immunoliposomes and use of plasmids encoding either pre- or pri-miRNA or short hairpin RNA [140,141].

Antagomirs were shown to efficiently target miRNAs when injected locally in the mouse cortex [142]. The potential for antagomir therapy has also been proven in the context of acute ischemic stroke in mice in which regulation of miR-181 affects the extent of brain injury [19]. In response to focal cerebral ischemia in the mouse we find that miR-181 increases in the core, where cells die, but decreases in the penumbra, where cells survive (Fig. 3A). Knockdown of miR-181 by intracerebroventricular infusion of its antagomir effectively reduced the infarction size and protected the penumbra (Fig. 4). So strategies reducing or blocking miR-181a protect the brain from stroke. Shi et al. [143] recently demonstrated that miR-29b is upregulated in rat brains following MCAO, and that targeted inhibition was cytoprotective in cultured neurons subjected to oxygen-glucose deprivation, functioning through the apoptotic regulator BCL2L2. Yin et al. [34] showed therapeutic efficacy of intracerebroventricular administration of miR-497 antagomir resulting in smaller infarct area following MCAO. Moreover, Selvamani *et al*. utilized antagomir-based therapy to demonstrate complex sex-specific miRNA interactions whereby intracerebroventricular infusion of Let7f antagomir exhibited neuroprotection only in intact female rats, but not males or ovariectomized females [144]. Using this miRNA antisense model, Buller et al. [77] demonstrated in a rat model of middle cerebral artery occlusion that miR-121 selectively downregulates Faslg, a TNFa family member, and that targeted inhibition of miR-121 was cytoprotective *in vivo*.

The viability of miRNAs as therapeutic targets is confirmed by the fact that a phase I clinical trial of an anti-miRNA was successfully concluded (santaris.com). In this case, a locked nucleic acid-based anti-miRNA targeting a liver specific miRNA, miR-122 (miravirsen or SPC3649), is being developed as a hepatitis C therapy. SPC3649 is currently

being tested is a Phase IIa clinical trial of previously untreated hepatitis C patients. In contrast to antagomir-directed miRNA silencing, miRNA overexpression strategies also exist and may be helpful in certain settings. Systemic delivery of miR-34a in a lipid-based delivery vehicle has been shown to block lung tumor growth *in vivo* [145]. Overexpressing miR-210 can improve cardiac function in a murine model of ischemic heart disease [122].

Because of their ability to simultaneously regulate many target genes, miRNAs are especially important candidates for stroke therapeutics. Recent evidence suggests that gene products targeted by one miRNA may belong to the same functional protein interaction network [146–148]. Hence it will be of utmost importance to gain a detailed knowledge of these targeted networks before interfering with the regulatory processes. Given the complexity of pathophysiological molecular signaling in the context of ischemic stroke, it comes as no surprise that targeting single genes for therapeutic intervention have failed in the clinic. Targeting noncoding genes such as miRNAs, which have the capacity to regulate large sets of evolutionary conserved coding genes, represents the future of gene therapy.

I. Conclusions

Ongoing research shows that miRNAs are key regulators in almost all cellular processes. miRNAs likely represent both new biomarkers and new therapeutic targets for many human diseases. The modulation of gene expression by miRNAs is an essential part of the coordinated gene regulation necessary for the complex dynamics of the central nervous system. Given that a single miRNA regulates several related mRNAs, it is not difficult to imagine a “silver bullet” treatment, whereby a single miRNA or possibly a small number of miRNAs, could coordinate several related pro-survival and pro-death pathways to improve outcome. However, the multiplicity of binding targets also poses potential difficulties in obtaining specificity of targeting leading to unexpected side effects. Although miRNAs represent a potentially powerful treatment paradigm, much work remains to be done in describing individual specific miRNA-mRNA interactions, understanding the regional and cell type specific distribution of miRNAs within the nervous system, and in developing organ-targeted delivery systems to optimize the therapeutic potential of miRNAs.

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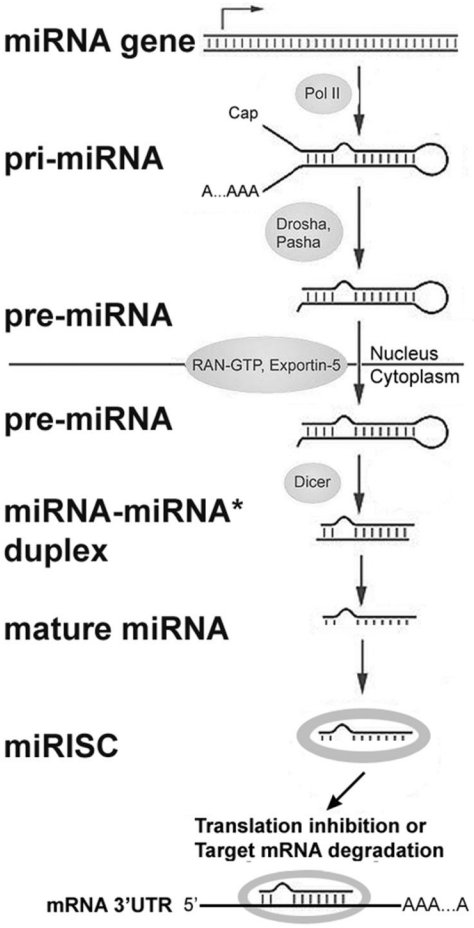

Fig. 1.
miRNA biogenesis and function

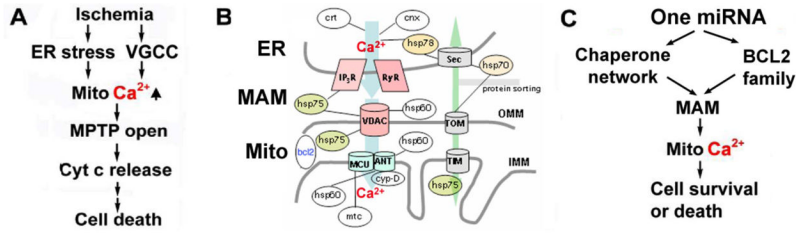

Fig. 2.

A. Diagram of cerebral ischemia induced cell death signaling cascade. B. Chaperone network and BCL2 family members control ER-mitochondria Ca^{2+} crosstalk (left) and protein import/sorting (right) at the mitochondrial associated ER membrane (MAM). C. miRNA can influence MAM and cell survival by targeting both the chaperone network and BCL2 family members. Abbreviations: OMM-mitochondrial outer membrane; IMM-mitochondrial inner membrane calcium binding proteins crt-calreticulin; cnx-calnexin; Sec protein import complex. Channels involved in calcium passage IP3R –inositol 1,4,5 trisphosphate receptor, VDAC- voltage dependent anion channel; Members of the HSP70 family are shown in shades of yellow (Hsp70, 75, 78); cyp-D cyclophilin-D; mtc mitocalcin; ANT adenine nucleotide translocase; TOM outermembrane translocase complex; TIM translocase complex of inner membrane; MCU mitochondrial calcium uniporter.

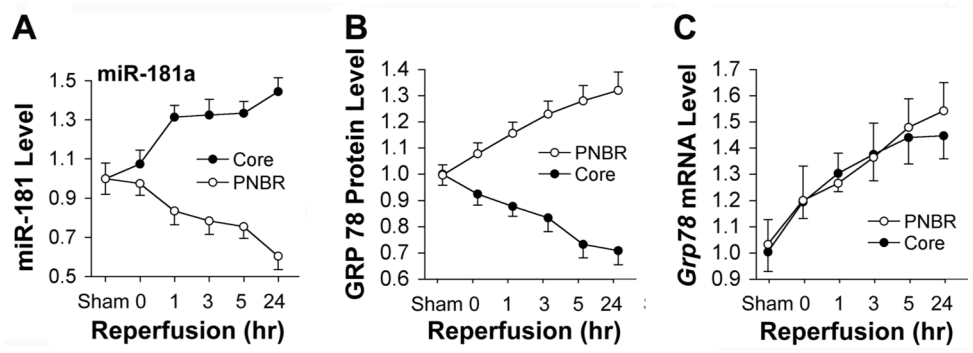

Fig. 3.

Expression of miR-181, GRP78 protein, and *Grp78* mRNA at different reperfusion time points after 1 hour transient focal cerebral ischemia. A. miR-181a expression in ischemic core and penumbra at different durations of reperfusion after middle cerrebral artery occlusion in mice shows increased levels in core but decreased levels in the penumbra (PNBR). B. GRP78 protein decreases in the ischemic core and increases in the penumbra with increasing durations of reperfusion after MCAO. Quantitation by densitometry of westerns for each time point. C. Expression of *Grp78* mRNA (RT-PCR) in ischemic core and penumbra increases with reperfusion time after MCAO. N=4 mice/group in all experiments. *P<0.05 by ANOVA and Newman-Keuls post hoc test. From [19].

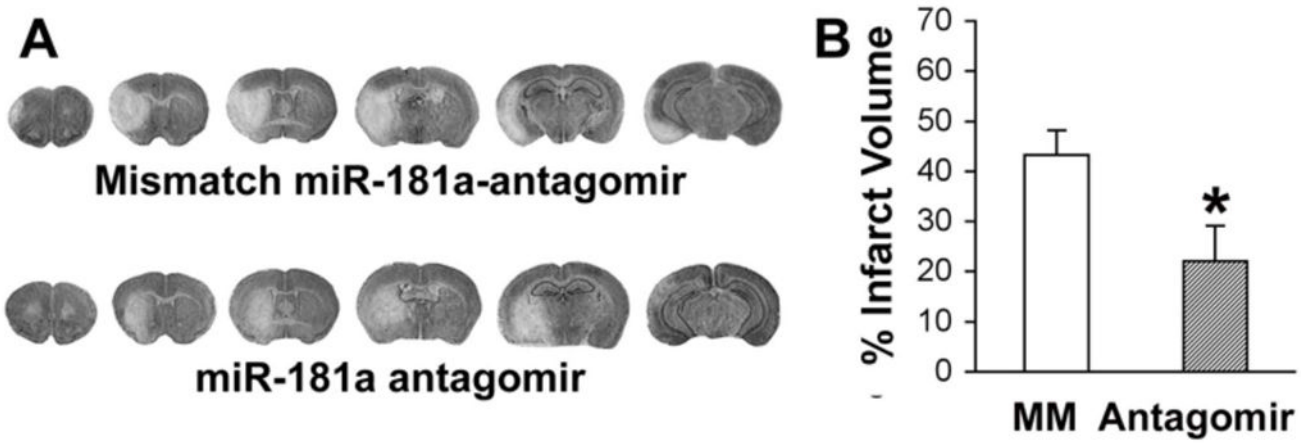

Fig. 4.

Effect of miR-181 down-regulation on infarction after focal ischemia. A. Representative cresyl violet-stained coronal sections demonstrate a decreased infarct size in a representative miR-181a antagomir transfected brain compared with the brain of a mismatch (MM) miR-181a-antagomir-injected animal also subjected to middle cerebral artery occlusion. B. The graph shows quantification of the infarct size. N=7 mice/group. *P<0.01 compared to MM control by T-test. From [19].

Table 1

miRNAs that may target both chaperones and BCL2 family members

miRNAs	Chaperones	BCL2 family
miR-181	GRP78	BCL2, MCL1, BIM
miR-30	GRP78	BCL2, BIM
miR-200	GRP75	BCL2
miR-17	HSP73	BCLw