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61/542,125 30 September 2011 (30.09.2011) US(71) Applicant (for all designated States except US): **THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY** [US/US]; Office Of Technology Licensing, 1705 El Camino Real, Palo Alto, CA 94306 (US).

(72) Inventors; and

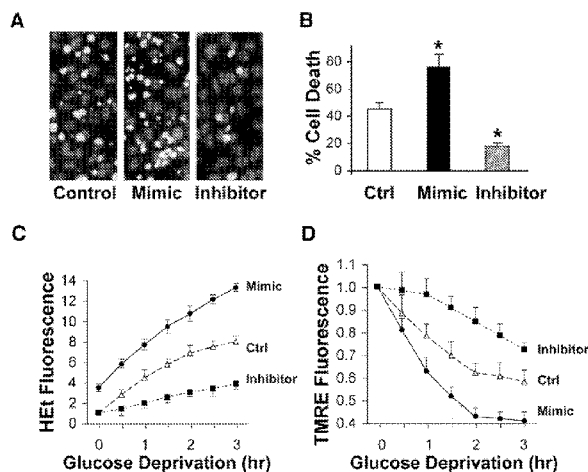
(75) Inventors/Applicants (for US only): **GIFFARD, Rona, G.** [US/US]; 770 Anderson Drive, Los Altos, CA 94024 (US).**OUYANG, Yibing** [US/US]; 38594 Whitman Terrace, Fremont, CA 94536 (US).(74) Agent: **BUCHBINDER, Jenny**; 29 Clinton Street #201, Redwood City, CA 94062 (US).

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(54) Title: MICRO RNAS TO TREAT STROKE, ISCHEMIC BRAIN INJURY, TRAUMATIC BRAIN INJURY, AND NEURODEGENERATIVE DISEASE



(57) Abstract: Compositions and methods for treating stroke, ischemic brain injury from cardiac arrest and resuscitation, traumatic brain injury, and neurodegenerative disease are disclosed. In particular, the invention relates to the use of miR-181, miR-29, and microRNA mimics and inhibitors thereof to modulate levels of Hsp-70 family chaperones and Bcl-2 family anti-apoptotic and pro-apoptotic proteins for treatment of neurological disorders.

Figure 4



MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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MICRO RNAS TO TREAT STROKE, ISCHEMIC BRAIN INJURY, TRAUMATIC BRAIN INJURY, AND NEURODEGENERATIVE DISEASE

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STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with Government support under contracts GM49831 and NS053898 awarded by the National Institutes of Health. The Government has certain rights in this invention.

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TECHNICAL FIELD

The present invention pertains generally to compositions and methods for treating stroke, ischemic brain injury from cardiac arrest and resuscitation, traumatic brain injury, and neurodegenerative disease. In particular, the invention relates to the use of miR-181, miR-29, and microRNA mimics and inhibitors thereof to modulate levels of Hsp-70 family chaperones and Bcl-2 family anti-apoptotic and pro-apoptotic proteins for treatment of neurological disorders.

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BACKGROUND

MicroRNAs are post-transcriptional regulators that provide tissue and cell type specific control of protein expression (Bartel (2009) Cell 136 (2):215–233; Bartel (2004) Cell 116 (2):281–297). Consisting of small, noncoding RNA molecules of approximately 21 to 23 nucleotides, microRNAs modulate protein expression by binding to complementary or partially complementary target messenger RNAs (mRNAs), thereby targeting mRNAs for degradation or translational inhibition. MicroRNA binding sites are frequently located in the 3'-untranslated region (UTR) of the target mRNA, but may also be located in coding regions of the mRNA. The 5'-region of the miRNA, typically nucleotides 2–7 of the miRNA, is termed the “seed sequence” and is particularly important for mRNA repression by miRNA. Because the seed sequence or sequence recognition site is short, generally only 6 base pairs long, each miRNA has many targets, and each mRNA can be targeted by multiple miRNAs.

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In mammals, specific miRNAs are known to control processes such as development, neuronal cell fate, apoptosis, proliferation, adipocyte differentiation,

hematopoiesis, and exocytosis, as well as diseases such as cancer and neuronal disorders. Recently, a few groups have shown the involvement of miRNAs in the pathogenesis of ischemic brain injury by using miRNA profiling techniques in a rat middle cerebral artery occlusion (MCAO) model (Dharap et al. (2009) J. Cereb.

- 5 Blood Flow Metab. 29:675-687; Jeyaseelan et al. (2007) Expert Opin. Ther. Targets 11:1119-1129; Liu et al. (2010) J. Cereb. Blood Flow Metab. 30:92-101) and a forebrain ischemic model (Yuan et al. (2010) J. Clin. Neurosci. 17:774-778), as well as in stroke patients (Tan et al. (2009) PLoS One 4, e7689). These findings suggest that several miRNAs may be good potential therapeutic targets for treatment of
- 10 stroke.

- The Bcl-2 family of pro- and anti-apoptotic proteins plays a key role in regulation of apoptosis through the mitochondrial pathway. Bcl-2 proteins can be subdivided into three groups: The first group consists of the anti-apoptotic multidomain members of the Bcl-2 family, such as the prototype B cell lymphoma-2
- 15 (Bcl-2), which contain BH1-4 domains. The second group consists of the pro-apoptotic multidomain proteins, which are commonly designated as belonging to the Bax subfamily and contain BH1-3 domains. The third group consists of proteins containing only the BH3 domain, and these proteins are pro-apoptotic (Adams and Cory (2007) Current Opinion in Immunology 19:488-496). Bcl-2 proteins control
- 20 apoptosis by regulating opening of the mitochondrial membrane permeability pore. In addition to their role in regulating mitochondrial permeability, Bcl-2 proteins may also play a role in regulating mitochondrial fission and fusion (Rolland and Conradt (2010) Current Opinion in Cell Biology 22:852-858) and cellular homeostasis, in particular, metabolism, calcium signaling, endoplasmic reticulum function, and
- 25 autophagy (Danial et al. (2010) Advances in Experimental Medicine and Biology 687:1-32).

- The regulation of the Bcl-2 family in response to cerebral ischemia is not fully understood. Bcl-2 normally decreases after brain ischemia (Martinez et al. (2007) Journal of Molecular Histology 38:295-302). Overexpression of pro-survival Bcl-2
- 30 family proteins has been shown to protect neural tissue against cerebral ischemia *in vivo* (Kitagawa et al. (1998) Stroke 29:2616-2621; Zhao et al. (2003) Journal of Neurochemistry 85:1026-1036) and *in vitro* (Xu et al. (1999) Neuroscience Letters 277:193-197). Pro-survival Bcl-2 proteins may provide neuroprotection, in part, by

helping to preserve mitochondrial function (for review see Ouyang and Giffard (2004) Cell Calcium 36:303-311).

Heat shock proteins of the 70 kD (HSP70) family include highly conserved chaperones that assist protein folding and help protect cells from stress. Regional and cellular distributions of HSP72 are altered in response to cerebral ischemia (Sharp et al. (1993) Stroke 24:172-75). In animal models of stroke, HSP70 family members have been shown to be protective. After global ischemia, HSP72 is induced primarily in CA3 pyramidal neurons and dentate granule cell neurons that survive ischemia, whereas HSP72 is not induced in CA1 pyramidal neurons that are destined to die (Vass et al. (1988) Acta Neuropathol. 77:128-135). After focal cerebral ischemia, expression of HSP72 is induced in the penumbra area surrounding the infarction (Kinouchi et al. (1993) J. Cereb. Blood Flow Metab. 13:105-115 and Kinouchi et al. (1993) Brain Res. 619:334-338). It appears that HSP72 is primarily induced in cells that survive ischemic injury and is protective to cells from subsequent lethal injuries.

Stroke is the third leading cause of death in the United States and the most common cause of neurological disability. The only currently approved therapy for stroke is clot lysis with a thrombolytic drug. This treatment is only effective in the first 3 to 4 hours after thrombosis, and cannot be used to treat hemorrhagic stroke. Currently, less than 5% of patients receive this treatment, so there is an urgent need for better acute treatments as well as treatments that will lead to improved neurological recovery.

SUMMARY

The present invention is based, in part, on the discovery that miR-181, miR-29, and microRNA mimics and inhibitors thereof can be used to modulate levels of Hsp-70 family chaperones and Bcl-2 family anti-apoptotic and pro-apoptotic proteins in the brain for treatment of neurological disorders. Thus, the present invention pertains generally to compositions and methods for using the mir-181 and mir-29 families of microRNAs, mimics, and inhibitors to increase levels of beneficial proteins, such as chaperones (e.g., Hsp70 family chaperones, such as Grp78) and anti-apoptotic proteins (e.g., Bcl-2 family anti-apoptotic proteins, such as Bcl-2 or Mcl-1), or to decrease levels of detrimental proteins, such as pro-apoptotic proteins (e.g., pro-apoptotic Bcl-2 family members, such as Bcl-2-L11, PUMA, or BMF) for treatment

of neurological disorders in which manipulating levels of mir-181 or mir-29 is therapeutic.

In one aspect, the invention includes a method for treating a subject having a neurological disorder by administering a therapeutically effective amount of one or more of a miR-181, a miR-181 mimic, a miR-181 inhibitor, a miR-29, a miR-29 mimic, or a miR-29 inhibitor to the subject. Neurological disorders that can be treated by methods of the invention include, but are not limited to, stroke, ischemic brain injury from cardiac arrest and resuscitation, traumatic brain injury, and neurodegenerative disease. MicroRNA or miRNA mimics can be used to reduce translation or increase degradation of the mRNA targeted by the miRNA or miRNA mimic. MicroRNA antagonists or inhibitors can be used to increase translation of the mRNA targeted by the miRNA that is inhibited by the miRNA inhibitor. In certain embodiments, the inhibitor is an antagomir, an antisense oligonucleotide, or an inhibitory RNA. In one embodiment, the inhibitor comprises the sequence of SEQ ID NO:9. In other embodiments, the miR-29 comprises a sequence selected from the group consisting of SEQ ID NOS:31-33. In some embodiments, the miR-181, miR-181 mimic, miR-181 inhibitor, miR-29, miR-29 mimic, or miR-29 inhibitor is expressed *in vivo* from an expression vector.

An effective amount of a miR-181, a miR-181 mimic, a miR-181 inhibitor, a miR-29, a miR-29 mimic, or a miR-29 inhibitor can be administered to a subject in one or more administrations, applications or dosages. By “therapeutically effective dose or amount” of a miRNA, miRNA mimic, or miRNA inhibitor is intended an amount that, when administered, as described herein, brings about a positive therapeutic response, such as improved neurological recovery from stroke or ischemic brain injury caused by cardiac arrest and resuscitation or traumatic brain injury or chronic neurodegenerative disease. Improved neurological recovery may include a reduction in cerebral infarction size, improved motor function, or improved cognition.

In certain embodiments, the invention includes a method for inhibiting miR-181 in a neuron cell, glial cell, or endothelial cell by introducing an effective amount of a miR-181 inhibitor into the cell. The glial cell may be microglia or macroglia (e.g., astrocytes, oligodendrocytes). As a result of inhibiting miR-181, the amount of Grp78, Bcl-2 or Mcl-1 in the cell is increased compared to the amount of Grp78, Bcl-2 or Mcl-1 in the cell in the absence of the inhibitor.

In certain embodiments, the invention includes a method for increasing the amount of a Bcl-2 family anti-apoptotic protein in a neuron cell, glial cell, or endothelial cell by introducing an effective amount of a miR-181 inhibitor into the cell. The glial cell may be microglia or macroglia (e.g., astrocytes, oligodendrocytes).
5 As a result of inhibiting miR-181, the amount of Grp78, Bcl-2 or Mcl-1 in the cell is increased compared to the amount of Grp78, Bcl-2 or Mcl-1 in the cell in the absence of the inhibitor.

In certain embodiments, the invention includes a method for decreasing the amount of a Bcl-2 family pro-apoptotic protein in a neuron cell, glial cell, or
10 endothelial cell by introducing an effective amount of a miR-29 or miR-29 mimic into the cell. The glial cell may be microglia or macroglia (e.g., astrocytes, oligodendrocytes). As a result of introducing miR-29 or a miR-29 mimic into the cell, the amount of PUMA or BMF in the cell is decreased compared to the amount of PUMA or BMF in the cell in the absence of the miR-29 or a miR-29 mimic. The
15 miR-29 may comprise a sequence selected from the group consisting of SEQ ID NOS:31-33.

In another aspect, the invention includes compositions for treatment of a neurological disorder. The compositions may comprise one or more of a miR-181, a miR-181 mimic, a miR-181 inhibitor, a miR-29, a miR-29 mimic, or a miR-29
20 inhibitor. In certain embodiments, compositions may further comprise a pharmaceutically acceptable carrier. Compositions may be administered into the brain or spinal cord of a subject. Compositions may be administered by any suitable method, including but not limited to, intracerebroventricularly, intraparenchymatously, intralesionally, intracerebrally, intraneurally, intraspinally,
25 intravenously, or intra-arterially. In one embodiment, compositions are administered by stereotactic injection into the brain.

These and other embodiments of the subject invention will readily occur to those of skill in the art in view of the disclosure herein.

30 **BRIEF DESCRIPTION OF THE FIGURES**

Figures 1A-1E show pri-miR-181, 3'UTRs of Bcl-2 family and vectors. Figure 1A shows a schematic representation of the genomic organization of the mouse miR-181. Figure 1B shows the sequences of mature wild type (WT) and seed

mutated (SM) miR-181a, miR-181b, miR-181c, and miR-181d (SEQ ID NOS:1-8). Figure 1C shows a schematic representation of the vector MWX-PGK-IRES-GFP containing cloned miR-181. Figure 1D shows the sequences of wild type (WT) and seed mutated (SM) 3'UTRs of Bcl-2, Bcl-2-L11, and Mcl-1 (SEQ ID NOS:17-22).

5 Figure 1E shows a schematic representation of the Renilla luciferase reporter vector phRL-TK containing cloned 3'UTR or SM-3'UTR.

Figures 2A-2C present data showing that miR-181 targets three members of the Bcl-2 family. Figure 2A shows sequence alignments of sequences SEQ ID NOS:59-110, produced using TargetScan. The alignments show that the seed
10 sequences of Bcl-2, Bcl-2-L11, and Mcl-1 3'UTRs, which are targeted by miR-181, are highly conserved across species. Figure 2B shows dual luciferase activity assays, which demonstrated that miR-181 recognizes all of these 3'UTRs. The assays were performed in BOSC23 cells cotransfected with the plasmid containing luciferase, the Bcl-2 or Bcl-2-L11 or Mcl-1 3'UTR (WT), and plasmids encoding either pri-miR-
15 181ab or pri-miR-181cd or their seed mutants (SM). Figure 2C shows dual luciferase assays, which were performed with the wild type 3'UTRs of Bcl-2, Bcl-2-L11, or Mcl-1 (WT) or their seed mutants (SM). These assays showed that miR-181ab and miR-181cd both reduced luciferase activity. Luciferase assays were performed in triplicate (*P<0.01 compared to the SM group).

20 Figures 3A-3E show expression of miR-181 and Bcl-2 family proteins after transfection of astrocytes with a miR-181a mimic or inhibitor. Figure 3A shows that transfection with increasing amounts of miR-181a mimic increased levels of miR-181a up to 16-fold. Figure 3B shows that transfection with increasing amounts of miR-181a inhibitor decreased levels of miR-181a by as much as 58%. Figure 3C
25 shows that mitochondrial morphology changed from a threadlike network (upper) to fragmented round dots (lower) after transfection with 50 pmol miR-181a mimic. Micrographs were taken after staining cells with tetramethylrhodamine methyl ester. Figure 3D shows relative miR-181a levels in astrocytes, which were measured after different durations *in vitro*. Figures 3E and 3F show that expression of Bcl-2 and
30 Mcl-1 protein, respectively, in primary astrocyte cultures was significantly decreased by transfection with miR-181a mimic and significantly increased by transfection with inhibitor (N=3). All experiments were performed in triplicate. *P<0.01 compared to the control (Ctrl).

Figures 4A-4D show the effects of miR-181a mimic and inhibitor on astrocyte ischemia-like injury *in vitro*. Figure 4A shows micrographs of cultures stained with propidium iodide (light gray, dead cells) and Hoechst dye (dark gray, live cells). The micrographs show that miR-181a mimic aggravated and the miR-181a inhibitor
 5 reduced injury induced by 24 hours of glucose deprivation (GD) in astrocytes. Figure 4B presents a bar graph showing quantitation of cell death by cell counting. The asterisk (*) indicates results that were significantly different in cultures treated with miR-181a mimic or inhibitor compared to untreated control (Ctrl) cultures subjected to the same injury. Figure 4C shows that transfection with miR-181a mimic or
 10 inhibitor affected the time course of reactive oxygen species (ROS) generation. Increasing ROS was detected as increasing H₂O₂ fluorescence due to GD stress. Figure 4D shows that increased miR-181a mimic and inhibitor altered the time course of change in mitochondrial membrane potential induced by GD as assessed by TMRE fluorescence.

Figures 5A-5C present data showing that HSPA5 is the target of miR-181. Figure 5A shows sequence alignments of sequences SEQ ID NOS:111-122 using TargetScan. The alignments show that the seed sequence of the HSPA5 3'UTR is highly conserved at nucleotides 86-92 across species. Figure 5B shows that dual luciferase activity assays of cultured astrocytes, co-transfected with the plasmid
 20 containing the HSPA5 3'UTR (HSPA5-WT) or its seed mutant (HSPA5-SM) and either miR-181 or their seed mutants, validated that miR-181 inhibited luciferase activity (*P<0.001 is statistically different from the control (Ctrl) group). Figure 5C shows expression levels of cerebral miR-181 in the cortex of normal control brains, which were measured for miR181a, miR181b, miR181c, and miR181d. The miR181a
 25 had the highest level of expression, which was greater than that of miR181b, which was greater than that of miR181d, which was greater than that of miR181c, which had the lowest level of expression.

Figures 6A and 6B show the expression of miR-181, GRP78 and grp78 mRNA after focal ischemia. Figure 6A shows expression of miR-181a in ischemic
 30 core and penumbra (PNBR), which was measured at different hours of reperfusion after one hour of middle cerebral artery occlusion (MCAO) in mice. Figure 6B shows expression of GRP78 and grp78 mRNA in ischemic core and penumbra, which was

measured at different hours of reperfusion after one hour of MCAO in mice (N=3 in each group).

Figures 7A-7C show the effects of miR-181 up- or down-regulation on ischemic infarction after focal ischemia. Figure 7A shows up- or down-regulation of miR-181a, which was measured in the brains of mice pretreated with either pri-miR-181a plasmid or miR-181a antagomir (N=4 in each group). Figure 7B shows GRP78 protein levels, which were measured in the brains of mice pretreated with either pri-miR-181a plasmid or miR-181a antagomir (N=4 in each group). Figure 7C shows representative cresyl violet-stained coronal sections, which demonstrated an enhancement in infarct size when miR-181a was overexpressed in brains compared with animals injected with the miR-181 seed mutant (SM) and a reduction in infarct size when miR-181a expression was inhibited in brains of animals injected with the miR-181a-antagomir compared with animals injected with the mismatched (MM) miR-181a-antagomir (negative control). Bar graphs show quantification of infarct volumes (*P<0.05 compared to the related control. N=7 in each group).

Figures 8A-8F show the effects of miR-181a up- or down-regulation on ischemia-like cell injury *in vitro*. Figure 8A shows micrographs of cultures stained with propidium iodide (light gray, dead cells) and Hoechst dye (dark gray, live cells). The micrographs show that the miR-181a mimic aggravated and the miR-181a inhibitor reduced injury induced by 24 hours of glucose deprivation (GD) in astrocytes. The bar graph shows the quantification of the percentage cell death. Figure 8B shows that pri-miR-181a, overexpressed in cells transfected with plasmid, also aggravated cell injury under 24 hours of GD. Figure 8C shows that transfection of miR-181a increased reactive oxygen species (ROS) significantly compared to its seed mutant (SM) control (Ctrl) in unstressed cells, which was determined by hydroethidine (Het) fluorescence measurements. Figure 8D shows that miR-181a does not influence mitochondrial membrane potential under unstressed conditions, which was determined using tetramethylrhodamine methyl ester (TMRE) fluorescence. Figure 8E shows time courses for the change of HET fluorescence under glucose deprivation stress for the miR-181a and its mutant and inhibitor. Figure 8F shows time courses for the change of TMRE fluorescence under glucose deprivation stress are shown for the miR-181a and its mutant and inhibitor. (*P<0.05 statistically different from control (Ctrl) group. Trans: GRP78 transfected. Scale bars, 25 μ m.)

Figures 9A-9C show that the miR-181 family does not target other chaperone genes. Dual luciferase activity assay of cultured astrocytes, co-transfected with a plasmid containing the 3'UTR of (Figure 9A) HSPA1A or (Figure 9B) HSPA9 or (Figure 9C) HSP90 and either miR-181ab, miR-181cd, or their seed mutants, showed that miR-181 does not inhibit luciferase activity.

Figure 10 shows miR-181b, miR-181c and miR-181d expression in ischemic core and penumbra at different hours of reperfusion after 1 hour of MCAO in mice.

Figures 11A-11C show the dose-response of miR-181 transfection with miR-181 mimic (Figure 11A), miR-181 inhibitor (Figure 11B), and miR-181a seed mutant and miR-181a plasmid (Figure 11C).

Figure 12A shows a schematic representation of the genomic organization of mouse miR-29. The two clusters are on chromosomes 1 and 6. Figure 12B shows the sequences of mature wild type (WT) and seed mutated (SM) miR-29a, miR-29b and miR-29c (SEQ ID NOS:31-36). SM are used as negative controls. Figure 12C shows a schematic of the vector MWX-PGK-IRES-GFP, which was used to express miR-29.

Figure 13 shows that miR-29 could potentially target 5 members of the Bcl-2 family. The seed sequences of BAK1, BBC3, BMF, BCL2L2 and MCL1 3'UTRs (SEQ ID NOS:37-48) are highly conserved across species (from TargetScan).

Figure 14 A shows the relative miR-29a levels in primary cultures of cortical neurons and astrocytes at 7 and 21 days *in vitro*, and in rat brain cortex at postnatal day 7 and 21. All values were normalized to the neuronal miR-29a level at 7 days. Figure 14B shows the relative levels of miR-29a, b, and c in normal rat hippocampus. Figure 14C shows the increase in levels of miR-29a in the hippocampal dentate gyrus (DG) area and the decrease in the *Cornu Ammonis* (CA) 1 area after 10 minutes of forebrain ischemia and 0 to 5 hours reperfusion. (N=4/group)

Figure 15A shows that pri-miR-29ab (ab) induced increased levels of miR-29a in cortical astrocytes following transfection compared to its seed mutant (ab-SM) and vector controls. Figure 15B shows the dose-response of miR-29a levels to transfection with increasing amounts of miR-29a mimic. Figure 15C shows the effects of the inhibitor on primary cultures of astrocytes relative to the control (Ctrl=1). Figure 15D shows that the miR-29a mimic reduces injury induced by 24 hours of glucose deprivation (GD) in primary astrocyte cultures. Figure 15E shows

that the miR-29a inhibitor aggravates cell injury induced by 24 hours of GD. All experiments were performed in triplicate (*P<0.01 compared to the control).

Figure 16A shows that rats treated with the pri-miR-29ab plasmid have elevated hippocampal levels of miR-29a. Levels of miR-29a were measured by RT-qPCR. Figure 16B shows that rats treated with the miR-29a antagomir have reduced hippocampal miR-29a (*P<0.01 compared to seed mutant (SM) or Ctrl group. N=3/group). Figure 16C shows that the pri-miR-29ab plasmid or miR-29a antagomir was injected stereotactically unilaterally just above CA1 two days before forebrain ischemia. Selective loss of CA1 hippocampal neurons (between white arrows) was observed at 6 days of reperfusion by cresyl violet staining (ischemia control). Loss of CA1 neurons was markedly reduced in the pri-miR-29ab injected brain and increased in the antagomir injected brain. Extensive loss of CA1-4 was observed in rats treated with the antagomir.

Figure 17A shows that increasing miR-29a with mimic reduces mitochondrial membrane depolarization in cortical astrocytes subjected to 3 hours of GD, whereas the miR-29a inhibitor increases depolarization. Depolarization is indicated by decreased fluorescence. Fluorescence values are normalized to the starting fluorescence of 1.0. Figure 17B shows that three hours of GD increased ROS in astrocytes. Transfection with the miR-29a mimic reduced ROS whereas transfection with the inhibitor increased ROS. Increasing H₂O₂ fluorescence indicates increasing ROS. Experiments were performed in triplicate (*P<0.01 compared to the control).

Figure 18A shows that PUMA (BBC3) and BMF are targets of miR-29. Dual luciferase activity assays using co-transfection with a plasmid containing luciferase followed by the BBC3 or BMF 3'UTR (WT) and plasmids encoding either pri-miR-29 or their seed mutants (SM) demonstrated that miR-29ab, but not miR-29c, recognizes both 3'UTRs. Figure 18B shows the same assay performed with the wild type 3'UTRs of BBC3 or BMF 3'UTR (WT) or their seed mutants (SM). The miR-29ab reduced luciferase activity. Assays were performed in triplicate (*P<0.01 compared to the miR-29ab-SM or 3'UTRs-SM group).

Figure 19A shows that PUMA protein levels are decreased in the hippocampus of rats pretreated with pri-miR-29ab plasmid. Figure 19B shows that PUMA protein levels increased in brains pretreated with miR-29a antagomir.

Representative immunoblots are shown above the graphs (N=4 rats in each group, *P<0.01 compared to SM or Ctrl group).

DETAILED DESCRIPTION

5 The practice of the present invention will employ, unless otherwise indicated, conventional methods of pharmacology, chemistry, biochemistry, recombinant DNA techniques and immunology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., Blackwell Scientific Publications); A.L. 10 Lehninger, *Biochemistry* (Worth Publishers, Inc., current addition); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (3rd Edition, 2001); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.).

 All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entireties.

15

I. DEFINITIONS

 In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

 It must be noted that, as used in this specification and the appended claims, the 20 singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a miRNA" includes a mixture of two or more miRNA, and the like.

 As used herein, the terms "microRNA," "miRNA," "mature microRNA," and "mature miRNA" refer to a non-coding single-stranded RNA molecule that is about 25 19 to about 25 nucleotides in length (including about 19, about 20, about 21, about 22, about 23, about 24, and about 25 nucleotides) that effectively reduces the expression level of target polynucleotides and polypeptides through the RNA interference pathway (i.e., through association with the RISC and subsequent degradation of target mRNA or translational inhibition). The term "microRNA" refers to both endogenous 30 miRNAs that have been found in any organism (e.g., plants, animals) and artificial miRNAs that include single-stranded RNA molecules with sequences of about 19-25 nucleotides in length other than those found in endogenous miRNAs that effectively reduce the expression of target polynucleotides through RNA interference.

A "target site" is the nucleic acid sequence recognized by a microRNA. A single target site typically has about six to about ten nucleotides. Typically, the target site is located within the 3'UTR of a mRNA, but the target site may also be located in the 5'UTR or the coding region of a mRNA.

5 "Administering" an expression vector, nucleic acid, microRNA, microRNA mimic, or microRNA inhibitor to a cell comprises transducing, transfecting, electroporating, translocating, fusing, phagocytosing, shooting or ballistic methods, etc., i.e., any means by which a nucleic acid can be transported across a cell membrane.

10 The term "derived from" is used herein to identify the original source of a molecule but is not meant to limit the method by which the molecule is made which can be, for example, by chemical synthesis or recombinant means.

By "isolated" when referring to a polynucleotide, such as a mRNA, microRNA, microRNA mimic, or microRNA inhibitor, is meant that the indicated
15 molecule is present in the substantial absence of other biological macromolecules of the same type. Thus, an "isolated microRNA molecule" refers to a polynucleotide molecule, which is substantially free of other polynucleotide molecules, e.g., other microRNA molecules that do not target the same RNA nucleotide sequence; however, the molecule may include some additional bases or moieties which do not
20 deleteriously affect the basic characteristics of the composition.

"Substantially purified" generally refers to isolation of a substance (compound, polynucleotide, protein, polypeptide, polypeptide composition) such that the substance comprises the majority percent of the sample in which it resides. Typically in a sample a substantially purified component comprises 50%, preferably
25 80%-85%, more preferably 90-95% of the sample. Techniques for purifying polynucleotides and polypeptides of interest are well-known in the art and include, for example, ion-exchange chromatography, affinity chromatography and sedimentation according to density.

The terms "polynucleotide," "oligonucleotide," "nucleic acid" and "nucleic
30 acid molecule" are used herein to include a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, the term includes triple-, double- and single-stranded DNA, as well as triple-, double- and single-stranded RNA. It also includes

modifications, such as by methylation and/or by capping, and unmodified forms of the polynucleotide. More particularly, the terms "polynucleotide," "oligonucleotide," "nucleic acid" and "nucleic acid molecule" include polydeoxyribonucleotides (containing 2-deoxy-D-ribose), polyribonucleotides (containing D-ribose), any other type of polynucleotide which is an N- or C-glycoside of a purine or pyrimidine base, and other polymers containing nonnucleotidic backbones, for example, polyamide (e.g., peptide nucleic acids (PNAs)) and polymorpholino (commercially available from the Anti-Virals, Inc., Corvallis, Oreg., as Neugene) polymers, and other synthetic sequence-specific nucleic acid polymers providing that the polymers contain nucleobases in a configuration which allows for base pairing and base stacking, such as is found in DNA and RNA. There is no intended distinction in length between the terms "polynucleotide," "oligonucleotide," "nucleic acid" and "nucleic acid molecule," and these terms will be used interchangeably. Thus, these terms include, for example, 3'-deoxy-2',5'-DNA, oligodeoxyribonucleotide N3' P5' phosphoramidates, 2'-O-alkyl-substituted RNA, double- and single-stranded DNA, as well as double- and single-stranded RNA, microRNA, DNA:RNA hybrids, and hybrids between PNAs and DNA or RNA, and also include known types of modifications, for example, labels which are known in the art, methylation, "caps," substitution of one or more of the naturally occurring nucleotides with an analog (e.g., 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, C5-propynylcytidine, C5-propynyluridine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-methylcytidine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, and 2-thiocytidine), internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, etc.), with negatively charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), and with positively charged linkages (e.g., aminoalkylphosphoramidates, aminoalkylphosphotriesters), those containing pendant moieties, such as, for example, proteins (including nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide or oligonucleotide. The term also includes

locked nucleic acids (e.g., comprising a ribonucleotide that has a methylene bridge between the 2'-oxygen atom and the 4'-carbon atom). See, for example, Kurreck et al. (2002) *Nucleic Acids Res.* 30: 1911-1918; Elayadi et al. (2001) *Curr. Opin. Invest. Drugs* 2: 558-561; Orum et al. (2001) *Curr. Opin. Mol. Ther.* 3: 239-243; Koshkin et al. (1998) *Tetrahedron* 54: 3607-3630; Obika et al. (1998) *Tetrahedron Lett.* 39: 5401-5404.

The terms "label" and "detectable label" refer to a molecule capable of detection, including, but not limited to, radioactive isotopes, fluorescers, chemiluminescers, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, chromophores, dyes, metal ions, metal sols, ligands (e.g., biotin or haptens) and the like. The term "fluorescer" refers to a substance or a portion thereof that is capable of exhibiting fluorescence in the detectable range. Particular examples of labels that may be used with the invention include, but are not limited to phycoerythrin, Alexa dyes, fluorescein, YPet, CyPet, Cascade blue, allophycocyanin, Cy3, Cy5, Cy7, rhodamine, dansyl, umbelliferone, Texas red, luminol, acradimum esters, biotin, green fluorescent protein (GFP), enhanced green fluorescent protein (EGFP), yellow fluorescent protein (YFP), enhanced yellow fluorescent protein (EYFP), blue fluorescent protein (BFP), red fluorescent protein (RFP), Dronpa, mCherry, mOrange, mPlum, Venus, firefly luciferase, Renilla luciferase, NADPH, beta-galactosidase, horseradish peroxidase, glucose oxidase, alkaline phosphatase, chloramphenical acetyl transferase, urease, MRI contrast agents (e.g., gadodiamide, gadobenic acid, gadopentetic acid, gadoteridol, gadofosveset, gadoversetamide, and gadoxetic acid), and computed tomography (CT) contrast agents (e.g., Diatrizoic acid, Metrizoic acid, Iodamide, Iotalamic acid, Ioxitalamic acid, Ioglicic acid, Acetrizoic acid, Iocarmic acid, Methiodal, Diodone, Metrizamide, Iohexol, Ioxaglic acid, Iopamidol, Iopromide, Iotrolan, Ioversol, Iopentol, Iodixanol, Iomeprol, Iobitridol, Ioxilan, Iodoxamic acid, Iotroxic acid, Ioglycamic acid, Adipiodone, Iobenzamic acid, Iopanoic acid, Iocetamic acid, Sodium iopodate, Tyropanoic acid, and Calcium iopodate).

"Recombinant" as used herein to describe a nucleic acid molecule means a polynucleotide of genomic, RNA, miRNA, cDNA, viral, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation is not associated with all or a portion of the polynucleotide with which it is associated in nature. The term "recombinant" as used with respect to a protein or polypeptide means a polypeptide

produced by expression of a recombinant polynucleotide. In general, the gene of interest is cloned and then expressed in transformed organisms, as described further below.

"Recombinant host cells," "host cells," "cells," "cell lines," "cell cultures," and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities, refer to cells which can be, or have been, used as recipients for recombinant vector or other transferred DNA or RNA, and include the original progeny of the original cell which has been transfected.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, a given promoter operably linked to a coding sequence is capable of effecting the expression of the coding sequence when the proper enzymes are present. Expression is meant to include the transcription of any one or more of transcription of an mRNA, microRNA, microRNA mimic, or microRNA antagonist from a DNA or RNA template and can further include translation of a protein from an mRNA template. The promoter need not be contiguous with the coding sequence, so long as it functions to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between the promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

Typical "control elements," include, but are not limited to, transcription promoters, transcription enhancer elements, transcription termination signals, polyadenylation sequences (located 3' to the translation stop codon), sequences for optimization of initiation of translation (located 5' to the coding sequence), and translation termination sequences.

The term "transfection" is used to refer to the uptake of foreign DNA or RNA by a cell. A cell has been "transfected" when exogenous DNA or RNA has been introduced inside the cell membrane. A number of transfection techniques are generally known in the art. See, e.g., Graham et al. (1973) *Virology*, 52:456, Sambrook et al. (2001) *Molecular Cloning*, a laboratory manual, 3rd edition, Cold Spring Harbor Laboratories, New York, Davis et al. (1995) *Basic Methods in Molecular Biology*, 2nd edition, McGraw-Hill, and Chu et al. (1981) *Gene* 13:197. Such techniques can be used to introduce one or more exogenous DNA or RNA

moieties into suitable host cells. The term refers to both stable and transient uptake of the genetic material, and includes uptake of microRNA.

"Pharmaceutically acceptable excipient or carrier" refers to an excipient that may optionally be included in the compositions of the invention and that causes no significant adverse toxicological effects to the patient.

"Pharmaceutically acceptable salt" includes, but is not limited to, amino acid salts, salts prepared with inorganic acids, such as chloride, sulfate, phosphate, diphosphate, bromide, and nitrate salts, or salts prepared from the corresponding inorganic acid form of any of the preceding, e.g., hydrochloride, etc., or salts prepared with an organic acid, such as malate, maleate, fumarate, tartrate, succinate, ethylsuccinate, citrate, acetate, lactate, methanesulfonate, benzoate, ascorbate, para-toluenesulfonate, palmoate, salicylate and stearate, as well as estolate, gluceptate and lactobionate salts. Similarly salts containing pharmaceutically acceptable cations include, but are not limited to, sodium, potassium, calcium, aluminum, lithium, and ammonium (including substituted ammonium).

The term "about," particularly in reference to a given quantity, is meant to encompass deviations of plus or minus five percent.

An "effective amount" of a miRNA, miRNA mimic, or miRNA inhibitor is an amount sufficient to effect beneficial or desired results, such as an amount that increases levels of beneficial proteins, such as chaperones (e.g., Hsp70 family chaperones, such as Grp78) and anti-apoptotic proteins (e.g., Bcl-2 family anti-apoptotic proteins, such as Bcl-2 or Mcl-1), or decreases levels of detrimental proteins, such as pro-apoptotic proteins (e.g., pro-apoptotic Bcl-2 family members, such as Bcl-2-L11 PUMA, or BMF). For a miRNA or miRNA mimic, an effective amount may reduce translation or increase degradation of the mRNA targeted by the miRNA or miRNA mimic. For a miRNA inhibitor, an effective amount may increase translation of the mRNA targeted by the miRNA that is inhibited by the miRNA inhibitor. An effective amount can be administered in one or more administrations, applications or dosages.

By "therapeutically effective dose or amount" of a miRNA, miRNA mimic, or miRNA inhibitor is intended an amount that, when administered as described herein, brings about a positive therapeutic response, such as improved neurological recovery from stroke or ischemic brain injury caused by cardiac arrest and resuscitation or

traumatic brain injury or chronic neurodegenerative disease. Improved neurological recovery may include a reduction in cerebral infarction size, improved motor function, or improved cognition. The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the condition being treated, the particular drug or drugs employed, mode of administration, and the like. An appropriate "effective" amount in any individual case may be determined by one of ordinary skill in the art using routine experimentation, based upon the information provided herein.

By "subject" is meant any member of the subphylum chordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like.

II. Modes of Carrying Out the Invention

Before describing the present invention in detail, it is to be understood that this invention is not limited to particular formulations or process parameters as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

Although a number of methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, the preferred materials and methods are described herein.

The present invention is based on the discovery that miR-181, miR-29, and microRNA mimics and inhibitors thereof can be used to modulate levels of Hsp-70 family chaperones and Bcl-2 family anti-apoptotic and pro-apoptotic proteins for treatment of neurological disorders. MiR-181 targets the HSPA5 gene of the Hsp70 family of chaperones and the Bcl-2, Bcl-2-L11, and Mcl-1 genes of the Bcl-2 family of pro-apoptotic and anti-apoptotic proteins. MiR-29 targets the PUMA and BMF genes of pro-apoptotic members of the Bcl-2 family. The inventors have further shown that the mir-181 family of microRNAs, miR-181 RNA mimics, and miR-181

inhibitors can be used to modulate levels of Grp78 and Bcl-2 and Mcl-1 proteins in the brain. Levels of all three proteins change inversely with changes in miR-181a (see Examples 5, 6, and 8), and reduction of miR-181a levels in astrocytes reduces cell death, lowers concentrations of reactive oxygen species, and preserves
5 mitochondrial function (see Examples 7 and 11). The inventors have further shown that in a mouse model of stroke, overexpression of miR-181 aggravates cerebral ischemic damage and increases cerebral infarct size, whereas inhibition of cerebral miR-181 is neuroprotective and decreases cerebral infarct size (see Examples 9 and 10). MiR-29 has been shown to regulate levels of the pro-apoptotic protein PUMA in
10 the brain (see Example 16). Increasing miR-29a levels in astrocytes reduces cell death, lowers concentrations of reactive oxygen species, and preserves mitochondrial function (see Example 15). Thus, the present invention pertains generally to compositions and methods for using miR-181, miR-29, and microRNA mimics and inhibitors thereof to modulate levels of Hsp-70 family chaperones and Bcl-2 family
15 pro-apoptotic and anti-apoptotic proteins for treatment of neurological disorders.

In one aspect, the invention provides a method for treating a neurological disorder by utilizing miR-181 or miR-29, agonists or mimics of miR-181 or miR-29, or inhibitors of miR-181 or miR-29. Such a neurological disorder may include, but is not limited to, stroke, ischemic brain injury from cardiac arrest and resuscitation,
20 traumatic brain injury, and neurodegenerative disease. Preferably, one or more symptoms of the neurological disorder are ameliorated or eliminated following administration of miR181 or miR-29, or an agonist or mimic of miR-181 or miR-29, or an inhibitor of miR-181 or miR-29, resulting in improved neurological recovery following treatment. Improved neurological recovery may include, for example, a
25 reduction in cerebral infarction size, improved motor function, or improved cognition.

In certain embodiments, a miR-181 or miR-29, an agonist of miR-181 or miR-29, or a mimic of miR-181 or miR-29 is used in the practice of the invention. The miR-181 or miR-29, agonist of miR-181 or miR-29, or mimic of miR-181 or miR-29 can be a polynucleotide comprising a mature miR-181 or miR-29, or a pri-miRNA or
30 pre-miRNA sequence, and may comprise one or more sequences from miR-181a, miR-181b, miR-181c or miR-181d or miR-29a, miR-29b, miR-29c. In certain embodiments, the polynucleotide comprises a sequence selected from the group consisting of SEQ ID NOS:1-4 and SEQ ID NOS:31-33. The polynucleotide can be

single stranded or double stranded and may contain one or more chemical modifications, such as, but not limited to, locked nucleic acids, peptide nucleic acids, sugar modifications, such as 2'-O-alkyl (e.g., 2'-O-methyl, 2'-O-methoxyethyl), 2'-fluoro, and 4'-thio modifications, and backbone modifications, such as one or more
5 phosphorothioate, morpholino, or phosphonocarboxylate linkages. In one embodiment, the polynucleotide is conjugated to cholesterol.

In another embodiment, an inhibitor of miR-181 or miR-29 is used in the practice of the invention. Inhibitors of miR-181 or miR-29 can include antagomirs, antisense oligonucleotides, and inhibitory RNA molecules. In one embodiment,
10 inhibition of microRNA function may be achieved by administering antisense oligonucleotides targeting a mature sequence of miR-181 or miR-29. The antisense oligonucleotides may be ribonucleotides or deoxyribonucleotides. Preferably, the antisense oligonucleotides have at least one chemical modification. Antisense oligonucleotides may be comprised of one or more "locked nucleic acids". "Locked
15 nucleic acids" (LNAs) are modified ribonucleotides that contain an extra bridge between the 2' and 4' carbons of the ribose sugar moiety resulting in a "locked" conformation that confers enhanced thermal stability to oligonucleotides containing the LNAs. Alternatively, the antisense oligonucleotides may comprise peptide nucleic acids (PNAs), which contain a peptide-based backbone rather than a sugar-
20 phosphate backbone. The antisense oligonucleotides may contain one or more chemical modifications, including, but are not limited to, sugar modifications, such as 2'-O-alkyl (e.g. 2'-O-methyl, 2'-O-methoxyethyl), 2'-fluoro, and 4' thio modifications, and backbone modifications, such as one or more phosphorothioate, morpholino, or phosphonocarboxylate linkages (see, for example, U.S. Pat. Nos. 6,693,187 and
25 7,067,641, which are herein incorporated by reference in their entirety). In some embodiments, suitable antisense oligonucleotides are 2'-O-methoxyethyl "gapmers" which contain 2'-O-methoxyethyl-modified ribonucleotides on both 5' and 3' ends with at least ten deoxyribonucleotides in the center. These "gapmers" are capable of triggering RNase H-dependent degradation mechanisms of RNA targets. Other
30 modifications of antisense oligonucleotides to enhance stability and improve efficacy, such as those described in U.S. Pat. No. 6,838,283, which is herein incorporated by reference in its entirety, are known in the art and are suitable for use in the methods of the invention. Antisense oligonucleotides, useful for inhibiting the activity of

microRNAs, are about 19 to about 25 nucleotides in length. Antisense oligonucleotides may comprise a sequence that is at least partially complementary to a mature miRNA sequence, e.g., at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% complementary to a mature miRNA sequence.

5 In some embodiments, the antisense oligonucleotide may be substantially complementary to a mature miRNA sequence, that is at least about 95%, 96%, 97%, 98%, or 99% complementary to a target polynucleotide sequence. In one embodiment, the antisense oligonucleotide comprises a sequence that is 100% complementary to a mature miRNA sequence.

10 In certain embodiments, the antisense oligonucleotides are antagomirs. "Antagomirs" are single-stranded, chemically-modified ribonucleotides that are at least partially complementary to the miRNA sequence. Antagomirs may comprise one or more modified nucleotides, such as 2'-O-methyl-sugar modifications. In some embodiments, antagomirs comprise only modified nucleotides. Antagomirs may also
15 comprise one or more phosphorothioate linkages resulting in a partial or full phosphorothioate backbone. To facilitate *in vivo* delivery and stability, the antagomir may be linked to a cholesterol or other moiety at its 3' end. Antagomirs suitable for inhibiting miRNAs may be about 15 to about 50 nucleotides in length, more preferably about 18 to about 30 nucleotides in length, and most preferably about 20 to
20 about 25 nucleotides in length. "Partially complementary" refers to a sequence that is at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% complementary to a target polynucleotide sequence. The antagomirs may be at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% complementary to a mature miRNA sequence. In some embodiments, the
25 antagomir may be substantially complementary to a mature miRNA sequence, that is at least about 95%, 96%, 97%, 98%, or 99% complementary to a target polynucleotide sequence. In other embodiments, the antagomirs are 100% complementary to the mature miRNA sequence.

In another embodiment, the inhibitor of miR-181 or miR-29 is an inhibitory
30 RNA molecule having a double stranded region that is at least partially identical and partially complementary to a mature sequence of miR-181 or miR-29. The inhibitory RNA molecule may be a double-stranded, small interfering RNA (siRNA) or a short hairpin RNA molecule (shRNA) comprising a stem-loop structure. The double-

stranded regions of the inhibitory RNA molecule may comprise a sequence that is at least partially identical and partially complementary, e.g., about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical and complementary, to the mature miRNA sequence. In some embodiments, the double-stranded regions of the inhibitory RNA comprise a sequence that is at least substantially identical and substantially complementary to the mature miRNA sequence. "Substantially identical and substantially complementary" refers to a sequence that is at least about 95%, 96%, 97%, 98%, or 99% identical and complementary to a target polynucleotide sequence. In other embodiments, the double-stranded regions of the inhibitory RNA molecule may contain 100% identity and complementarity to the target miRNA sequence.

The inhibitory nucleotide molecules described herein may target a sequence of miR-181, including but not limited to, miR-181a (SEQ ID NO:1), miR-181b (SEQ ID NO:2), miR-181c (SEQ ID NO:3), or miR-181d (SEQ ID NO:4) or a sequence of miR-29, including but not limited to, miR-29a (SEQ ID NO:31), miR-29b (SEQ ID NO:32), or miR-29c (SEQ ID NO:33). In some embodiments, inhibitors of miR-181 are antagomirs comprising a sequence that is perfectly complementary to a mature sequence of miR-181. In one embodiment, an inhibitor of miR-181 is an antagomir comprising the sequence of SEQ ID NO: 9.

In certain embodiments, inhibitors of miR-181 or miR-29 are chemically-modified antisense oligonucleotides. In one embodiment, an inhibitor of miR-181 or miR-29 is a chemically-modified antisense oligonucleotide comprising a sequence substantially complementary to a sequence selected from the group consisting of SEQ ID NOS:1-4 or a sequence selected from the group consisting of SEQ ID NOS:31-33, respectively. As used herein, "substantially complementary" refers to a sequence that is at least about 95%, 96%, 97%, 98%, 99%, or 100% complementary to a target polynucleotide sequence (e.g., mature or precursor miRNA sequence).

Antisense oligonucleotides may comprise a sequence that is substantially complementary to a precursor miRNA sequence (pre-miRNA). In some embodiments, the antisense oligonucleotide comprises a sequence that is substantially complementary to a sequence located outside the stem-loop region of the pre-miRNA sequence.

In other embodiments of the invention, inhibitors of miR-181 or miR-29 may be inhibitory RNA molecules, such as ribozymes, siRNAs, or shRNAs. In one embodiment, an inhibitor of miR-181 or miR-29 is an inhibitory RNA molecule comprising a double-stranded region, wherein the double-stranded region comprises a sequence having 100% identity and complementarity to a mature sequence of miR-181 (e.g., SEQ ID NOS:1-4) or a mature sequence of miR-29 (e.g., SEQ ID NOS:31-33). In some embodiments, inhibitors of miR-181 or miR-29 are inhibitory RNA molecules which comprise a double-stranded region, wherein said double-stranded region comprises a sequence of at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity and complementarity to a mature sequence of miR-181 or miR-29.

In certain embodiments, the invention includes a method of regulating expression of Grp78, Bcl-2, or Mcl-1 in a cell comprising introducing into the cell miR-181 or a modulator of miR-181, which may be an agonist or antagonist of miR-181. In one embodiment, the expression of Grp78, Bcl-2, or Mcl-1 is increased in the cell following administration of an inhibitor of miR-181. In another embodiment, the expression of Bcl-1-L11 is decreased in the cell following administration of miR-181 or a miR-181 mimic. The cell may be a neuron cell, glial cell, or endothelial cell. In certain embodiments, the glial cell is microglia or macroglia (e.g., an astrocyte or an oligodendrocyte).

In other embodiments, the invention includes a method of regulating expression of PUMA or BMF in a cell comprising introducing into the cell miR-29 or a modulator of miR-29, which may be an agonist or antagonist of miR-29. In one embodiment, the expression of PUMA or BMF is increased in the cell following administration of an inhibitor of miR-29. In another embodiment, the expression of PUMA or BMF is decreased in the cell following administration of miR-29 or a miR-29 mimic. The cell may be a neuron cell, glial cell, or endothelial cell. In certain embodiments, the glial cell is microglia or macroglia (e.g., an astrocyte or an oligodendrocyte).

In certain embodiments, the miR-181, miR-29, agonist of miR-181 or miR-29, mimic of miR-181 or miR-29, or inhibitor of miR-181 or miR-29 is expressed *in vivo* from a vector. A "vector" is a composition of matter which can be used to deliver a nucleic acid of interest to the interior of a cell. Numerous vectors are known in the art

including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "vector" includes an autonomously replicating plasmid or a virus. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, lentiviral vectors, and the like. An expression construct can be replicated in a living cell, or it can be made synthetically. For purposes of this application, the terms "expression construct," "expression vector," and "vector," are used interchangeably to demonstrate the application of the invention in a general, illustrative sense, and are not intended to limit the invention.

In one embodiment, an expression vector for expressing miR-181, miR-29, an agonist of miR-181 or miR-29, a mimic of miR-181 or miR-29, or an inhibitor of miR-181 or miR-29 comprises a promoter "operably linked" to a polynucleotide encoding the miR-181, miR-29, agonist of miR-181 or miR-29, mimic of miR-181 or miR-29, or inhibitor of miR-181 or miR-29. The phrase "operably linked" or "under transcriptional control" as used herein means that the promoter is in the correct location and orientation in relation to a polynucleotide to control the initiation of transcription by RNA polymerase and expression of the polynucleotide.

In certain embodiments, the nucleic acid encoding a polynucleotide of interest is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase I, II, or III. Typical promoters for mammalian cell expression include the SV40 early promoter, a CMV promoter such as the CMV immediate early promoter (see, U.S. Patent Nos. 5,168,062 and 5,385,839, incorporated herein by reference in their entireties), the mouse mammary tumor virus LTR promoter, the adenovirus major late promoter (Ad MLP), and the herpes simplex virus promoter, among others. Other nonviral promoters, such as a promoter derived from the murine metallothionein gene, will also find use for mammalian expression. These and other promoters can be obtained from commercially available plasmids, using techniques well known in the art. See, e.g., Sambrook et al., *supra*. Enhancer elements may be used in association with the promoter to increase expression levels of the constructs. Examples include the SV40

early gene enhancer, as described in Dijkema et al., *EMBO J.* (1985) 4:761, the enhancer/promoter derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus, as described in Gorman et al., *Proc. Natl. Acad. Sci. USA* (1982b) 79:6777 and elements derived from human CMV, as described in Boshart et al., *Cell* (1985)

5 41:521, such as elements included in the CMV intron A sequence.

Typically, transcription terminator/polyadenylation signals will also be present in the expression construct. Examples of such sequences include, but are not limited to, those derived from SV40, as described in Sambrook et al., *supra*, as well as a bovine growth hormone terminator sequence (see, e.g., U.S. Patent No. 5,122,458).

10 Additionally, 5'- UTR sequences can be placed adjacent to the coding sequence in order to enhance expression of the same. Such sequences include UTRs which include an Internal Ribosome Entry Site (IRES) present in the leader sequences of picornaviruses such as the encephalomyocarditis virus (EMCV) UTR (Jang et al. *J. Virol.* (1989) 63:1651-1660. Other picornavirus UTR sequences that will also find
15 use in the present invention include the polio leader sequence and hepatitis A virus leader and the hepatitis C IRES.

In certain embodiments of the invention, the cells containing nucleic acid constructs of the present invention may be identified *in vitro* or *in vivo* by including a marker in the expression construct. Such markers would confer an identifiable
20 change to the cell permitting easy identification of cells containing the expression construct. Usually the inclusion of a drug selection marker aids in cloning and in the selection of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. Alternatively, enzymes such as herpes simplex virus thymidine kinase (tk) or
25 chloramphenicol acetyltransferase (CAT) may be employed. Fluorescent markers (e.g., GFP, EGFP, Dronpa, mCherry, mOrange, mPlum, Venus, YPet, phycoerythrin), or immunologic markers can also be employed. The selectable marker employed is not believed to be important, so long as it is capable of being expressed
simultaneously with the nucleic acid encoding a gene product. Further examples of
30 selectable markers are well known to one of skill in the art.

There are a number of ways in which expression vectors may be introduced into cells. In certain embodiments of the invention, the expression construct comprises a virus or engineered construct derived from a viral genome. A number of

viral based systems have been developed for gene transfer into mammalian cells. These include adenoviruses, retroviruses (γ -retroviruses and lentiviruses), poxviruses, adeno-associated viruses, baculoviruses, and herpes simplex viruses (see e.g., Warnock et al. (2011) *Methods Mol. Biol.* 737:1-25; Walther et al. (2000) *Drugs* 60(2):249-271; and Lundstrom (2003) *Trends Biotechnol.* 21(3):117-122; herein incorporated by reference in their entireties). The ability of certain viruses to enter cells via receptor-mediated endocytosis, to integrate into host cell genomes and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells.

For example, retroviruses provide a convenient platform for gene delivery systems. Selected sequences can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either *in vivo* or *ex vivo*. A number of retroviral systems have been described (U.S. Pat. No. 5,219,740; Miller and Rosman (1989) *BioTechniques* 7:980-990; Miller, A. D. (1990) *Human Gene Therapy* 1:5-14; Scarpa et al. (1991) *Virology* 180:849-852; Burns et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:8033-8037; Boris-Lawrie and Temin (1993) *Cur. Opin. Genet. Develop.* 3:102-109; and Ferry et al. (2011) *Curr Pharm Des.* 17(24):2516-2527). Lentiviruses are a class of retroviruses that are particularly useful for delivering polynucleotides to mammalian cells because they are able to infect both dividing and nondividing cells (see e.g., Lois et al (2002) *Science* 295:868-872; Durand et al. (2011) *Viruses* 3(2):132-159; herein incorporated by reference).

A number of adenovirus vectors have also been described. Unlike retroviruses which integrate into the host genome, adenoviruses persist extrachromosomally thus minimizing the risks associated with insertional mutagenesis (Haj-Ahmad and Graham, *J. Virol.* (1986) 57:267-274; Bett et al., *J. Virol.* (1993) 67:5911-5921; Mittereder et al., *Human Gene Therapy* (1994) 5:717-729; Seth et al., *J. Virol.* (1994) 68:933-940; Barr et al., *Gene Therapy* (1994) 1:51-58; Berkner, K. L. *BioTechniques* (1988) 6:616-629; and Rich et al., *Human Gene Therapy* (1993) 4:461-476). Additionally, various adeno-associated virus (AAV) vector systems have been developed for gene delivery. AAV vectors can be readily constructed using techniques well known in the art. See, e.g., U.S. Pat. Nos. 5,173,414 and 5,139,941; International Publication Nos. WO 92/01070 (published 23 January 1992) and WO

93/03769 (published 4 March 1993); Lebkowski et al., *Molec. Cell. Biol.* (1988) 8:3988-3996; Vincent et al., *Vaccines* 90 (1990) (Cold Spring Harbor Laboratory Press); Carter, B. J. *Current Opinion in Biotechnology* (1992) 3:533-539; Muzyczka, N. *Current Topics in Microbiol. and Immunol.* (1992) 158:97-129; Kotin, R. M. Human Gene Therapy (1994) 5:793-801; Shelling and Smith, *Gene Therapy* (1994) 1:165-169; and Zhou et al., *J. Exp. Med.* (1994) 179:1867-1875.

Another vector system useful for delivering the polynucleotides of the present invention is the enterically administered recombinant poxvirus vaccines described by Small, Jr., P. A., et al. (U.S. Pat. No. 5,676,950, issued Oct. 14, 1997, herein incorporated by reference).

Additional viral vectors which will find use for delivering the nucleic acid molecules of interest include those derived from the pox family of viruses, including vaccinia virus and avian poxvirus. By way of example, vaccinia virus recombinants expressing a nucleic acid molecule of interest (e.g., encoding miR-181, miR-29, an agonist of miR-181 or miR-29, a mimic of miR-181 or miR-29, or an inhibitor of miR-181 or miR-29) can be constructed as follows. The DNA encoding the particular nucleic acid sequence is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the sequences of interest into the viral genome. The resulting TK-recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

Alternatively, avipoxviruses, such as the fowlpox and canarypox viruses, can also be used to deliver the nucleic acid molecules of interest. The use of an avipox vector is particularly desirable in human and other mammalian species since members of the avipox genus can only productively replicate in susceptible avian species and therefore are not infective in mammalian cells. Methods for producing recombinant avipoxviruses are known in the art and employ genetic recombination, as described above with respect to the production of vaccinia viruses. See, e.g., WO 91/12882; WO 89/03429; and WO 92/03545.

Molecular conjugate vectors, such as the adenovirus chimeric vectors described in Michael et al., J. Biol. Chem. (1993) 268:6866-6869 and Wagner et al., Proc. Natl. Acad. Sci. USA (1992) 89:6099-6103, can also be used for gene delivery.

Members of the Alphavirus genus, such as, but not limited to, vectors derived from the Sindbis virus (SIN), Semliki Forest virus (SFV), and Venezuelan Equine Encephalitis virus (VEE), will also find use as viral vectors for delivering the polynucleotides of the present invention. For a description of Sindbis-virus derived vectors useful for the practice of the instant methods, see, Dubensky et al. (1996) J. Virol. 70:508-519; and International Publication Nos. WO 95/07995, WO 96/17072; as well as, Dubensky, Jr., T. W., et al., U.S. Pat. No. 5,843,723, issued Dec. 1, 1998, and Dubensky, Jr., T. W., U.S. Patent No. 5,789,245, issued Aug. 4, 1998, both herein incorporated by reference. Particularly preferred are chimeric alphavirus vectors comprised of sequences derived from Sindbis virus and Venezuelan equine encephalitis virus. See, e.g., Perri et al. (2003) J. Virol. 77: 10394-10403 and International Publication Nos. WO 02/099035, WO 02/080982, WO 01/81609, and WO 00/61772; herein incorporated by reference in their entireties.

A vaccinia based infection/transfection system can be conveniently used to provide for inducible, transient expression of the polynucleotides of interest (e.g., encoding miR-181, miR-29, an agonist of miR-181 or miR-29, a mimic of miR-181 or miR-29, or an inhibitor of miR-181 or miR-29) in a host cell. In this system, cells are first infected *in vitro* with a vaccinia virus recombinant that encodes the bacteriophage T7 RNA polymerase. This polymerase displays exquisite specificity in that it only transcribes templates bearing T7 promoters. Following infection, cells are transfected with the polynucleotide of interest, driven by a T7 promoter. The polymerase expressed in the cytoplasm from the vaccinia virus recombinant transcribes the transfected DNA into RNA. The method provides for high level, transient, cytoplasmic production of large quantities of RNA. See, e.g., Elroy-Stein and Moss, Proc. Natl. Acad. Sci. USA (1990) 87:6743-6747; Fuerst et al., Proc. Natl. Acad. Sci. USA (1986) 83:8122-8126.

As an alternative approach to infection with vaccinia or avipox virus recombinants, or to the delivery of nucleic acids using other viral vectors, an amplification system can be used that will lead to high level expression following introduction into host cells. Specifically, a T7 RNA polymerase promoter preceding

the coding region for T7 RNA polymerase can be engineered. Translation of RNA derived from this template will generate T7 RNA polymerase which in turn will transcribe more template. Concomitantly, there will be a cDNA whose expression is under the control of the T7 promoter. Thus, some of the T7 RNA polymerase generated from translation of the amplification template RNA will lead to transcription of the desired gene. Because some T7 RNA polymerase is required to initiate the amplification, T7 RNA polymerase can be introduced into cells along with the template(s) to prime the transcription reaction. The polymerase can be introduced as a protein or on a plasmid encoding the RNA polymerase. For a further discussion of T7 systems and their use for transforming cells, see, e.g., International Publication No. WO 94/26911; Studier and Moffatt, J. Mol. Biol. (1986) 189:113-130; Deng and Wolff, Gene (1994) 143:245-249; Gao et al., Biochem. Biophys. Res. Commun. (1994) 200:1201-1206; Gao and Huang, Nuc. Acids Res. (1993) 21:2867-2872; Chen et al., Nuc. Acids Res. (1994) 22:2114-2120; and U.S. Pat. No. 5,135,855.

In order to effect expression of sense or antisense gene constructs, the expression construct must be delivered into a cell. This delivery may be accomplished *in vitro*, as in laboratory procedures for transforming cells lines, or *in vivo* or *ex vivo*, as in the treatment of certain disease states. One mechanism for delivery is via viral infection where the expression construct is encapsidated in an infectious viral particle.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These include the use of calcium phosphate precipitation, DEAE-dextran, electroporation, direct microinjection, DNA-loaded liposomes, lipofectamine-DNA complexes, cell sonication, gene bombardment using high velocity microprojectiles, and receptor-mediated transfection (see, e.g., Graham and Van Der Eb (1973) Virology 52:456-467; Chen and Okayama (1987) Mol. Cell Biol. 7:2745-2752; Rippe et al. (1990) Mol. Cell Biol. 10:689-695; Gopal (1985) Mol. Cell Biol. 5:1188-1190; Tur-Kaspa et al. (1986) Mol. Cell. Biol. 6:716-718; Potter et al. (1984) Proc. Natl. Acad. Sci. USA 81:7161-7165; Harland and Weintraub (1985) J. Cell Biol. 101:1094-1099; Nicolau and Sene (1982) Biochim. Biophys. Acta 721:185-190; Fraley et al. (1979) Proc. Natl. Acad. Sci. USA 76:3348-3352; Fechheimer et al. (1987) Proc Natl. Acad. Sci. USA 84:8463-8467; Yang et al. (1990) Proc. Natl. Acad. Sci. USA 87:9568-9572; Wu and

Wu (1987) J. Biol. Chem. 262:4429-4432; Wu and Wu (1988) Biochemistry 27:887-892; herein incorporated by reference). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

5 Once the expression construct has been delivered into the cell the nucleic acid encoding the gene of interest may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the
10 nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

15 In yet another embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is particularly applicable for transfer *in vitro* but it may be applied to *in vivo* use as well. Dubensky et al. (Proc. Natl. Acad. Sci. USA (1984) 81:7529-7533) successfully injected polyomavirus DNA in the form of
20 calcium phosphate precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (Proc. Natl. Acad. Sci. USA (1986) 83:9551-9555) also demonstrated that direct intraperitoneal injection of calcium phosphate-precipitated plasmids results in
25 expression of the transfected genes. It is envisioned that DNA encoding a gene of interest may also be transferred in a similar manner *in vivo* and express the gene product.

In still another embodiment, a naked DNA expression construct may be transferred into cells by particle bombardment. This method depends on the ability to
30 accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein et al. (1987) Nature 327:70-73). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current,

which in turn provides the motive force (Yang et al. (1990) Proc. Natl. Acad. Sci. USA 87:9568-9572). The microprojectiles may consist of biologically inert substances, such as tungsten or gold beads.

5 In a further embodiment, the expression construct may be delivered using liposomes. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat (1991) Liver Diseases, Targeted Diagnosis and Therapy Using Specific Receptors and Ligands, Wu et al. (Eds.), Marcel Dekker, NY, 87-104). Also contemplated is the use of lipofectamine-DNA complexes.

15 In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda et al. (1989) Science 243:375-378). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-I) (Kato et al. (1991) J. Biol. Chem. 266(6):3361-3364). In yet 20 further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-I. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*, then they are applicable for the present invention. Where a bacterial promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate 25 bacterial polymerase.

Other expression constructs which can be employed to deliver a nucleic acid encoding a particular gene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific 30 distribution of various receptors, the delivery can be highly specific (Wu and Wu (1993) Adv. Drug Delivery Rev. 12:159-167).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several

ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) and transferrin (see, e.g., Wu and Wu (1987), *supra*; Wagner et al. (1990) Proc. Natl. Acad. Sci. USA 87(9):3410-3414). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol et al. (1993) FASEB J. 7:1081-1091; Perales et al. (1994) Proc. Natl. Acad. Sci. USA 91(9):4086-4090), and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau et al. (Methods Enzymol. (1987) 149:157-176) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that a nucleic acid encoding a particular gene also may be specifically delivered into a cell type by any number of receptor-ligand systems with or without liposomes. For example, epidermal growth factor (EGF) may be used as the receptor for mediated delivery of a nucleic acid into cells that exhibit upregulation of EGF receptor. Mannose can be used to target the mannose receptor on liver cells. Also, antibodies to CD5 (CLL), CD22 (lymphoma), CD25 (T-cell leukemia) and MAA (melanoma) can similarly be used as targeting moieties.

In a particular example, an oligonucleotide may be administered in combination with a cationic lipid. Examples of cationic lipids include, but are not limited to, lipofectin, DOTMA, DOPE, and DOTAP. The publication of WO/0071096, which is specifically incorporated by reference, describes different formulations, such as a DOTAP:cholesterol or cholesterol derivative formulation that can effectively be used for gene therapy. Other disclosures also discuss different lipid or liposomal formulations including nanoparticles and methods of administration; these include, but are not limited to, U.S. Patent Publication 20030203865, 20020150626, 20030032615, and 20040048787, which are specifically incorporated by reference to the extent they disclose formulations and other related aspects of administration and delivery of nucleic acids. Methods used for forming particles are also disclosed in U.S. Pat. Nos. 5,844,107, 5,877,302, 6,008,336, 6,077,835, 5,972,901, 6,200,801, and 5,972,900, which are incorporated by reference for those aspects.

In certain embodiments, gene transfer may more easily be performed under *ex vivo* conditions. *Ex vivo* gene therapy refers to the isolation of cells from an animal, the delivery of a nucleic acid into the cells *in vitro*, and then the return of the modified cells back into an animal. This may involve the surgical removal of tissue/organs from an animal or the primary culture of cells and tissues.

The microRNA, microRNA mimic, or microRNA inhibitor may comprise a detectable label in order to facilitate detection of binding of the microRNA, microRNA mimic, or microRNA inhibitor to a target nucleic acid. Detectable labels suitable for use in the present invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical, or chemical means. Useful labels in the present invention include biotin or other streptavidin-binding proteins for staining with labeled streptavidin conjugate, magnetic beads (e.g., Dynabeads), fluorescent dyes (e.g., phycoerythrin, YPet, fluorescein, texas red, rhodamine, green fluorescent protein, and the like, see, e.g., Molecular Probes, Eugene, Oreg., USA), radiolabels (e.g., ^3H , ^{125}I , ^{35}S , ^{14}C , or ^{32}P), enzymes (e.g., horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold (e.g., gold particles in the 40-80 nm diameter size range scatter green light with high efficiency) or colored glass or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads. In addition, magnetic resonance imaging (MRI) contrast agents (e.g., gadodiamide, gadobenic acid, gadopentetic acid, gadoteridol, gadofosveset, gadoversetamide, gadoxetic acid), and computed tomography (CT) contrast agents (e.g., Diatrizoic acid, Metrizoic acid, Iodamide, Iotalamic acid, Ioxitalamic acid, Ioglicic acid, Acetrizoic acid, Iocarmic acid, Methiodal, Diodone, Metrizamide, Iohexol, Ioxaglic acid, Iopamidol, Iopromide, Iotrolan, Ioversol, Iopentol, Iodixanol, Iomeprol, Iobitridol, Ioxilan, Iodoxamic acid, Iotroxic acid, Ioglycamic acid, Adipiodone, Iobenzamic acid, Iopanoic acid, Iocetamic acid, Sodium iopodate, Tyropanoic acid, Calcium iopodate) are useful as labels in medical imaging. Patents teaching the use of such labels include U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; 4,366,241; 5,798,092; 5,695,739; 5,733,528; and 5,888,576.

The present invention also encompasses pharmaceutical compositions comprising one or more of a miR-181, a miR-29, an agonist of miR-181 or miR-29, a mimic of miR-181 or miR-29, or an inhibitor miR-181 or miR-29 and a

pharmaceutically acceptable carrier. Where clinical applications are contemplated, pharmaceutical compositions will be prepared in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

5 Colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes, may be used as delivery vehicles for the agonists or inhibitors of microRNA function described herein. Commercially available fat emulsions that are suitable for delivering the nucleic acids of the
10 invention to tissues, such as cardiac muscle tissue and smooth muscle tissue, include Intralipid, Liposyn, Liposyn II, Liposyn III, Nutrilipid, and other similar lipid emulsions. A preferred colloidal system for use as a delivery vehicle *in vivo* is a liposome (i.e., an artificial membrane vesicle). The preparation and use of such systems is well known in the art. Exemplary formulations are also disclosed in U.S.
15 Pat. No. 5,981,505; U.S. Pat. No. 6,217,900; U.S. Pat. No. 6,383,512; U.S. Pat. No. 5,783,565; U.S. Pat. No. 7,202,227; U.S. Pat. No. 6,379,965; U.S. Pat. No. 6,127,170; U.S. Pat. No. 5,837,533; U.S. Pat. No. 6,747,014; and WO 03/093449, which are herein incorporated by reference in their entireties.

One will generally desire to employ appropriate salts and buffers to render
20 delivery vehicles stable and allow for uptake by target cells. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the delivery vehicle, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. The phrases "pharmaceutically acceptable" or "pharmacologically
25 acceptable" refers to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes solvents, buffers, solutions, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like acceptable for use in formulating
30 pharmaceuticals, such as pharmaceuticals suitable for administration to humans. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredients of the present invention, its use in therapeutic compositions is

contemplated. Supplementary active ingredients also can be incorporated into the compositions, provided they do not inactivate the nucleic acids of the compositions.

Compositions for use in the invention will comprise a therapeutically effective amount of the desired miRNA, miRNA mimic, or miRNA inhibitor. By

5 “therapeutically effective dose or amount” of a miRNA, miRNA mimic, or miRNA inhibitor is intended an amount that, when administered as described herein, brings about a positive therapeutic response, such as improved neurological recovery from stroke, or ischemic brain injury caused by cardiac arrest and resuscitation, or traumatic brain injury, or chronic neurodegenerative disease. Improved neurological
10 recovery may include a reduction in cerebral infarction size, improved motor function, or improved cognition.

An “effective amount” of a miRNA, miRNA mimic, or miRNA inhibitor is an amount sufficient to effect beneficial or desired results, such as an amount that increases levels of beneficial proteins, such as chaperones (e.g., Hsp70 family
15 chaperones, such as Grp78) and anti-apoptotic proteins (e.g., Bcl-2 family anti-apoptotic proteins, such as Bcl-2 or Mcl-1), or decreases levels of detrimental proteins, such as pro-apoptotic proteins (e.g., pro-apoptotic Bcl-2 family members, such as Bcl-2-L11, PUMA, or BMF). For a miRNA or miRNA mimic, an effective
20 amount may reduce translation or increase degradation of the mRNA targeted by the miRNA or miRNA mimic. For a miRNA inhibitor, an effective amount may increase translation of the mRNA targeted by the miRNA that is inhibited by the miRNA inhibitor. An effective amount can be administered in one or more administrations, applications or dosages.

Once formulated, the compositions are conventionally administered
25 parenterally, e.g., by injection, intracerebroventricularly, intraparenchymatously, intracephalically, intracerebrally, intracerebellarly, intracranially, intraneurally, intraspinally, subcutaneously, intraperitoneally, intramuscularly, intra-arterially, or intravenously. In one embodiment, compositions are administered by stereotactic injection into the brain. Compositions may be injected directly into lesions or into the
30 arterial blood supply of a lesion. Additional formulations suitable for other modes of administration include oral and pulmonary formulations, suppositories, and transdermal formulations, aerosol, intranasal, and sustained release formulations.

Dosage treatment may be a single dose schedule or a multiple dose schedule. The exact amount necessary will vary depending on the desired response; the subject being treated; the age and general condition of the individual to be treated; the severity of the condition being treated; the mode of administration, among other factors. An appropriate effective amount can be readily determined by one of skill in the art. A “therapeutically effective amount” will fall in a relatively broad range that can be determined through routine trials using *in vitro* and *in vivo* models known in the art.

The pharmaceutical forms suitable for injectable use or catheter delivery include, for example, sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. Generally, these preparations are sterile and fluid to the extent that easy injectability exists. Preparations should be stable under the conditions of manufacture and storage and should be preserved against the contaminating action of microorganisms, such as bacteria and fungi. Appropriate solvents or dispersion media may contain, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions may be prepared by incorporating the active compounds in an appropriate amount into a solvent along with any other ingredients (for example as enumerated above) as desired, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the desired other ingredients, e.g., as enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation

include vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient(s) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The compositions of the present invention generally may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include, for example, acid addition salts (formed with the free amino groups of the protein) derived from inorganic acids (e.g., hydrochloric or phosphoric acids, or from organic acids (e.g., acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups of the protein can also be derived from inorganic bases (e.g., sodium, potassium, ammonium, calcium, or ferric hydroxides) or from organic bases (e.g., isopropylamine, trimethylamine, histidine, procaine and the like).

Upon formulation, solutions are preferably administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations may easily be administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution generally is suitably buffered and the liquid diluent first rendered isotonic for example with sufficient saline or glucose. Such aqueous solutions may be used, for example, for intravenous, intramuscular, subcutaneous and intraperitoneal administration. Preferably, sterile aqueous media are employed as is known to those of skill in the art, particularly in light of the present disclosure. By way of illustration, a single dose may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, and general safety and purity standards as required by FDA Office of Biologics standards.

Any of the compositions described herein may be included in a kit. For example, at least one miR-181, agonist of miR-181, mimic of miR-181, inhibitor of miR-181, miR-29, agonist of miR-29, mimic of miR-29, or inhibitor of miR-29, or

any combination thereof, may be included in a kit. The kit may also include one or more transfection reagents to facilitate delivery of polynucleotides to cells.

The components of the kit may be packaged either in aqueous media or in lyophilized form. The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which a component may be placed, and preferably, suitably aliquoted. Where there is more than one component in the kit (labeling reagent and label may be packaged together), the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a vial. The kits of the present invention also will typically include a means for containing the nucleic acids, and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

When the components of the kit are provided in one and/or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means.

The container means will generally include at least one vial, test tube, flask, bottle, syringe and/or other container means, into which the nucleic acid formulations are placed, preferably, suitably allocated. The kits may also comprise a second container means for containing a sterile, pharmaceutically acceptable buffer and/or other diluent.

Such kits may also include components that preserve or maintain the miRNAs/polynucleotides or that protect against their degradation. Such components may be RNase-free or protect against RNases. Such kits generally will comprise, in suitable means, distinct containers for each individual reagent or solution.

A kit will also include instructions for employing the kit components as well the use of any other reagent not included in the kit. Instructions may include variations that can be implemented. A kit may also include utensils or devices for administering the miRNA agonist or inhibitor by various administration routes, such as parenteral or catheter administration or coated stent.

III. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

Example 1

Materials and Methods

Materials

Swiss Webster mice were obtained from Charles River (Wilmington, MA, USA) or Simonsen (Gilroy, CA). Rat astrocyte C6 cells and human BOSC 23 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The MWX-PGK-IRES-GFP vector was purchased from Addgene (Cambridge, MA, USA). The following reagents were used in assays: fluorescent-tagged miRNA transfection control, negative control, and miR-181a and miR-29a mimic and inhibitor, which were purchased from Thermo Scientific via Dharmacon (Lafayette, CO, USA); miRNA reverse transcriptase primers, which were purchased from Applied Biosystems; and miR-181a Antagomir and Mismatch, which were purchased from Alnylam Pharmaceuticals. The catalogue numbers of these products are listed in Table 1.

Table 1. Catalog Numbers of Materials Bought from Companies

Name	Catalog Number	Company
miR-181a Primer	000480	Applied Bioscience (Foster City, CA)
miR-181b Primer	001098	
miR-181c Primer	000482	
miR-181d Primer	001099	
U6 snRNA Primer	001973	

Name	Catalog Number	Company
miR-181a Mimic	C-310435-05-0005	Thermo Scientific via Dharmacon (Chicago, IL)
miR-181a Inhibitor	C-310435-07-0005	
Positive Control	CP-004500-01-05	
Negative Control	CN-001000-01-05	
miR-181a Antagomir	AL-SQ#3869	Alnylam Pharmaceuticals (Cambridge, MA, USA)
miR-181a Mismatch	AL-SQ#30048	
miR-29a Primer	002112	Applied Bioscience (Foster City, CA, USA)
miR-29b Primer	000413	
miR-29c Primer	000587	
U6 snRNA Primer	001973	
miR-29a Mimic	C-310521-07-0005	Thermo Scientific via Dharmacon (Chicago, IL, USA)
miR-29a Inhibitor	IH-310521-08-0005	
miR-29a Antagomir	IH-120642-00-30	
Positive Control	CP-004500-01-05	
Negative Control	CN-001000-01-05	

Luciferase Reporter Assay

The luciferase reporter assay was performed according to the method described by Trujillo et al. (EMBO J. (2010) 29:3272–3285). Cells were plated at a density of $1.2\text{--}1.5 \times 10^4$ cells/well in 96-well plates one day before transfection. Cells were co-transfected with the firefly luciferase control reporter plasmid, Renilla luciferase target reporter, and miRNA expression vector, which were added to each well in amounts of 0.25 ng, 0.05 ng and 40 ng, respectively, with Fugene reagent (Roche, New Jersey, USA) according to the manufacturer's instructions. At 24 hours after transfection, 100 μl of culture medium was added to each well. Cells were harvested 48 hours after transfection and assayed using the Dual-Luciferase system (Promega). Results are expressed as relative luciferase activity by first normalizing to the firefly luciferase transfection control, then to the Renilla/firefly value of the empty control vector and finally to the corresponding seed mutant reporter control.

Cell Cultures and Transfection

Primary astrocyte cultures were prepared from postnatal day 1–3 Swiss Webster mice (Charles River, Wilmington, MA, USA) as described previously

(Ouyang et al. (2006) Stress Chaperones 11:180-186; herein incorporated by reference). Neocortices were dissected, treated with trypsin, and plated as a single-cell suspension. Cell lines of rat astrocyte cell C6 and human BOSC 23, purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), were grown in Dulbecco's Modified Eagle Medium (DMEM, #11995, Gibco, Carlsbad, CA, USA) supplemented with 10% FBS and 100 µg/ml penicillin/streptomycin. Astrocyte cell line or 5 day old primary astrocytes in 24-well plates were transfected with pri-miR-181 plasmids, miR-181a mimic, or inhibitor, or their controls using FuGeneHD (Roche, Branford, CT, USA) according to the manufacturer's instructions.

Assessment of Cell Injury and Cell Death, and Live Cell Imaging

Glucose deprivation (GD) was performed on primary astrocyte cultures as described previously (Ouyang et al. (2011) Mitochondrion 11(2):279-86); Ouyang et al. (2006) Cell Stress Chaperones 11, 180-186; herein incorporated by reference in their entirety). Cell injury was quantified after GD by microscopic evaluation and cell counting after Hoechst 33342 (5 µM) and propidium iodide (PI, 5 µM) staining. PI stains dead cells but does not cross intact plasma membranes. Hoechst dye is a cell permeable nucleic acid stain that labels all nuclei.

Mitochondrial membrane potential was monitored using tetramethylrhodamine methyl ester (TMRE) and reactive oxygen species were monitored using hydroethidine (HET), as previously described (Ouyang et al. (2011) Mitochondrion 11, 279-286), using a Zeiss Axiovert 200M fluorescence microscope (Zeiss, Jena, Germany).

Reverse Transcription Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated with TRIzol® (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Equal amounts of total RNA (200 ng) were reverse-transcribed with 1.3 mM dNTPs (with dTTP), 50 U reverse transcriptase, 10 U RNase inhibitor, and specific miRNA reverse transcriptase primers (Applied Biosystems) at 16°C for 30 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. PCR reactions were then conducted using the TaqMan® MicroRNA Assay Kit (Applied Biosystems) at 95°C for 10 minutes, followed by 40 cycles of

95°C for 15 seconds and 60°C for 1 min. Each reaction contained 0.75 µl of the RT reaction product, 5 µl TaqMan® 2×Universal PCR Master Mix (Applied Biosystems) in a total volume of 10 µl using the 7900HT (Applied Biosystems). Predesigned primer/probes (Applied Biosystems) for miRNAs and mouse U6 were from Applied Biosystems; catalogue numbers are listed in Table 1. The expression of miR-181a, b, c, or d was normalized using U6 as the internal control. Measurements were normalized to U6 (ΔC_t) and comparisons calculated as the inverse log of $\Delta\Delta C_t$ to give the relative fold change for all miRNA levels (Livak and Schmittgen, (2001) Methods 25, 402-408.). Liu et al. have validated U6 as not changing in cerebral ischemia (Liu et al. (2010) Cereb. Blood Flow Metab. 30, 92-101). The PCR experiments were repeated three times, each using separate sets of samples.

Immunoblotting

Immunoblotting was performed as previously described (Han et al. (2009) Anesth. Analg. 108, 280-287; Ouyang et al. (2007) J. Neurosci. 27, 4253-4260; herein incorporated by reference in their entireties). Equal amounts (about 50-75 µg) of protein were separated on a polyacrylamide gel and electrotransferred to Immobilon polyvinylidene fluoride membrane (Millipore Corp., Billerica, MA, USA). Membranes were blocked and incubated overnight with primary antibody against Bcl-2 (1:1000, #2870, Cell Signaling, Danvers, MA, USA), Bim (1:500, ALX-804-527, Enzo, Plymouth Meeting, PA, USA), Mcl-1 (1:1000, 600-401-394, Rockland, Gilbertsville, PA, USA), or GRP-78 (PA1-37806, Affinity BioReagents, Golden, CO, USA) and β -actin (926-42210, LiCOR Bioscience, NE, USA), and β -actin (1:1000, 926-42210, LiCOR Bioscience, Lincoln, NE, USA), washed and incubated with 1:15000 anti-rabbit antibody (926-32221, LiCOR Bioscience) and anti-mouse antibody (926-32220, LiCOR Bioscience). Immunoreactive bands were visualized using the LICOR Odyssey infrared imaging system according to the manufacturer's protocol. Densitometric analysis was performed using ImageJ software (NIH). Band intensities were normalized to β -actin.

Intracerebroventricular Infusion of miRNA Plasmid and Antagomir

All experimental protocols performed on animals were performed according to protocols approved by the Stanford University Animal Care and Use Committee and

in accordance with the NIH guide for the care and use of laboratory animals. Plasmid embedding pri-miR-181a1b1 was injected intracerebroventricularly (ICV) in mice according to the method of Xiong et al. (Stroke (2011) 42:2026). Adult male C57BL/6J mice (25-30 g) were anesthetized with 2% isoflurane in 70% N₂O balance O₂ by facemask and placed in a stereotaxic frame with a mouse head holder. The brain infusion cannula was stereotaxically implanted into the left lateral ventricle of the brain (bregma: -0.58 mm; dorsoventral: 2.1 mm; lateral: 1.2 mm) as described previously (Xiong et al (2011) Stroke 42(7):2026-32). and was affixed to the skull. One µg of plasmid or antogomir or their controls was mixed with the cationic lipid DOTAP (1:3 µg/µl; Roche Applied Science, Indianapolis, IN, USA). After mixing for 5 seconds and incubating at 37 °C for 15 minutes, the mixture was infused into the left lateral cerebral ventricle at a speed of 1 µl/minute via a burr hole. After that the bone wound was closed with bone wax, anesthesia was discontinued, and mice were returned to their cages.

15

Focal cerebral ischemia

Two days after the injection, mice were anesthetized, as described above, and focal cerebral ischemia was produced by 1 hour of middle cerebral artery occlusion (MCAO) with a silicone-coated 6-0 monofilament followed by reperfusion, as described before (Han et al, *supra*; Xiong et al, *supra*). Sham-operated mice underwent an identical procedure, without inserting the suture. Rectal temperature was maintained at 37 ± 1°C with a heating pad. Heart rate, oxygen saturation and respiratory rate were monitored continuously. At different time points of reperfusion, mouse brains were removed and used for assessing infarct volume, RT-qPCR and Western blot analysis. 1 hour of MCAO results in infarction in the core and penumbral areas at the borders of infarction, which can be rescued by agents. To explore the possible regional changes of miRNA expression, brain tissues corresponding to the ischemic core and penumbra were dissected for RT-qPCR and Western blot analysis as described by Gao et al. (J. Neurochem. (2008) 105, 943-955; see Figure 1).

30

Measurement of Cerebral Infarction Area

At 24 hours after MCAO, the mice were deeply anesthetized with isoflurane, and brains were harvested rapidly after perfusion with cold phosphate buffered saline and cold 4% paraformaldehyde. For histological analysis, brains were sectioned coronally into 40 micron coronal sections with a vibratome. Coronal sections were assessed by cresyl violet staining. Infarction volume was determined using four slices per mouse and was analyzed by a blinded observer and corrected for edema using the NIH Image program as described previously (Han et al, *supra*).

Statistics

All data reported represent at least 3 independent experiments for n=3-6 cultures in each experiment. Data reported are means $\pm$ SD. Statistical difference was determined using the T test for comparison of two groups or ANOVA followed by Mann-Whitney test for experiments with greater than two groups using Prism 5.0a software (Graphpad, La Jolla, CA, USA) or StatView software (SAS Institute, Cary, NC, U.S.A.). $P < 0.05$ was considered significant.

Example 2

Constructs Comprising MicroRNA-181

Several constructs comprising microRNA-181 were prepared (see Figures 1A-1C), including the pri-miR-181ab construct comprising the sequences of wild-type mature miR-181a (SEQ ID NO:1) and mir-181b (SEQ ID NO:2); the miR-181cd construct comprising the sequences of wild-type mature miR-181c (SEQ ID NO:3) and mir-181d (SEQ ID NO:4), the miR-181ab-SM construct comprising the seed mutant sequences of miR-181a-SM (SEQ ID NO:5) and mir-181b-SM (SEQ ID NO:6); and the miR-181cd-SM construct comprising the seed mutant sequences of miR-181c-SM (SEQ ID NO:7) and mir-181d-SM (SEQ ID NO:8). MicroRNA sequences were cloned into the MWXPGKIREs-GFP plasmid. This plasmid included a PGK promoter, an internal ribosomal entry site (IRES), and a green fluorescent protein (GFP) reporter gene. DNA fragments containing the pri-miR-181ab, the pri-miR-181cd hairpin, or their corresponding seed mutant sequences and

about 250 nucleotides of flanking sequence were cloned downstream of the PGK promoter of the MWXPGKIREs-GFP plasmid (Figure 1C).

5

Example 3

Constructs Comprising 3'UTR Sequences of Bcl-2, Bcl-2-L11, and Mcl-1

Several constructs comprising the 3'UTR sequences of Bcl-2 family genes were prepared (see Figures 1D and 1E), including the Bcl-2-3'UTR construct comprising the sequence of the mouse Bcl-2 3'UTR (SEQ ID NO:17); the Bcl-2-L11-3'UTR construct comprising the sequence of the mouse Bcl-2-L11 3'UTR (SEQ ID NO:18); and the Mcl-1-3'UTR construct comprising the sequence of the mouse Mcl-1 3'UTR (SEQ ID NO:19). The 3'UTRs of Bcl-2, Bcl-2-L11, and Mcl-1 were cloned into the phRL-TK vector (Promega, Madison, WI, USA), which contains a thymidine kinase (TK) promoter, a Renilla luciferase reporter gene, and an SV40 polyadenylation sequence (see Figure 1E). The PCR primer sets used to generate specific 3'UTR fragments are shown in Table 2.

Table 2. Primer sets used to generate 3'UTRs of Bcl-2 family genes

	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
BCL2	TCAGGCAAATGGTCGAATC (SEQ ID NO:11)	CAAGAAGCAGCTTGAAGGAG (SEQ ID NO:12)
BCL2L11	GTCATGTCCCTCTCTTGGTG (SEQ ID NO:13)	GTCCTACCAAAAACGAGAGAG (SEQ ID NO:14)
MCL1	GAACATAAGAGGGAAGTCTTGGA (SEQ ID NO:15)	GGGTTATTTAGGTTCAAAGTTTTG (SEQ ID NO:16)

Mutant 3'UTRs of the Bcl-2, Bcl-2-L11, and Mcl-1 genes with 6 base substitutions were generated (SEQ ID NOS:20-22; also see Figures 1D and 1E, mutated nucleotides are indicated in bold and underlined). Both wild type and mutant inserts were confirmed by sequencing.

Example 4**Constructs Comprising 3'UTR Sequences of HSPA5, HSPA9 and HSPA1A**

Several constructs comprising the 3'UTR sequences of HSP70 family genes were prepared. The full length mouse 3'UTRs of HSPA5, HSPA9 and HSPA1A were cloned into the Renilla luciferase reporter vector phRL-TK from Promega (Madison, WI, USA). The primer sets used to generate specific 3'UTR fragments are shown in Table 3

Table 3. Primer sets used to generate 3'UTRs of HSP70 family genes

	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
HSPA5	GTGCACTGATCTGCTAGAGC (SEQ ID NO:23)	CTTAAATTTTAGTTATCTAAAATAAAAGATGG (SEQ ID NO:24)
HSPA9	TAATAGCAGAAATTTGAAGCCAGAAG (SEQ ID NO:25)	GGAACCCAGATTCTTTCCCTA (SEQ ID NO:26)
HSPA1A	TAGAGGCCTCTGCTGGCTCT (SEQ ID NO:27)	TGGCAAGTGATTTGAAGTTTATTTTC (SEQ ID NO:28)

A mutant 3'UTR of the HSPA5 gene with 6 base substitutions was also generated. The sequences of the wild-type and mutant HSPA5 3'UTR segments from nucleotides 83 to 89 are the following:

HSPA5 3'UTR: 5'- G-UCUCGAAUGUAA-UU-3' (SEQ ID NO:29)

Mutant HSPA5 3'UTR: 5'- G-UCUC**CUUACAA**A-UU-3' (SEQ ID NO:30)

The six nucleotides shown in bold and underlined are mutated. Both wild-type and mutant inserts were confirmed by sequencing.

Example 5**MiR-181 Targets the 3'UTRs of Three Bcl-2 Family Members**

Using computational miRNA target prediction algorithms, as detailed at TargetScan (targetscan.org, Release 5.1) and Microcosm Targets (ebi.ac.uk/enrightsrv/microcosm), we identified three members of the Bcl-2 family

with mRNA 3'UTRs that were potential targets: Bcl-2 and Mcl-1 in the pro-survival subgroup and Bim/Bcl-2-like 11 (Bcl-2-L11) a BH3-only pro-apoptotic protein. The seed sequences of all these 3'UTR targeted by miR-181 are highly evolutionarily conserved (Figure 1A), suggesting a critical role in normal physiology.

5 To validate these 3'UTRs as targets of miR-181, cells were cotransfected with constructs of luciferase control reporter, luciferase target reporter containing the 3'UTR of Bcl-2, Bcl-2-L11/Bim, or Mcl-1 (wild type, WT) and pri-miR-181 (WT or pri-miR-181 mutant). As frequently observed, there is some difference in luciferase activity with the addition of even the pri-miR-181 SM compared to the vector control.
10 These differences generally reflect non-specific effects; in this case the BOSC23 cells used do not normally express miR-181. Others have seen similar changes with miRNA SM (Arnold et al. (2011) Genome Research 21, 798-810). We also tested additional negative controls for miR-181, scrambled RNAi and an irrelevant miRNA mmu-let-7b and saw little effect on luciferase activity with any of these three 3'UTRs
15 (data not shown). As shown in Figure 2B, both pri-miR-181ab and pri-miR-181cd repress all three 3'UTRs compared to their pri-miR-181 mutant controls. To exclude off-target effects, we also mutated the seed sequences of the 3'UTRs of Bcl-2, Bcl-2-L11, and Mcl-1 as shown in Figure 1D.

 Cells were cotransfected with constructs of luciferase control reporter,
20 luciferase target reporter containing the 3'UTR of wild type or its mutant, and miR-181ab or cd WT. These results validated Bcl-2, Bcl-2-L11, and Mcl-1 as targets of both miR-181ab and miR-181cd (Figure 2C).

 We initially only mutated the miR-181 binding site that is predicted to be conserved across species. This led to an increase in luciferase activity, but did not
25 reach the level seen in the vector control. In the case of the mouse BCL2 3'UTR, a second binding site exists (at position 1480-1486, 220 bp before the conserved target site). When both BCL2 3'UTR binding sites are mutated full luciferase activity is restored (Figure 3), suggesting that this second site may also be active.

Example 6

MiR-181 Alters Pro-Survival Bcl-2 Family Protein Levels in Astrocytes

5

Since miR-181 is highly expressed in brain, we studied its effects on these proteins in astrocytes. Our preliminary results showed that in mouse brain miR-181a was present at the highest level, with miR-181b about 10%, miR-181c about 34%, and miR-181d about 8% of miR-181a levels (data not shown). We chose to focus on the most highly expressed family member, miR-181a. First, we titrated mimic and inhibitor and measured levels of miR-181a (Figures 3A and 3B). We noted that with 50 pmol mimic, the configuration of mitochondria in the cells changed from the usual filamentous pattern, upper panel, to a fragmented pattern (Figure 3C), so we chose a lower concentration of mimic for the subsequent studies. We used transfection with either 10 pmol mimic or 40 pmol inhibitor, which resulted in an increase of 16-fold in miR-181a levels with mimic (Figure 3A) or a decrease of 58% with inhibitor (Figure 3B). We previously found that the ability to undergo apoptosis decreased with time in culture for astrocytes (Xu et al. (2004) Neurological Research 26, 632-643), so we assessed miR-181a levels in astrocytes with time in culture, using RT-qPCR (Figure 3D). Levels increased between day 3 and 7, and then were fairly stable through 60 days. If changes in miR-181a are biologically relevant they should result in changes in protein levels. We examined the protein levels of Bcl-2, Bim, and Mcl-1 by Western blot after transfection of astrocytes with either mimic or inhibitor. Protein levels of Mcl-1 and Bcl-2 changed significantly in transfected cells, decreasing in the presence of mimic and increasing after transfection with inhibitor (Figures 3 E and F), but effects were stronger for Bcl-2, decreasing more than 50% with mimic, while Mcl-1 decreased about 30%. Bim protein was not detectable in our primary cultured astrocytes using commercially available antibodies.

30

Example 7**MiR-181 Influences Apoptosis, Mitochondrial Function, and Oxidative Stress
in Glucose Deprived Astrocytes**

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Cells were transfected with miR-181a mimic or inhibitor one day before GD. We examined the effects of changing miR-181a levels on astrocyte survival of 24 hours of glucose deprivation (GD). As demonstrated in Figures 4A and 4B, elevated expression of miR-181a reduced cell survival by 31%, whereas knockdown of

10 endogenous miR-181a levels increased survival by 27% compared to control cells. Increased levels of miR-181a were associated with increased numbers of cells showing condensed nuclear morphology, typical of apoptosis in astrocytes (Xu et al., *supra*). Reactive oxygen species (ROS) generation was then assessed during glucose deprivation using hydroethidine. ROS increased with mimic and decreased with

15 inhibitor relative to control astrocytes (Figure 4C). We also note that the starting level of ROS was higher in cells treated with miR-181a mimic compared with the control (Figure 4C).

Mitochondrial membrane potential was assessed with tetramethylrhodamine during glucose deprivation. More rapid depolarization was observed in cells

20 transfected with mimic, and slower depolarization was observed in cells transfected with inhibitor (Figure 4D). Thus reduction of miR-181a is associated with reduced cell death, reduced oxidative stress, and preserved mitochondrial function while increased miR-181a levels have the opposite effects (Table 4).

25 Table 4. Summary of related changes in miR-181a and target protein levels, cell survival and mitochondrial function

Transfection	miR-181a level	Bcl-2 or Mcl-1 levels	Cell survival	Mitochondrial function
miR-181a mimic	↑	↓	↓	↓
miR-181a inhibitor	↓	↑	↑	↑

Discussion

We have identified three targets of miR-181a in the Bcl-2 family, Bcl-2, Mcl-1, and Bcl-2-L11/Bim. Bcl-2 and Mcl-1 are antiapoptotic, whereas Bcl-2-L11/Bim is

30 proapoptotic. We examined the effects of altering miR-181a levels in primary culture

astrocytes, and observed effects on Bcl-2 and Mcl-1 protein levels, but were unable to detect Bim protein in our cells. In astrocytes, differential expression of the proteins leads to increased vulnerability with increased miR-181a and decreased vulnerability when miR-181a is reduced, consistent with changes in the protein levels of Bcl-2 and Mcl-1. Since control of mRNA by miRNA is combinatorial, it is likely that the complement of miRNA expressed at a given time in a given cell will determine the overall changes in mRNA function. Other miRNA will also target these mRNAs and therefore affect final protein levels.

Overexpression of any of the five pro-survival members protects cells against apoptosis induced by a variety of cytotoxic stimuli (Cory et al. (2003) *Oncogene* 22, 8590-8607). Gene targeting studies, however, reveal some specificity *in vivo*, including differences in relative expression by cell types and tissue (Cory et al., *supra*; Ranger et al. (2001) *Nat. Genet.* 28, 113-118.). Mcl-1 is a key regulator of apoptosis during CNS development and after DNA damage (Arbour et al. (2008) *The Journal of Neuroscience* 28, 6068-6078). Mcl-1 deficiency results in peri-implantation embryonic lethality (Rinkenberger et al. (2000) *Genes & Development* 14, 23-27), the most severe phenotype among knockouts of the anti-apoptotic Bcl-2 family members.

The reduction of hsa-miR-181a and b in gliomas negatively correlates with tumor grade (Shi et al. (2008) *Brain Res* 1236, 185-193) and ability to sensitize to radiation (Chen et al. (2010) *Oncol. Rep.* 23, 997-1003), consistent with the ability found here for reduction of miR-181 to increase resistance to apoptosis in normal astrocytes. For ischemia we seek to increase resistance to apoptosis, the opposite of the desired effect in cancer. Bcl-2 has previously been studied as a protein that can protect from cerebral ischemia, including *in vivo* and *in vitro* ischemia (Lawrence et al. (1996) *Journal of Neuroscience* 16, 486-496; Zhao et al. (2003) *Journal of Neurochemistry* 85, 1026-1036). A prior report demonstrated a correlation between miR-181 levels and Bcl-2 levels, but did not use a luciferase assay to validate the putative target sequence (Chen et al. (2010) *Oncol. Rep.* 23, 997-1003). In the case of Mcl-1, Zimmerman and colleagues (Zimmerman et al. (2010) *Molecular Pharmacology* 78, 811-817) reported positive luciferase results for human miR-181b against Mcl-1, and further observed regulation of miR-181 by Lyn kinase in leukemia. We confirmed their results with mouse miR-181a.

The results reported here suggest that miR-181 regulation of Bcl-2 and Mcl-1 contributes to mitochondrial dysfunction observed with *in vitro* ischemic stress, in this case glucose deprivation, in astrocytes. Bcl-2, Bcl-xL and Mcl-1, when overexpressed, lead to a controlled increase in oxidative stress and antioxidant defense, with increased levels of superoxide dismutase and glutathione peroxidase (Kowaltowski et al. (2004) Free Radical Biology and Medicine 37, 1845-1853; Papadopoulos et al. (1998) European Journal of Neuroscience 10, 1252-1260). The observed effects of altered levels of miR-181 are consistent with prior observations on the effects of overexpression of Bcl-2 or Bcl-xL alone, namely a decrease in oxidative stress and preserved mitochondrial membrane potential (Kowaltowski et al., *supra*; Ouyang and Giffard (2004) Cell Calcium 36, 303-311; Ouyang and Giffard (2004) Neurochemistry International 45, 371-379; Papadopoulos et al., *supra*).

In summary, Bcl-2, Mcl-1, and Bim are targets of mouse miR-181. Protein levels of Bcl-2 and Mcl-1 change inversely with changes in miR-181a. Reduction of miR-181a levels is associated with reduced cell death, reduced oxidative stress and preserved mitochondrial function in astrocytes.

Example 8

MiR-181 Targets the 3'UTR of HSPA5

From computational miRNA target prediction algorithms, as detailed at TargetScan (targetscan.org, Release 5.1) and Microcosm Targets (ebi.ac.uk/enright-srv/microcosm), we found that a broadly conserved miRNA family (conserved across most vertebrates, usually to zebrafish), miR-181, could potentially target mRNA 3'UTRs of HSPA5 (GRP78), HSPA9 (GRP75) and HSPA1A (HSP72). The seed sequence of HSPA5 3'UTR that is targeted by miR-181 is highly conserved at nucleotides 86-92 (Figure 5A), suggesting a critical role in their physiology and pathology.

To validate whether miR-181 directly recognizes the 3'UTRs of HSP70 family genes, we cotransfected astrocytes with constructs of luciferase control reporter, luciferase target reporter containing the 3'UTRs of HSPA5, HSPA9 and HSPA1A (wild type or mutant) and miRNA (miR-181 or miR-181sm). As showed in Figure 9,

none of the miR-181 family members (a to d) have a direct effect on the 3' UTRs of HSPA9 and HSPA1A, but all significantly decrease luciferase activity by about 60% when the 3'UTR of HSPA5 was applied. MiR-181 acts directly at the HSPA5 3'UTR (Figure 5B).

5

Example 9

Differential Expression of MiR-181 and GRP78 in Infarct Core and Penumbra After MCAO

10

As reported earlier (Chen et al, (2004) Science 303, 83-86), miR-181 was very strongly expressed in the brain. In normal control brain miR-181a has the highest level, miR-181b is 56%, miR-181c about 13% and miR-181d about 22% of miR-181a levels (Figure 5C). Since miR-181a is the most highly expressed miR-181 family member in the brain (Figure 6A), the contribution of miR-181a to total amount of miR-181 should be higher than the other three members.

15

To explore the possible regional and spatial changes of miRNA expression, brain tissue was dissected into ischemic core and ischemic penumbra at 0, 1, 3, 5 and 24 hours of reperfusion after 1 hour of MCAO. Following 1 hour of MCAO, the levels of all miR-181 family members changed, increasing in the ischemic core area and decreasing in the penumbra (Figure 6A and Figure 10). At 24 hours of reperfusion, the miR-181a level in the ischemic core increased to $144 \pm 7\%$, whereas that of the penumbra decreased to $60 \pm 8\%$ compared with control animals (Figure 6A). To confirm that miR-181 acts as a negative regulator of GRP78 translation under ischemic conditions, we examined the time course for both GRP78 protein and grp78 mRNA expression at the same reperfusion time points as in the miR-181a measurement above.

20

25

The differential regional expression of miR-181 indicates that miR-181 plays a role in the development of the ischemic core as well as in protection of the penumbra from further injury after cerebral ischemia.

30

Example 10

Effect of MiR-181 Overexpression and Inhibition on I/R Cerebral Injury *in Vivo*

5 To further evaluate the biological role of miR-181 in ischemic brain injury *in vivo*, we injected either pri-miR-181 plasmid or its antagomir ICV. Two days after successful administration, greater than 2-fold overexpression or nearly complete knockdown of miR-181 levels in the brain by miR-181a or its antagomir was confirmed by quantitative RT-PCR analysis (Figure 7A). In contrast, the pri-miR-
10 181 seed mutant plasmid or mutant antagomir-181 had no effect on the expression level of miR-181 compared with the saline control (data not shown). The expression levels of GRP78 were inversely correlated with the expression of miR-181 (Figure 7B). Overexpression of miR-181 aggravated cerebral ischemic damage and increased the infarct size at 24 hours of reperfusion after 1 hour of MCAO, and knockdown of
15 miR-181 effectively attenuated ischemic injury (Figure 7C). These data demonstrate that inhibition of cerebral miR-181 provides a neuroprotective role in ischemic brain insult, probably by targeting GRP78 chaperone.

Example 11

20

Altered MiR-181 Expression Regulates GD-Induced Cell Death in Cultured Astrocytes

 Gain-of function or loss-of-function was achieved by transfecting a miR-181
25 mimic or inhibitor into cultured astrocytes in order to investigate the functional significance of miR-181 in cerebral ischemia. The dose-response of miR-181 transfection is shown in Figures 11A and 11B. 10 pmol mimic and 40 pmol inhibitor were used in experiments. The effects of miR-181a on cell survival with 24 hours of GD were examined. As demonstrated in Figure 8A, increased expression of miR-181
30 reduced cell survival by 31%, whereas knockdown of endogenous miR-181 increased survival by 27% compared to control cells (Figure 8B). We repeated the results with pri-miR-181ab constructs. RT-qPCR analyses revealed that the miR-181a expression level was more than 7-fold greater than miR-181b in human BOSC 23 cells, which

express negligible amounts of miR-181 under normal conditions (data not shown). Figure 11C shows that the level of expression of miR-181a approximately doubled after transfection in the astrocyte cell line. Figure 8B demonstrates that under this moderate and more naturally overexpressing condition, miR-181 reduced cell survival by 17%. These results further support the conclusion from *in vivo* cerebral ischemia that inhibition of cerebral miR-181 provides neuroprotection.

To further examine if functions known to be influenced by the GRP78 chaperone (Ouyang et al. (2011), *supra*) are also influenced by miR-181, ROS and mitochondrial membrane potential in cultured cells were measured before and after GD. Cells were transfected with the miR-181 mimic or inhibitor one day before being subjected to 24 hours of GD. In normal transfected primary astrocytes and cell lines, treatment with the miR-181 mimic markedly increased generation of ROS within 24 or 48 hours after transfection (Figure 8C). Meanwhile, miR-181 had no noticeable effect on mitochondrial membrane potential in both cells within 48 hours after transfection (Figure 8D). Mitochondrial membrane potential is an important parameter of mitochondrial function and has been widely used as an indicator of cell health. Our data indicate that although cells produce more ROS, there was no apparent cell death caused by increased miR-181 levels under normal conditions. Under GD conditions, we noted a highly significant increase in ROS (Figure 8E) and decreased mitochondrial membrane potential (Figure 8F) in miR-181 overexpressing astrocytes, which was reversed by miR-181 inhibitor (Figures 8E and 8F). These results indicate that miR-181 regulates mitochondrial activity and ROS generation, which may contribute to cell death versus survival in the setting of cell stress.

Discussion

The major finding in this report is that a brain enriched miRNA, miR-181, regulates GRP78 expression and further regulates cerebral cell death in response to ischemic insults. The data indicated that miR-181 regulates the molecular chaperone family of proteins through gene silencing and modulating stroke induced brain injury.

MicroRNAs are evolutionarily selected post-transcriptional gene regulatory molecules that play an important role in cell physiology and pathology. The putative targets of miRNAs can be identified using prediction algorithms. Accurate prediction is complicated, however, by the secondary structure of the target mRNA, which

controls the accessibility of miRNA binding, and other factors. Thus, some predicted targets turn out not to be real targets.

Using TargetScan and Microcosm Targets algorithms, we found that the broadly conserved miRNA family miR-181 could potentially target mRNA 3'UTRs of some HSP70 family chaperone members. However, our results showed that grp78 mRNA, in particular, the HSPA5 3'UTR is the real target of miR-181. The results from computational miRNA target prediction algorithms revealed that the GRP78 gene HSPA5 3'UTR has only three conserved sites for miRNA families broadly conserved among vertebrates, and miR-181 is one of them (Targetscan.org).

The miR-181 family, especially miR-181a and miR-181b, are well known brain-enriched miRNAs (Miska et al. (2004) Genome Biol. 5, R68), and their aberrant expression has been associated with brain diseases. Hsa-miR-181a and hsa-miR-181b have low expression in human gliomas and glioma cell lines and their expression is negatively correlated with tumor grade (Shi et al. (2008) Brain Res. 1236, 185-193). Down-regulation of miR-181a-c was statistically significant in a glioblastoma patient and the expression level of the miR-181 family can predict responses to treatment (Slaby et al, (2010) Neoplasma 57, 264-269). The miR-181a sensitizes human malignant glioma cells to radiation (Chen et al, *supra*).

However, extremely little information has been reported regarding the relationship of the miR-181 family to cerebral ischemia. Two seemingly contradictory miRNA profiling studies have demonstrated aberrant expression of miR-181 in both global and focal ischemic models. In the first study, after 20 minutes of global ischemia in rat, hippocampal miR-181a was markedly upregulated after 30 minutes of reperfusion and further increased after 24 hours of reperfusion, but miR-181d during this period decreased more than 5-fold (Yuan et al. (2010) J. Clin. Neurosci. 17, 774-778). In the second study, after transient focal ischemia by MCAO, miR-181b was downregulated after 24 hours of reperfusion (Jeyaseelan et al. (2008) Stroke 39, 959-966). In our examination of differential expression of miR-181 in infarct core and penumbra after MCAO, we find that in the earlier reperfusion period after MCAO, increased miR-181 indicates an injured area that is destined to die and decreased miR-181 indicates an area that is potentially salvageable and a potential target for rescue treatments.

As a master regulator of unfolded protein response in ER, GRP78 plays a key role in development, cancer and neurological disorders (Wang et al. (2009) Antioxid Redox Signal 11, 2307-2316). While there is a modest increase in GRP78 mRNA after focal brain ischemia (Wang et al. (1993) Neurochem Int 23, 575-582), GRP78 mRNA expression after global ischemia and normothermic reperfusion is significantly suppressed in hippocampal pyramidal cells (Aoki et al. (2001) Brain Res Mol Brain Res 95, 117-128). Reports show that prior induction of increased levels of GRP78 with a pharmacological inducer reduces neuronal loss in both forebrain (Oida et al. (2008) Brain Res 1208, 217-224) and focal cerebral ischemia (Kudo et al. (2008) Cell Death Differ 15, 364-375).

We have recently shown that overexpressing GRP78 protects astrocytes against ischemic injury by maintaining relatively normal cellular functions (Ouyang et al. (2011), *supra*). Here we show that temporal and spatial changes of GRP78 after *in vitro* and *in vivo* cerebral ischemia are regulated by a specific miRNA, miR-181. Our data further indicate that downregulation of miR-181 represents an important adaptive mechanism to upregulate the expression levels of GRP78 during ischemia or recovery.

In conclusion, our data suggest that dysregulation of miR-181 expression contributes to cerebral ischemic injury. Knockdown of endogenous miR-181 provides protection against ischemia/reperfusion-induced brain cell death by targeting GRP78, a well-studied important molecular chaperone and neuroprotector.

Example 12

Constructs Comprising MicroRNA-29

Several constructs comprising microRNA-29 were prepared (see Figures 12A-12C), including the pri-miR-29ab construct comprising the sequence of wild-type mature miR-29a (SEQ ID NO:31) and the sequence of wild-type mature miR-29b (SEQ ID NO:32); the miR-29c construct comprising the sequence of wild-type mature miR-29c (SEQ ID NO:33); the miR-29ab-SM construct comprising the seed mutant sequence of miR-29a-SM (SEQ ID NO:34) and the seed mutant sequence of miR-29b-SM (SEQ ID NO:35); the miR-29a-SM construct comprising the seed mutant

sequence of miR-29a-SM (SEQ ID NO:34); the miR-29b-SM construct comprising the seed mutant sequence of miR-29b-SM (SEQ ID NO:35); and the miR-29c-SM construct comprising the seed mutant sequence of miR-29c-SM (SEQ ID NO:36). DNA fragments containing the pri-miR-29a, pri-miR-29b, pri-miR-29c, or their corresponding seed mutant sequences and about 250 nucleotides of flanking sequence were cloned into the MWXPGKIREs-GFP plasmid downstream of the PGK promoter (Figure 12C).

Example 13

Constructs Comprising 3'UTR Sequences of BAK1, BBC3, BMF, Bcl-2-L2 and Mcl-1

Several constructs comprising the 3'UTR sequences of Bcl-2 family genes were prepared (see Figure 13), including the Bak-1-3'UTR construct comprising the sequence of the mouse Bak-1 3'UTR (SEQ ID NO:37); the BBC3-1-3'UTR construct comprising the sequence of the mouse BBC3-1 3'UTR (SEQ ID NO:38); the BBC3-2-3'UTR construct comprising the sequence of the mouse BBC3-2 3'UTR (SEQ ID NO:39); the BMF-3'UTR construct comprising the sequence of the mouse BMF 3'UTR (SEQ ID NO:40); the Bcl-2-L2-3'UTR construct comprising the sequence of the mouse Bcl-2-L2 3'UTR (SEQ ID NO:41); and the Mcl-1-3'UTR construct comprising the sequence of the mouse Mcl-1 3'UTR (SEQ ID NO:42). The 3'UTRs of Bak-1, BBC3, Bmf, Bcl-2-L2 and Mcl-1 were cloned into the phRL-TK vector (Promega, Madison, WI, USA) downstream of the TK promoter (see Figure 13). The primer sets used to generate specific 3'UTR fragments are shown in Table 5.

Table 5. Primer sets used to generate specific fragments or full length of 3'UTRs of Bcl-2 family genes

3'UTR	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
BAK1	CTCCCAAGGGTGCCCTTTG (SEQ ID NO:49)	CAGGGTGAGGGGATTGCAC (SEQ ID NO:50)
BBC3	ACTTTTCTGCACCATGTAGC (SEQ ID NO:51)	TGTCCTTACAGGTAGTGCCAG (SEQ ID NO:52)
BMF	GATACAGGAAGAAGCTAGGACTG (SEQ ID NO:53)	CATCAGGATGTGCAGCCTAG (SEQ ID NO:54)
BCL2L2	GACCATTCCAGCTCTGAGTC (SEQ ID NO:55)	AGTGCAACCTTCTCTCAAAG (SEQ ID NO:56)
MCL1	TTGTGAGTGCAATAGGGGAC	CATTAACCTGCAGTTGGTCCT

	(SEQ ID NO:57)	(SEQ ID NO:58)
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Constructs containing mutant 3'UTRs of the Bak-1, BBC3, Bmf, Bcl-2-L2 and Mcl-1 genes with 8 base substitutions (SEQ ID NOS:43-48) were also generated. See Figure 13 (mutated nucleotides are underlined). Both wild type and mutant
5 inserts were confirmed by sequencing.

Example 14

Levels of MicroRNA-29a in Cortical Neurons, Astrocytes, and Brain 10 and Effects of Ischemia

The levels of miR-29a in primary cultures of cortical neurons and astrocytes were measured *in vitro* after 7 and 21 days in culture. The levels of miR-29a were also measured in the brain cortex of rats at postnatal day 7 and 21. All values were
15 normalized to the neuronal miR-29a level at 7 days. As shown in Figure 14A, miR-29a is expressed in neurons, astrocytes and brain cortex, though at much higher levels in astrocytes and brain cortex than in neurons.

Next, the relative expression levels of miR-29a, miR-29b, and miR-29c were measured in normal rat hippocampus (Figure 14B). The miR29a had the highest level
20 of expression in rat hippocampus, its expression level being about 9-fold greater than that of miR29c and 50-fold greater than that of miR181b. In order to determine the effects of ischemia on expression of miR-29 in rat brain, levels of miR-29a were measured before and after forebrain ischemia and reperfusion (Figure 14C). Levels of miR-29a increased in the hippocampal DG area and decreased in the CA1 area
25 after 10 minutes of forebrain ischemia followed by 0 to 5 hours of reperfusion.

We then examined the effects of treating rats with miR29 or antagomir before inducing ischemia. Levels of miR-29a were determined by RT-qPCR. As shown in Figure 16, rats treated with the pri-miR-29ab plasmid showed elevated hippocampal levels of miR-29a. Rats treated with miR-29a antagomir showed reduced
30 hippocampal miR-29a. Either pri-miR-29ab plasmid or miR-29a antagomir was injected stereotactically unilaterally just above the CA1 section of the hippocampus two days before forebrain ischemia. Brain tissue was stained with cresyl violet. Selective loss of CA1 hippocampal neurons was observed after 6 days of reperfusion

(ischemia control). Loss of CA1 neurons was markedly reduced in the pri-miR-29ab injected brain and increased in the antagomir injected brain. Extensive loss of CA1-4 neurons was observed with antagomir.

5

Example 15

MiR-29 Influences Cell Survival in Glucose Deprived Astrocytes

The dose-response of miR-29 transfection of astrocytes in the presence and
10 absence of mimic and inhibitor is shown in Figures 15A-15C. Transfection with the pri-miR-29ab (ab) construct induced increased levels of miR-29a in cortical astrocytes compared to the seed mutant (ab-SM) and vector controls (Figure 15A). The effects of increasing amounts of miR-29a mimic or inhibitor in primary cultures of astrocytes are shown in Figures 15B and 15C.

15 We then studied the effects of miR-29 mimic and inhibitor on cell survival with 24 hours of GD. Astrocytes were transfected with the miR-29a mimic or inhibitor one day before GD. As demonstrated in Figures 15D and 15E, the miR-29a mimic increased cell survival by about 40%, whereas the miR-29a inhibitor increased cell death by about 90% compared to control cells. Thus, the miR-29a mimic reduces
20 cell injury and the miR-29a inhibitor aggravates cell injury induced by 24 hours of GD.

Mitochondrial membrane potential was assessed with tetramethylrhodamine (TMRE) during glucose deprivation. Membrane depolarization was determined by measuring decreases in TMRE fluorescence. Fluorescence values were normalized
25 to the starting fluorescence of 1.0. As shown in Figure 17, increasing miR-29a with mimic reduced mitochondrial membrane depolarization, whereas the miR29a inhibitor increased membrane depolarization in cortical astrocytes subjected to 3 hours of GD.

Reactive oxygen species (ROS) generation was then assessed during glucose
30 deprivation using hydroethidine (HET). Three hours of GD increased ROS in astrocytes. Transfection with the miR-29a mimic reduced ROS generation and transfection with the miR-29a inhibitor increased ROS generation relative to control

astrocytes. These results indicate that miR-29a influences mitochondrial activity and functional state with stress, as well as influencing apoptosis.

Example 16

5

MiR-29 Targets the 3'UTRs of Two Bcl-2 Family Members

Using computational miRNA target prediction algorithms, as detailed at TargetScan (targetscan.org, Release 5.1), we identified five members of the Bcl-2 family with mRNA 3'UTRs that were potential targets of miR29: Bak-1, BBC3, Bmf, Bcl-2-L2 and Mcl-1 (see Figure 13). In order to determine which 3'UTRs were targets of miR-29, we performed dual luciferase activity assays. As shown in Figure 18, PUMA (also known as BBC3) and BMF were validated as targets of miR-29. Dual luciferase activity assays were performed using co-transfection with a plasmid containing luciferase followed by the BBC3 or BMF 3'UTR (WT) and plasmids encoding either pri-miR-29 or their seed mutants (SM). The results show that miR-29ab, but not miR-29c, recognizes both 3'UTRs (Figure 18A). The same assay performed with the wild type 3'UTRs of BBC3 or BMF 3'UTR (WT) or their seed mutants (SM) shows that miR-29ab reduces luciferase activity for wild-type BBC3 and BMF 3'UTRs. Assays were performed in triplicate.

20

Example 17

MiR-29 Alters Pro-Apoptotic Bcl-2 Family Protein Levels in Brain

25

We examined the protein levels of PUMA by Western blot after treatment of rats with either miR29 or antagomir. PUMA protein levels were decreased in the hippocampus of rats pretreated with the pri-miR-29ab plasmid, whereas PUMA protein levels increased in brains pretreated with the miR-29a antagomir (Figure 19). Representative immunoblots are shown above the graphs (N=4 rats in each group, *P<0.01 compared to SM or Ctrl group).

30

While the preferred embodiments of the invention have been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.

Claims

What is claimed is:

1. A method for treating a subject having a neurological disorder, the method
5 comprising administering a therapeutically effective amount of a miR-181 inhibitor, a miR-29, or a miR-29 mimic to the subject.
2. The method of claim 1, wherein the neurological disorder is selected from the
group consisting of stroke, ischemic brain injury, traumatic brain injury, and
10 neurodegenerative disease.
3. The method of claim 2, wherein the subject shows improved recovery of
motor function after treatment.
- 15 4. The method of claim 2, wherein the subject shows improved cognition after
treatment.
5. The method of claim 2, wherein cerebral infarction size is reduced in the
subject after treatment.
20
6. The method of claim 1, wherein the subject is a mammal.
7. The method of claim 6, wherein the subject is human.
- 25 8. The method of claim 1, wherein the miR-181 inhibitor, miR-29, or miR-29
mimic is administered intracerebroventricularly.
9. The method of claim 1, wherein the miR-181 inhibitor, miR-29, or miR-29
mimic is administered intralesionally.
30
10. The method of claim 1, wherein the miR-181 inhibitor, miR-29, or miR-29
mimic is administered stereotactically into the brain of the subject.
11. The method of claim 1, wherein the miR-181 inhibitor, miR-29, or miR-29
35 mimic is administered into the spinal cord of the subject.

12. The method of claim 1, wherein the miR-181 inhibitor, miR-29 or miR-29 mimic is administered into cerebrospinal fluid of the subject.
- 5 13. The method of claim 1, wherein the miR-181 inhibitor, miR-29 or miR-29 mimic is administered intra-arterially.
14. The method of claim 13, wherein the miR-181 inhibitor, miR-29 or miR-29 mimic is administered intra-arterially into the blood supply of a lesion in the subject.
- 10 15. The method of claim 1, wherein the miR-181 inhibitor is selected from the group consisting of an antagomir, an antisense oligonucleotide, and an inhibitory RNA.
- 15 16. The method of claim 15, wherein the miR-181 inhibitor comprises the sequence of SEQ ID NO:9.
17. The method of claim 1, wherein the miR-29 comprises a sequence selected from the group consisting of SEQ ID NOS:31-33.
- 20 18. The method of claim 1, wherein the miR-181 inhibitor, miR-29, or miR-29 mimic comprises a detectable label.
19. A method for inhibiting miR-181 in a neuron cell, glial cell, or endothelial
25 cell, the method comprising introducing an effective amount of a miR-181 inhibitor into the cell.
20. The method of claim 19, wherein the glial cell is microglia or macroglia.
- 30 21. The method of claim 20, wherein the glial cell is an astrocyte or oligodendrocyte.
22. The method of claim 19, wherein the amount of Grp78 in the cell is increased compared to the amount of Grp78 in the cell in the absence of the inhibitor.

35

23. The method of claim 19, wherein the amount of a Bcl-2 family anti-apoptotic protein in the cell is increased compared to the amount of the Bcl-2 family anti-apoptotic protein in the cell in the absence of the inhibitor.
- 5 24. The method of claim 23, wherein the protein is Bcl-2 or Mcl-1.
25. The method of claim 19, wherein the inhibitor is selected from the group consisting of an antagomir, an antisense oligonucleotide, and an inhibitory RNA.
- 10 26. The method of claim 25, wherein the inhibitor comprises the sequence of SEQ ID NO:9.
27. A method for increasing the amount of Grp78 protein in a neuron cell, glial cell, or endothelial cell, the method comprising introducing an effective amount of a
15 miR-181 inhibitor into the cell.
28. The method of claim 27, wherein the glial cell is microglia or macroglia.
29. The method of claim 28, wherein the glial cell is an astrocyte or
20 oligodendrocyte.
30. The method of claim 27, wherein the inhibitor is selected from the group consisting of an antagomir, an antisense oligonucleotide, and an inhibitory RNA.
- 25 31. The method of claim 30, wherein the inhibitor comprises the sequence of SEQ ID NO:9.
32. A method for increasing the amount of a Bcl-2 family anti-apoptotic protein in a neuron cell, glial cell, or endothelial cell, the method comprising introducing an
30 effective amount of a miR-181 inhibitor into the cell.
33. The method of claim 32, wherein the glial cell is microglia or macroglia.
34. The method of claim 33, wherein the glial cell is an astrocyte or
35 oligodendrocyte.

35. The method of claim 32, wherein the inhibitor is selected from the group consisting of an antagomir, an antisense oligonucleotide, and an inhibitory RNA.
36. The method of claim 35, wherein the inhibitor comprises the sequence of SEQ
5 ID NO:9.
37. The method of claim 32, wherein the protein is Bcl-2 or Mcl-1.
38. A method for increasing the amount of a Bcl-2 family anti-apoptotic protein in
10 a neuron cell, glial cell, or endothelial cell of a subject, the method comprising introducing an effective amount of a miR-181 inhibitor into the neuron cell, glial cell, or endothelial cell of the subject.
39. The method of claim 38, wherein the neuron cell, glial cell, or endothelial cell
15 is located in the brain or spinal cord of the subject.
40. The method of claim 39, wherein the glial cell is microglia or macroglia.
41. The method of claim 40, wherein the glial cell is an astrocyte or
20 oligodendrocyte.
42. The method of claim 38, wherein the inhibitor is selected from the group consisting of an antagomir, an antisense oligonucleotide, and an inhibitory RNA.
- 25 43. The method of claim 42, wherein the inhibitor comprises the sequence of SEQ ID NO:9.
44. The method of claim 38, wherein the protein is Bcl-2 or Mcl-1.
- 30 45. A method for increasing the amount of a Grp78 protein in a neuron cell, glial cell, or endothelial cell of a subject, the method comprising introducing an effective amount of a miR-181 inhibitor into the neuron, glial cell, or endothelial cell of the subject.

46. The method of claim 45, wherein the neuron cell, glial cell, or endothelial cell is located in the brain or spinal cord of the subject.
47. The method of claim 46, wherein the glial cell is microglia or macroglia.
- 5 48. The method of claim 47, wherein the glial cell is an astrocyte or oligodendrocyte.
- 10 49. The method of claim 45, wherein the inhibitor is selected from the group consisting of an antagomir, an antisense oligonucleotide, and an inhibitory RNA.
50. The method of claim 49, wherein the inhibitor comprises the sequence of SEQ ID NO:9.
- 15 51. A method for decreasing the amount of a Bcl-2 family pro-apoptotic protein in a neuron cell, glial cell, or endothelial cell, the method comprising introducing an effective amount of a miR-29 or a miR-29 mimic into the cell.
- 20 52. The method of claim 51, wherein the glial cell is microglia or macroglia.
53. The method of claim 52, wherein the glial cell is an astrocyte or oligodendrocyte.
- 25 54. The method of claim 51, wherein the miR-29 comprises a sequence selected from the group consisting of SEQ ID NOS:31-33.
55. The method of claim 51, wherein the Bcl-2 family pro-apoptotic protein is PUMA or BMF.
- 30 56. A method for decreasing the amount of a Bcl-2 family pro-apoptotic protein in a neuron cell, glial cell, or endothelial cell of a subject, the method comprising introducing an effective amount of a miR-29 or miR-29 mimic into the neuron cell, glial cell, or endothelial cell of the subject.
- 35 57. The method of claim 56, wherein the neuron cell, glial cell, or endothelial cell is located in the brain or spinal cord of the subject.

58. The method of claim 57, wherein the glial cell is microglia or macroglia.
59. The method of claim 58, wherein the glial cell is an astrocyte or
5 oligodendrocyte.
60. The method of claim 56, wherein the miR-29 comprises a sequence selected
from the group consisting of SEQ ID NOS:31-33.
- 10 61. The method of claim 56, wherein the Bcl-2 family pro-apoptotic protein is
PUMA or BMF.

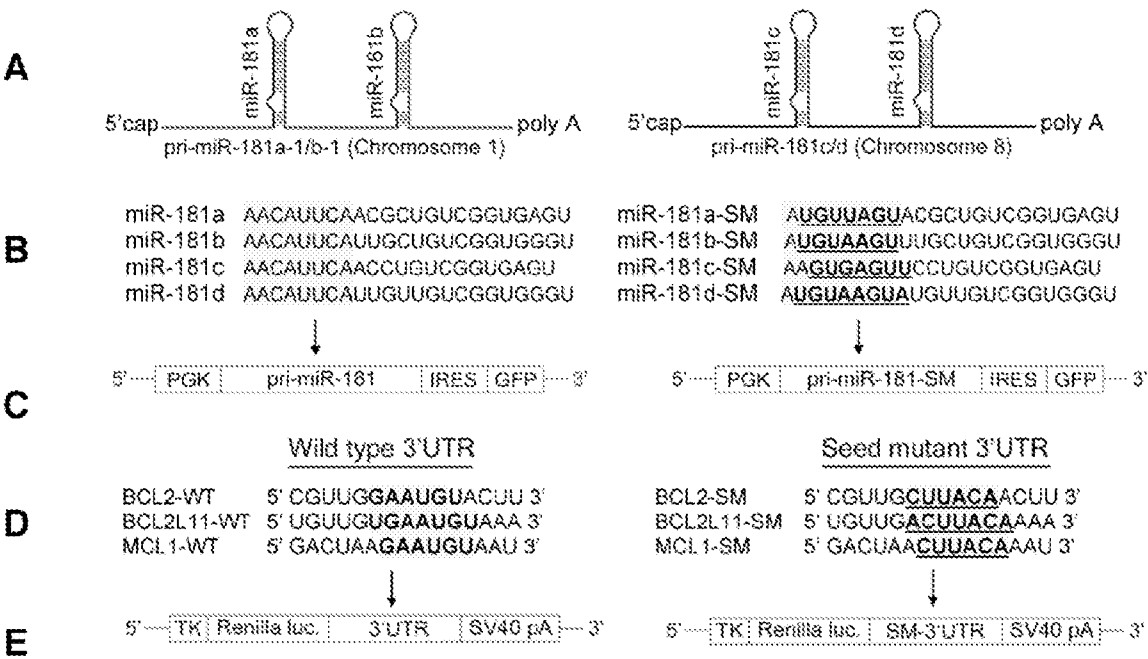


Figure 1

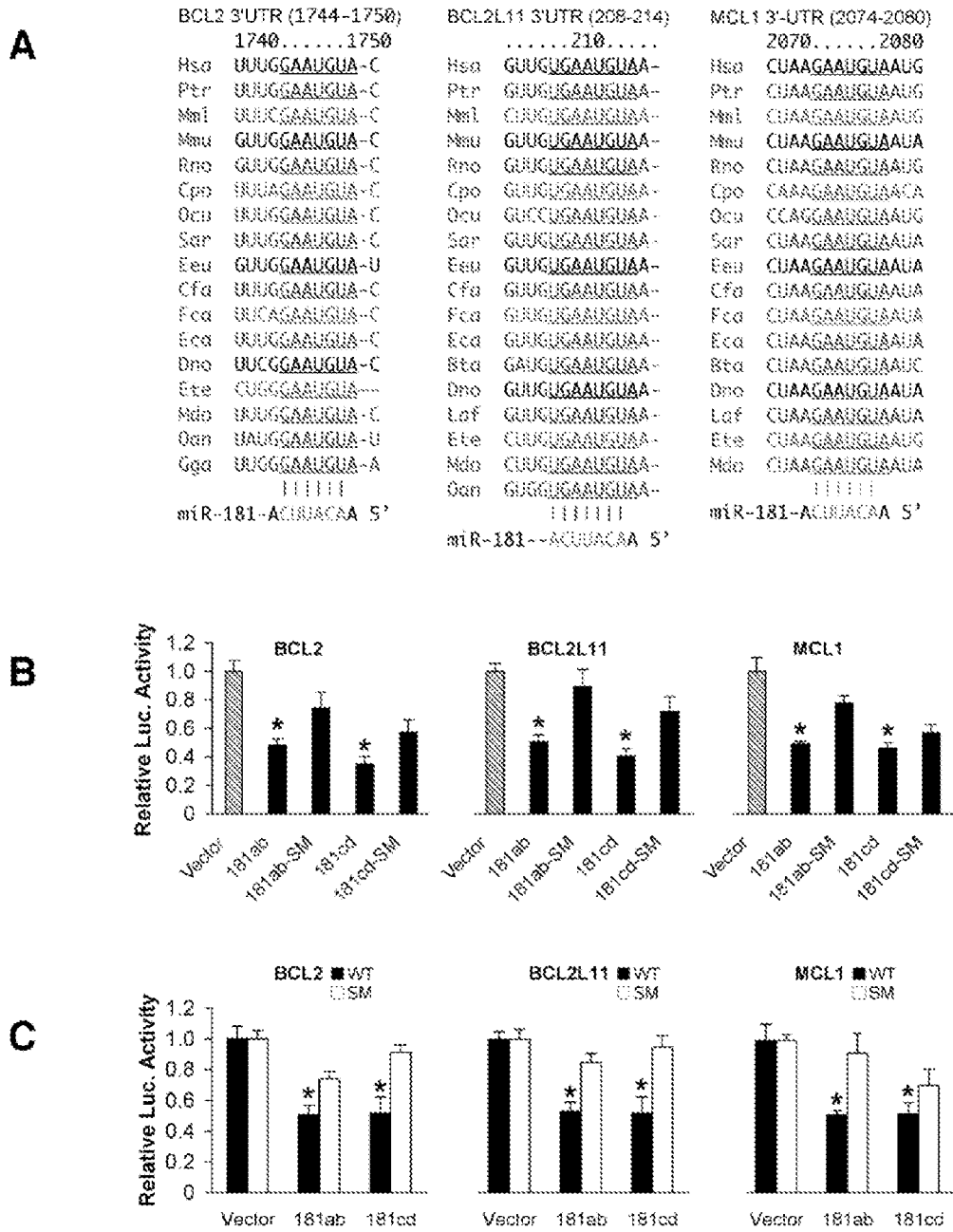


Figure 2

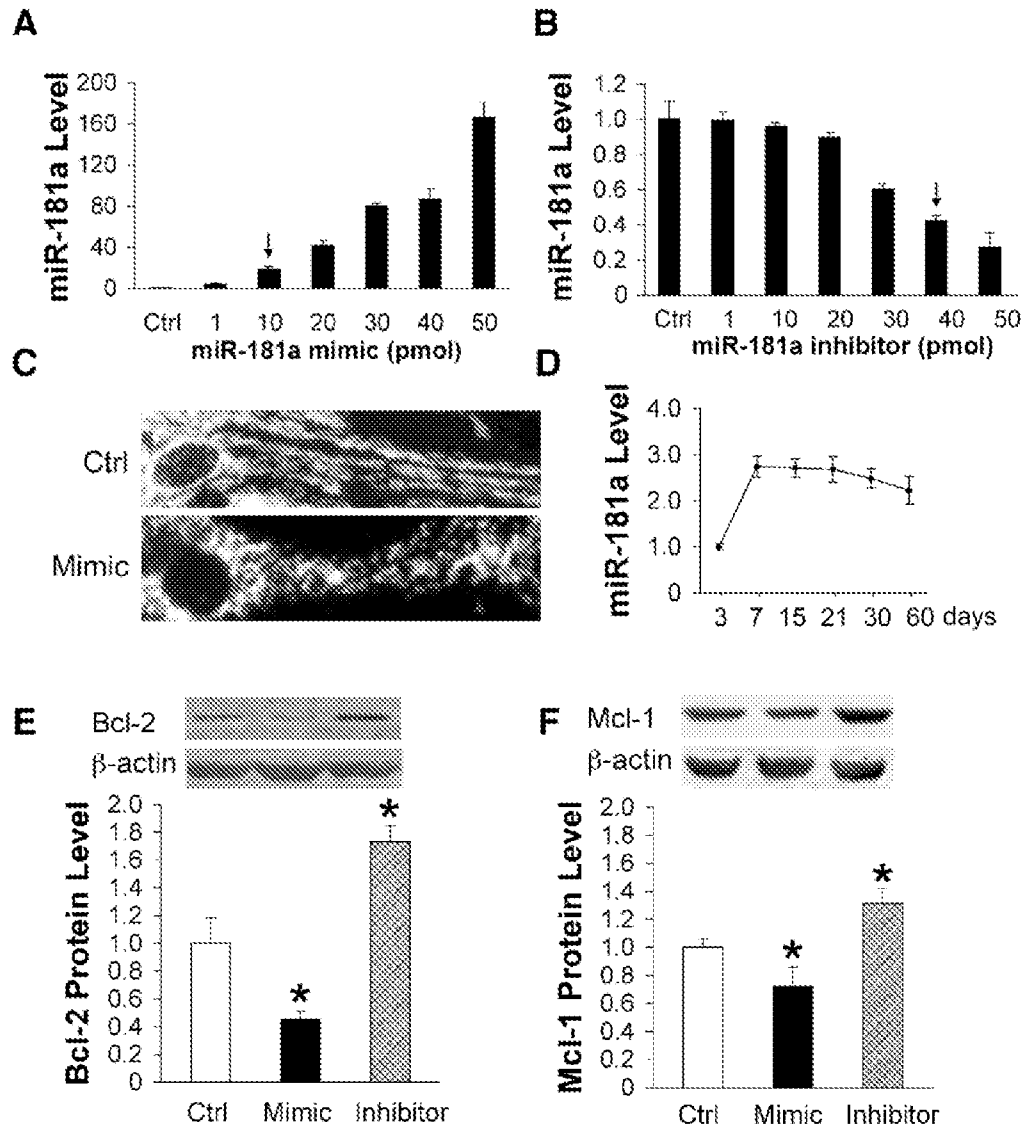


Figure 3

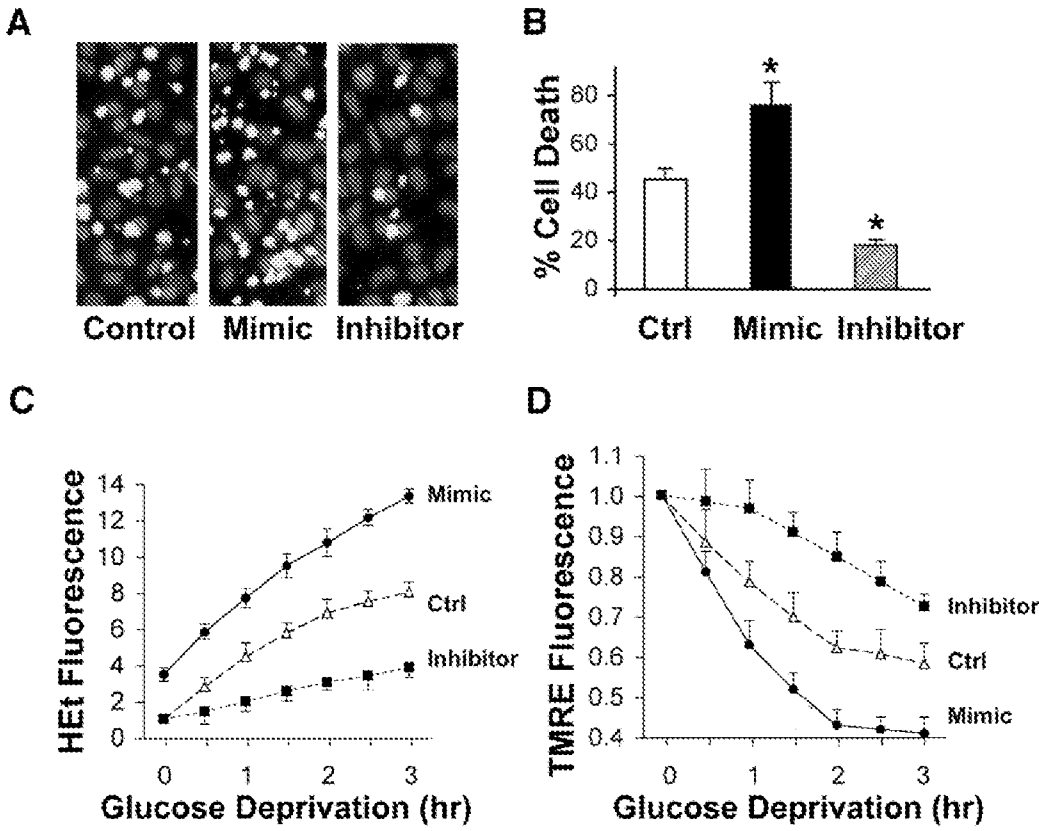


Figure 4

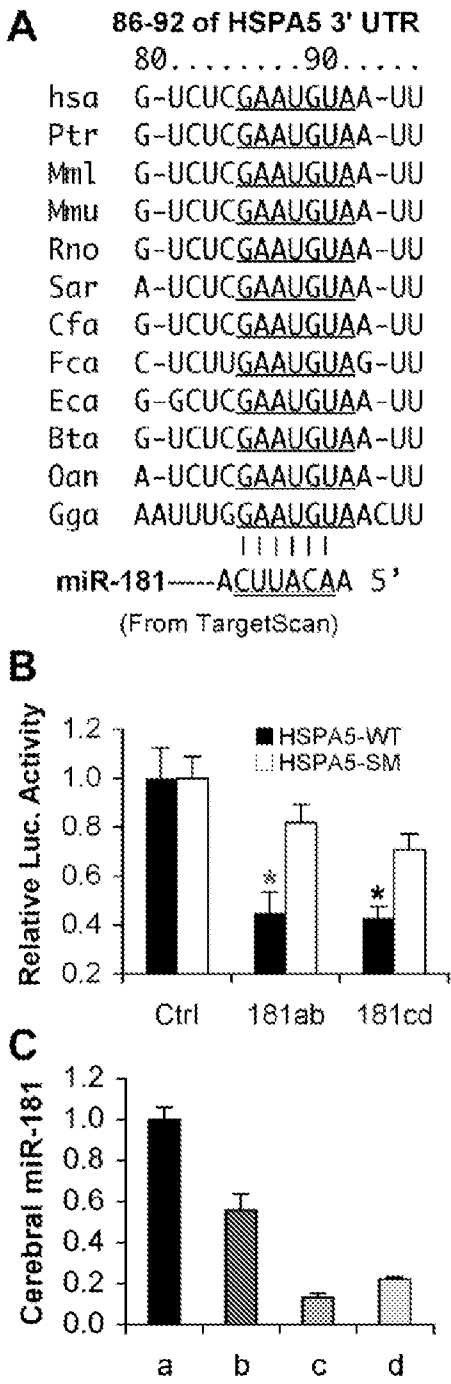


Figure 5

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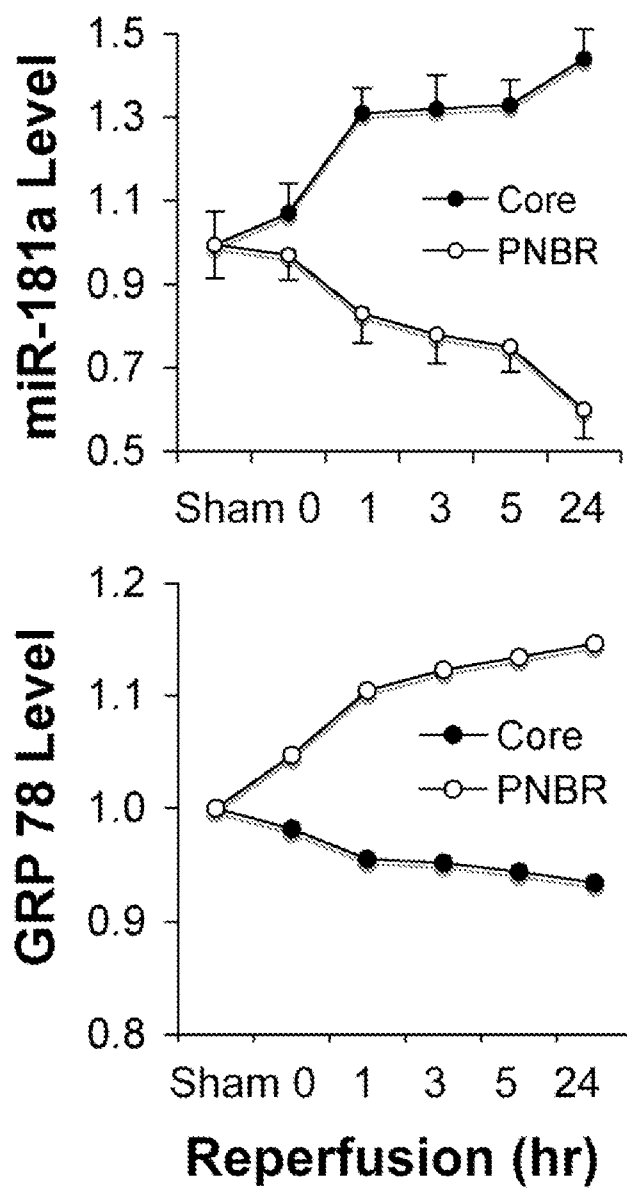


Figure 6

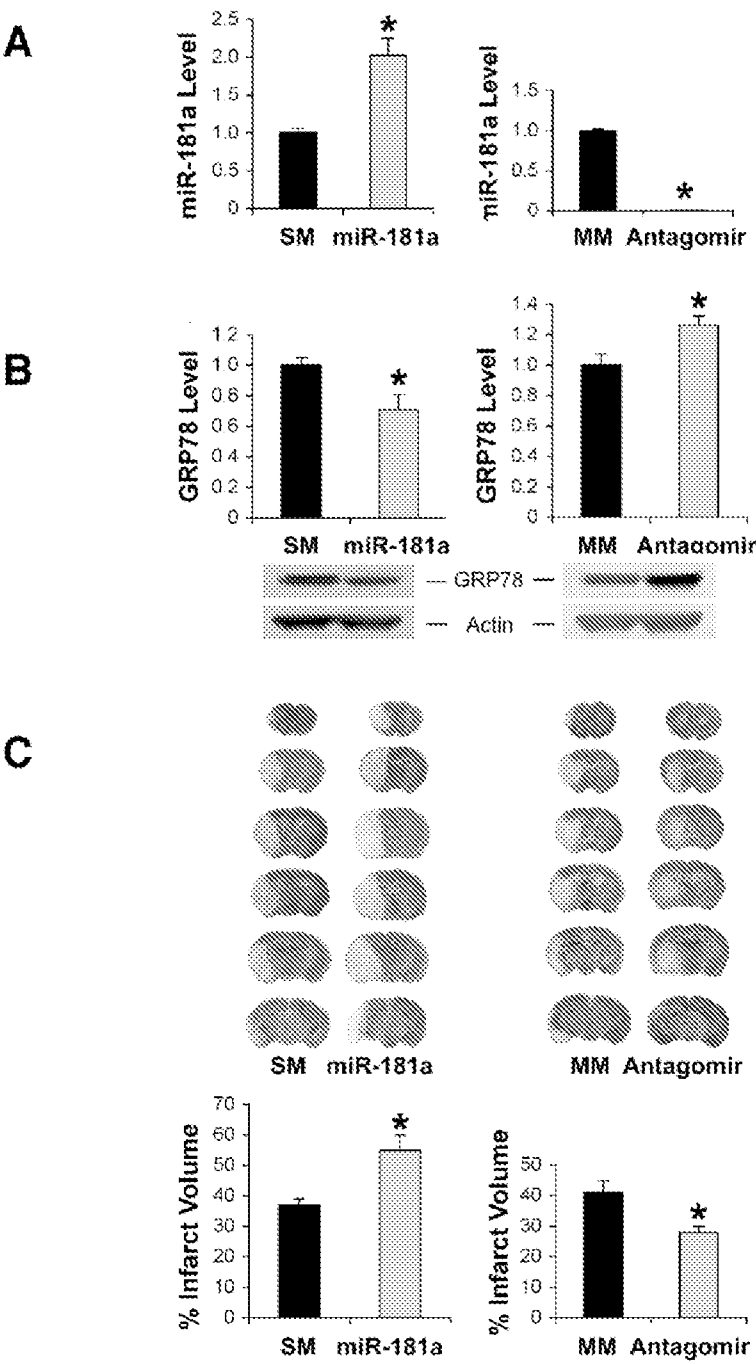


Figure 7

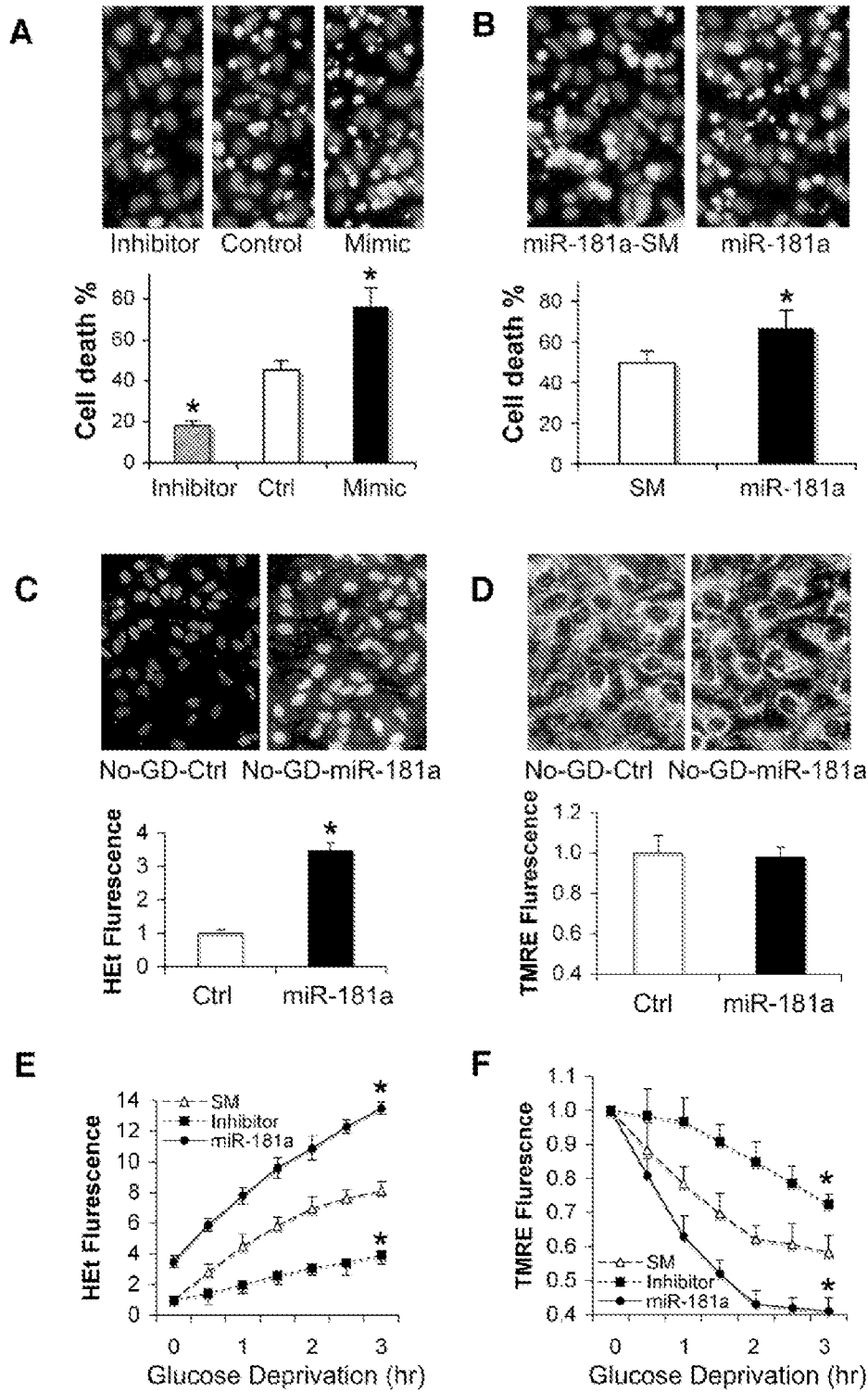


Figure 8

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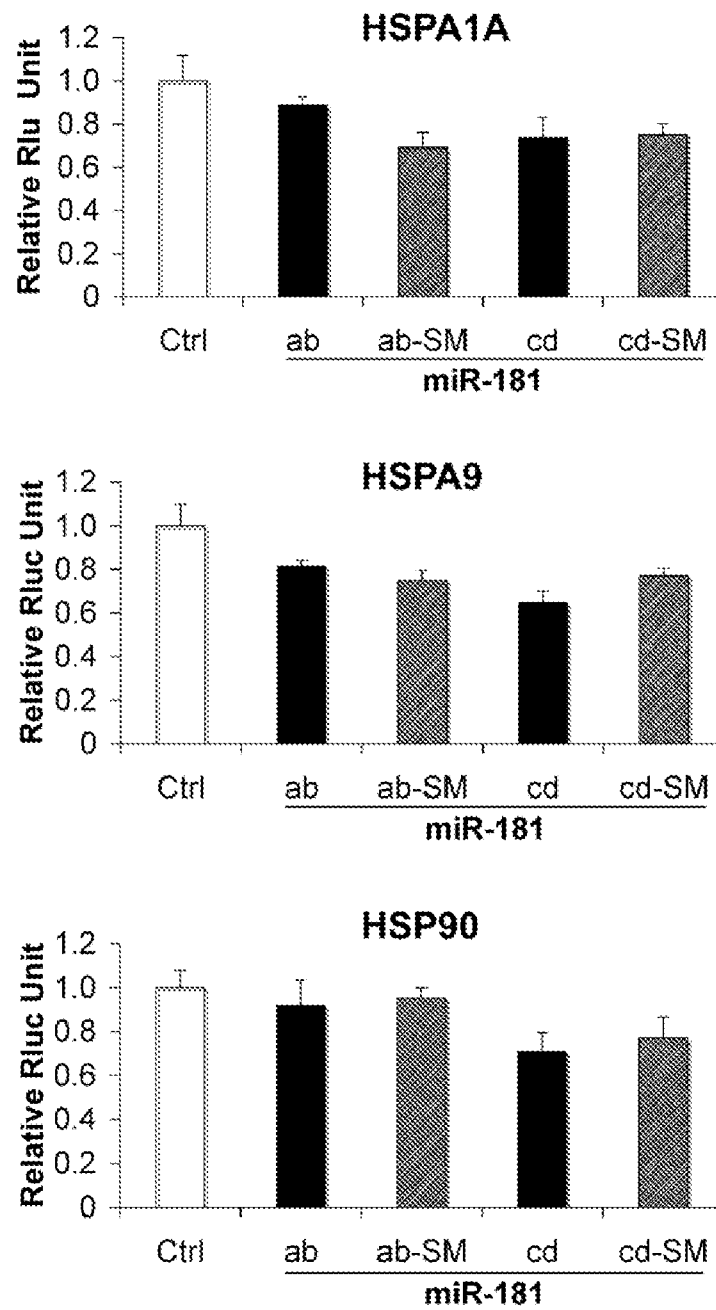


Figure 9

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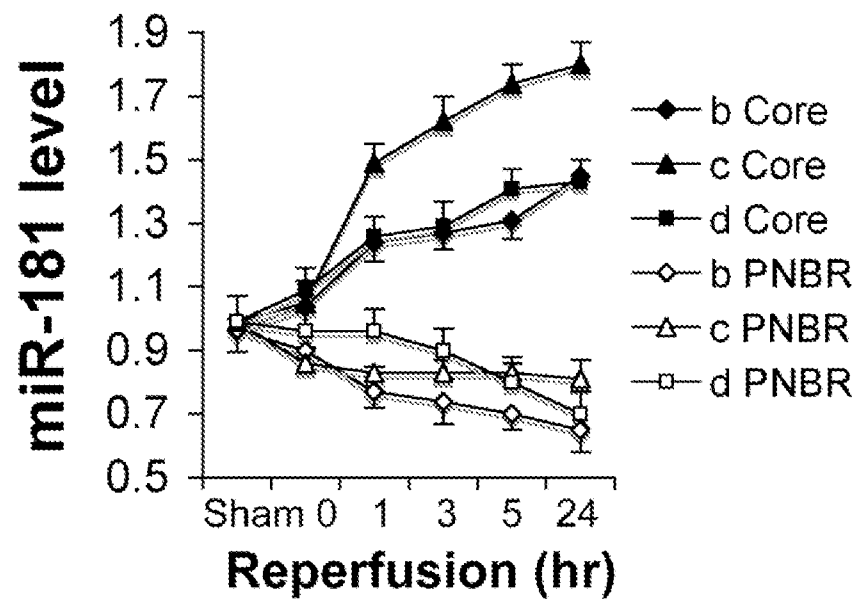


Figure 10

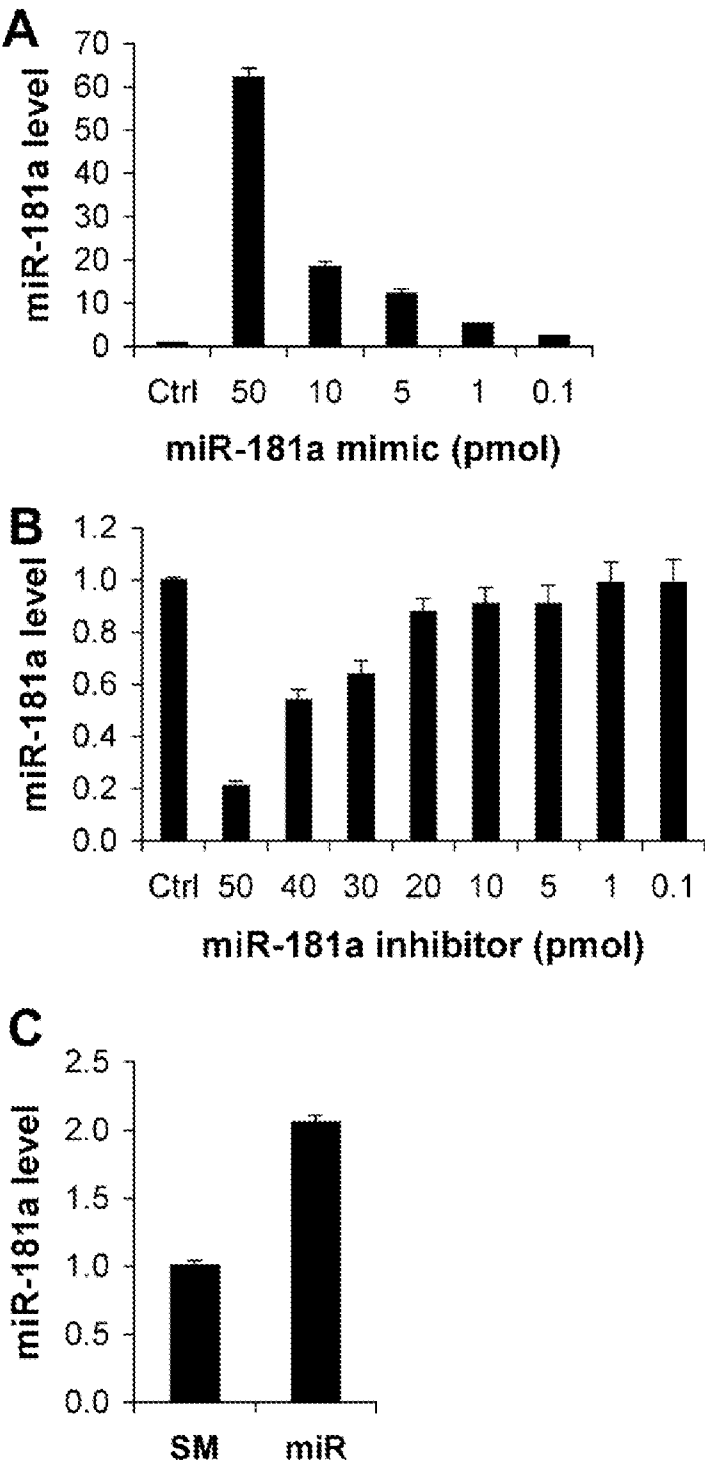


Figure 11

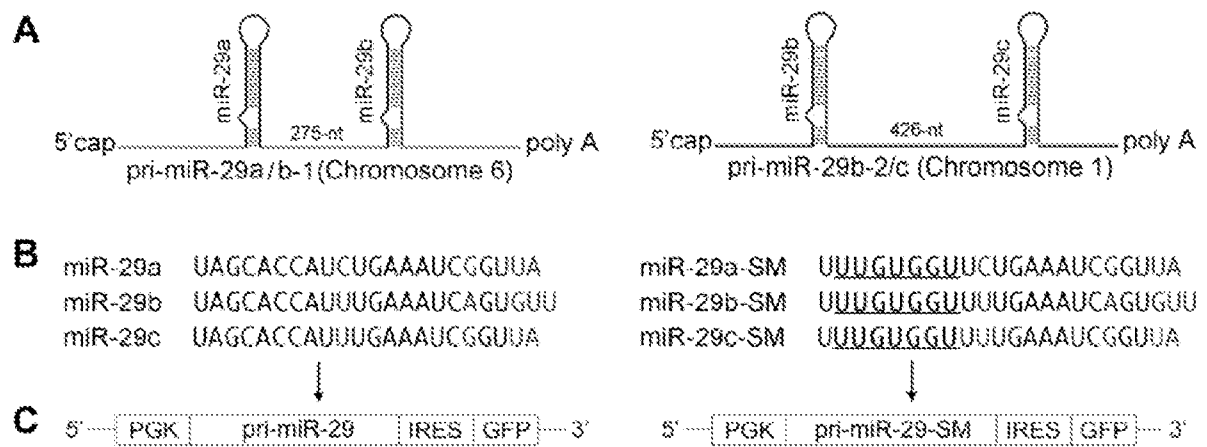


Figure 12

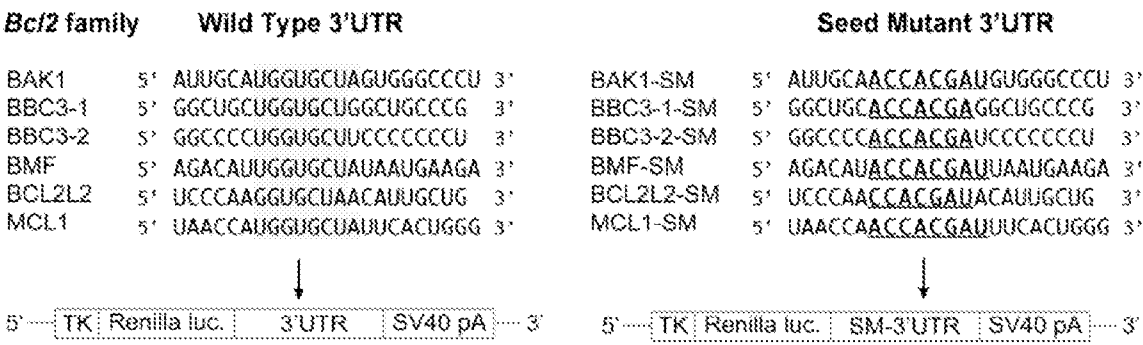


Figure 13

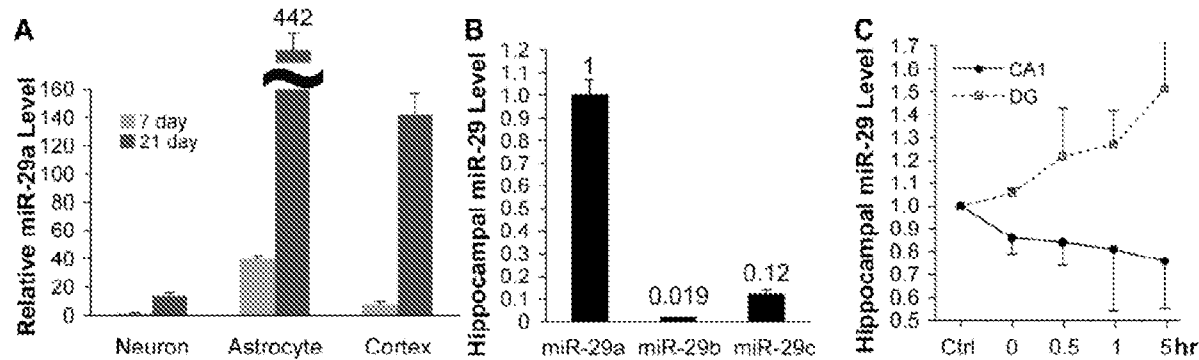


Figure 14

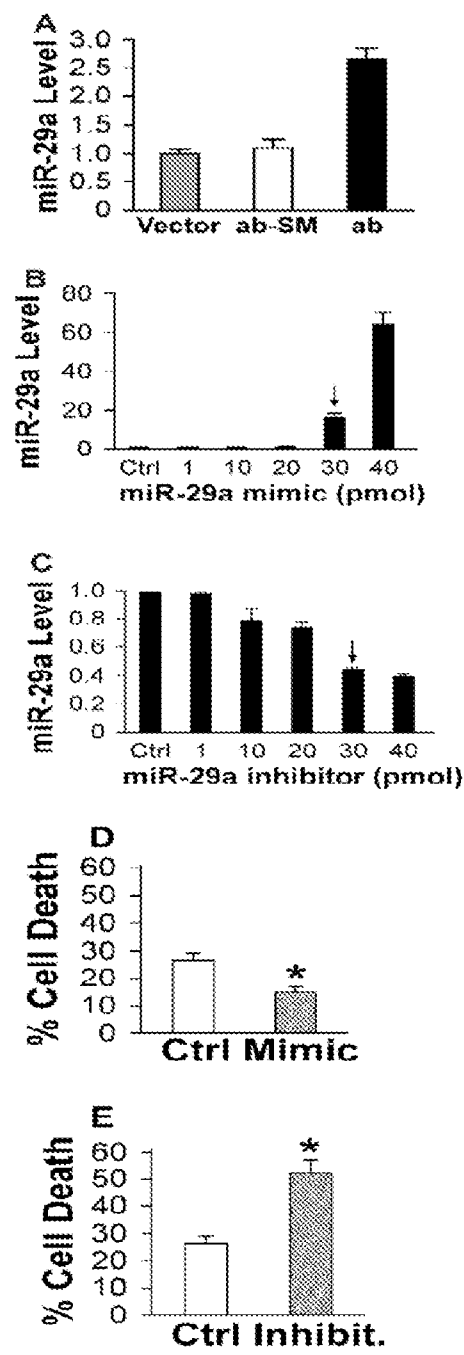


Figure 15

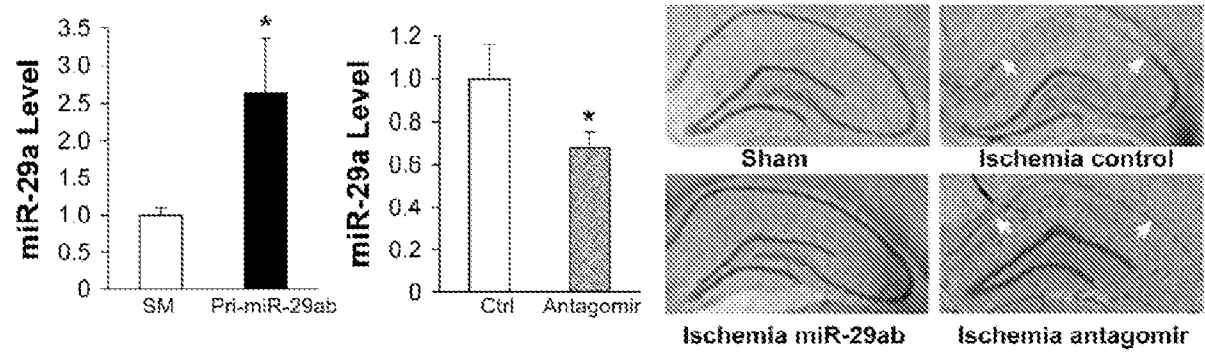


Figure 16

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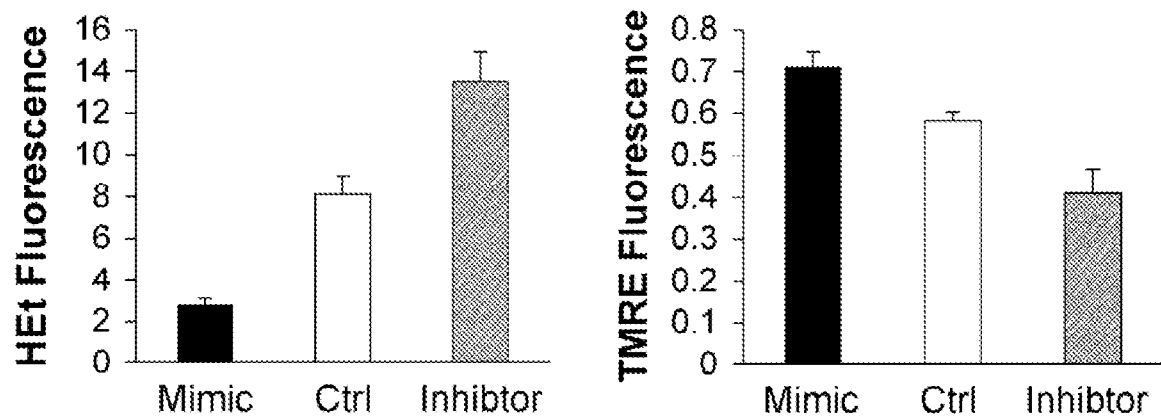


Figure 17

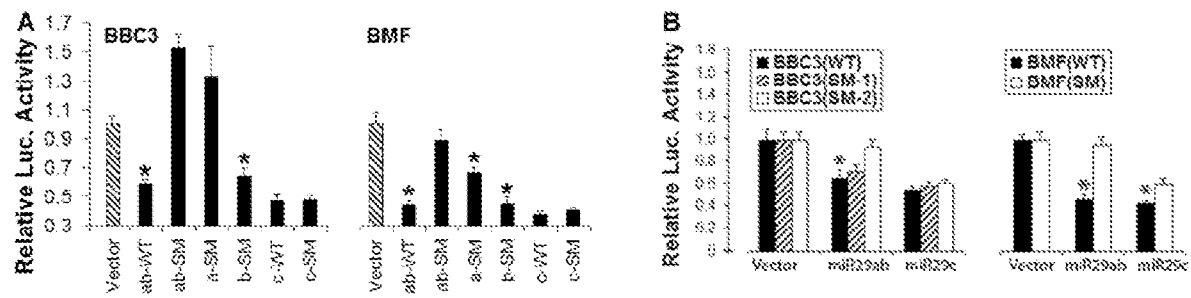


Figure 18

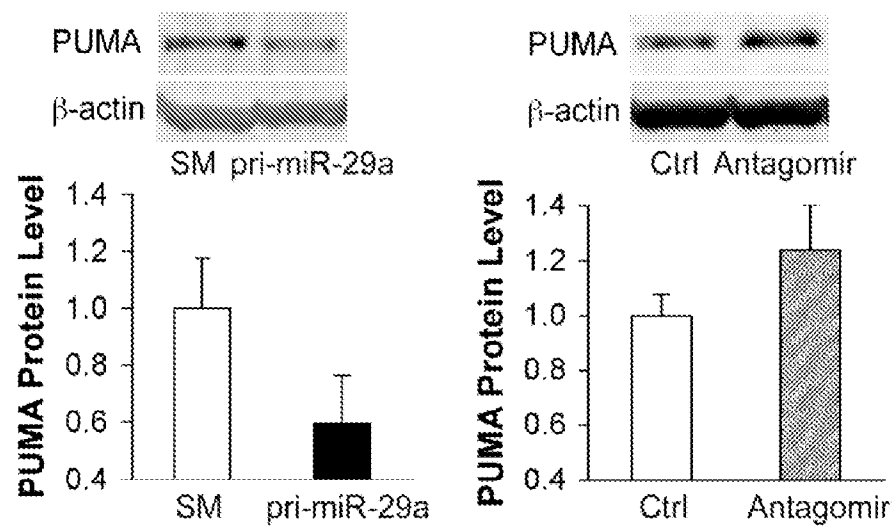


Figure 19