

EPIGENETIC REGULATION OF STROKE RECOVERY

CHANGES IN DNA METHYLATION AND MICRO-RNA REGULATION FOLLOWING
STROKE AND EGF/EPO NEUROGENESIS THERAPY

MICHAEL D. LOWINGS
B.Sc., University of Lethbridge, 2007

A Thesis
Submitted to the School of Graduate Studies
of the University of Lethbridge
in Partial Fulfilment of the
Requirements for the Degree

MASTER OF SCIENCE

Department of Biological Sciences
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

© Michael D. Lowings, 2009-2010

In memory of Dr. Ludvik Pahulje Sr.

Had you not taught your children the value of education, this thesis wouldn't exist.

Abstract

Stroke is one of the most common, and damaging, neurological afflictions. Stroke causes widespread and variable chronic effects, due to the limited regenerative ability of the adult brain. Altered gene expression induces neuronal changes necessary for plasticity-dependent recovery, effects which can be enhanced by growth hormone-based pharmaceuticals. These processes are driven by alterations in the informational capacity of the genome – changes driven by epigenetic regulators. Following experimental strokes, and treatment with EGF and EPO, this study shows that two epigenetic regulatory mechanisms, DNA methylation and microRNA regulation, are significantly altered, both in treated and untreated animals. Specifically, treatment induces a net global suppression of miRNA activity, which appears to modify the physical behaviour of neurons in domains ranging from plasticity and memory formation, growth and replication, and potentially even to neurological disease signalling. The confirmation of epigenetic alterations following a stroke indicates a future role for epigenetic neuro-pharmacology in stroke management.

Acknowledgements

To everyone that made this paper possible:

Many thanks to Dr. Igor Koturbash, Dr. Fabiola Zucchi, Rocio Rodriguez, James Meservy, Yaroslav Ilnytsky, and Arif Muhammad for their invaluable technical assistance, Dr. Gerlinde Metz for use of equipment, and John Duffin, Malcolm Lowings, Courtney Palmer and Anastasia Kulpa for assistance with editing and publication.

Thank you to my supervisors and committee: Dr. Olga Kovalchuk, Dr. Bryan Kolb, Dr. Ute Kothe-Wieden and Dr. Stewart Rood.

Thanks you to my family for their support and encouragement.

Thank you to the CCBN and the University of Lethbridge for the use of space.

Thank you AHFMR for providing the funding necessary to perform this experiment.

And a special thank you to my support group. You know who you are, and you know exactly why I'm thanking you.

Table of Contents

1 – Introduction	1
2 – Ischemic Stroke	4
2.1 – Causes, Risk Factors	4
2.2 – Pathology	5
2.2.1 – Mechanisms of Cell Damage in the Penumbra	8
2.2.2 – Inflammation and the Immune Response	11
2.2.3 – The Blood-Brain Barrier	12
2.2.4 – Reperfusion Injury	13
3 – The Study of Stroke	15
3.1 – Experimental Models of Stroke	16
3.1.1 – Methods not requiring Craniectomy	16
3.1.2 – Methods requiring Craniectomy	19
4 – Post-Stroke Gene Expression and Neural Plasticity	21
4.1 – Epigenetics	21
4.1.1 – DNA Methylation	23
4.1.2 – Histone Modification	25
4.2 – Short RNA Epigenetic Modulation	27
4.2.1 – A Brief History of microRNA	27
4.2.2 – MicroRNA Genomics	29
4.2.3 – miRNA Biogenesis	30
4.2.4 – Target Recognition	34
4.2.5 – Analysis of miRNA Expression	38
5 – Statement of Purpose	41
6 – Materials and Methods	42
6.1 – Animals	42
6.2 – Surgery	42
6.3 – Tissue Collection	43
6.4 – miRNA microarray Expression Analysis	43
6.5 – Confirmation of a Putative miRNA target	45
6.6 – Western Blot analysis of Protein Expression	44
7 – Results	47
7.1 – miRNA microarray Expression Analysis	47
7.1.1 – General Trends	47
7.1.2 – Specific miRNAs of probable biological significance	48
7.1.3 – Specific miRNAs of possible biological significance	xx
7.2 – Confirmation of a Putative miRNA target	xx
7.3 – Analysis of Protein Expression	52
7.3.1 – Noggin and BMP4	53
7.3.2 – MeCP2 and <i>de novo</i> DNA Methyltransferases	53
8 – Discussion	55
8.1 – miRNA microarray Expression Analysis	55
8.1.1 – General Trends	55
8.1.2 – Specific miRNAs of probable biological significance	57
8.1.3 – Specific miRNAs of possible biological significance	xx

8.2 – Alterations in DNA Methylation Machinery	72
8.2.1 – MeCP2	72
8.2.2 – DNMT3a and DNMT3b	74
8.3 – Confirming a putative miRNA target – miR-152 and Noggin	77
9 – Concusions, General Trends, and Future Outlook	80
10 – Works Cited	82
11 – Appendix I – Figures	92

List of Figures

Figure 1. Schematic representation of the biogenesis and mode of action of microRNA.	93
Figure 2. Confirmation of miR-152 / NogginUTR interaction	94
Figure 3. miRNA microarray analysis	95
Figure 4. miRNA microarray analysis	96
Figure 5. miRNA microarray analysis	97
Figure 6. Western Blot analysis of Noggin and BMP4	98
Figure 7. Western Blot analysis of MeCP2	99
Figure 8 Western Blot analysis of DNMT3a and DNMT3b	99

List of Abbreviations

3'UTR – Untranslated region, 3' end
ACA – anterior cerebral artery
AD – Alzheimer's disease
Ago – Argonaute protein
AMPA – a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP – adenosine triphosphate
BBB – blood-brain barrier
BID – BCL interacting domain
BMP – bone morphogenic protein
CNS – central nervous system
CREB – c-element response binding protein
CRP – C-reactive protein
DNMT1 – DNA Methyltransferase I
DNMT3a – DNA Methyltransferase 3a
DNMT3b – DNA Methyltransferase 3b
dsRNA – double stranded RNA
EGF – epithelial growth factor
EPO – erythropoietin
ERK – Extracellular signal-Related Kinase
FADD – Fas-Associated death domain
FasL – Fas ligand
HDAC – histone deacetylase
HEK – human embryonic kidney
IL-1 – interleukin 1
IRS1 – insulin receptor substrate 1
ISH – in-situ hybridization
LAMC2 – laminin gamma 2
LNA – Locked Nucleic Acid
LV – lateral ventricle
MBD – methyl-CpG binding domain
MCA – middle cerebral artery
MCAO – middle cerebral artery occlusion
MET – hepatocyte growth factor receptor
miRNA – micro-RNA
miRNA* - antisense strand of a micro-RNA
MMP – matrix metalloprotease
mPT – mitochondrial permeability transition
mRNA – messenger RNA
NMDA – n-methyl-d-aspartic acid
PAF – platelet-activating factor
PARP – poly(ADP-ribose) polymerase
PK – protein kinase
PTGS – post transcriptional gene silencing
RISC – RNA-induced silencing complex

RNAi – RNA interference
ROS – reactive oxygen species
RT-PCR – reverse transcriptase polymerase chain reaction
siRNA – short interfering RNA
SOD – superoxide dismutase
TERT – telomerase reverse transcriptase
TPA – Tissue Plasminogen Activator
VAMP – vesicle-associated membrane protein
vWF – von Willebrand factor

1 - Introduction

Cerebrovascular accident, or, as it is more commonly known, stroke, is one of the most widespread and common human medical afflictions. Briefly put, stroke is a disruption of neurological function caused by the interruption of blood supply to the brain. The resultant hypoxic conditions lead to cell damage and death, and result in a loss of neurological function. According to the most recent data, stroke is the second most common cause of death globally (accounting for some 9% of all human deaths (Donnan, Fisher, Macleod, & Davis, 2008)), and as the population of the developing world ages, it may soon overtake heart attack as the most common cause (Feigin, 2005). Furthermore, and perhaps more relevant to the purpose of this work, stroke is the leading cause of adult disability in both the United States and Europe, estimated to account for 4% of all direct health care costs in industrialised nations (an estimated 40.9 billion USD per year) (Donnan, et al., 2008). Given the vast cost to both our health care systems and our life expectancies, stroke has become a major focus of medical neuroscience research, with research concentrating on three major areas. The first of these, as always, is prevention. Stroke rates are in decline in the industrialised world, due to both our greater understanding of the risk factors associated with it (high blood pressure, cigarette use, and the like) and our improving quality of life (Strong, Mathers, & Bonita, 2007). However, increasing life expectancy in most regions of the world mitigates the effects of preventative research somewhat, as an aging population will undoubtedly experience more and more strokes. The second major thrust of stroke research, acute treatment, seeks to reduce the physiological impacts of the damage caused by the stroke, by either removing the source of the

stroke (using for example, thrombolytic ‘clot buster’ drugs such as Tissue Plasminogen Activator (TPA) ("Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group," 1995)) or simply placing stroke patients in an environment containing sufficient medical expertise (Langhorne, Williams, Gilchrist, & Howie, 1993). Acute therapy, though, has a major drawback – it has limited effectiveness outside of very precise therapeutic windows. Studies indicate that TPA therapy is only effective within three hours of stroke onset (Cronin, Weisman, & Llinas, 2008), and aspirin, which has shown considerable promise as an acute treatment, within 48 hours ("CAST: randomised placebo-controlled trial of early aspirin use in 20,000 patients with acute ischaemic stroke. CAST (Chinese Acute Stroke Trial) Collaborative Group," 1997). Given these factors, it is unreasonable to presume that we will ever ‘stop stroke in its tracks’. Instead, stroke research consists of a third major focus, which will be the topic of this work: methods whereby the damage caused by stroke can be compensated for, and perhaps even repaired.

Before examining options for neural recovery and repair, it is important to note that stroke is divided into two clinical subtypes, both of which cause similar symptoms, but present different pathology. Ischemic stroke (which accounts for roughly 80% of all clinical strokes (Grysiewicz, Thomas, & Pandey, 2008)) results from the occlusion of an afferent blood vessel and the subsequent reduction of the blood and oxygen supply to the affected brain regions (Donnan, et al., 2008). Hemorrhagic stroke, on the other hand, is caused by ruptures in afferent blood vessels, leading to hypoxia-based cell death and presentation of stroke symptoms

(Auer & Sutherland, 2005). The difference between these subtypes is critical in the acute therapeutic window, as their successful clinical management is vastly different (Donnan, et al., 2008). It remains to be seen whether a recovery based treatment strategy is as sensitive to the underlying pathology of a stroke.

2 – Ischemic Stroke

2.1 – Causes, Risk Factors

There are a number of factors that are capable of contributing to the onset of an ischemic stroke. An ischemic event can be caused by anything ranging from a thrombotic blockage of an artery to the collapse of arterial walls due to hypoplasia or arterial weakening. It is impossible to conclude that a certain constellation of physical markers is directly responsible for ischemic events, but a number of common factors can be identified from the literature. Perhaps the most important factor is atherosclerotic vascular disease, as it has been shown to be one of the key contributors to both myocardial and cerebral ischemia (Adams, 2009). Indeed, most of the risk factors associated with stroke (hypertension, hyperlipidemia, and diabetes mellitus) are identical to those associated with atherosclerosis.

It has also long been known that migraine (and especially migraine with aura (Pezzini, et al., 2009)) is highly correlated to incidence of ischemic stroke. Though a direct causative link between the two has yet to be shown (Katsarava, Rabe, & Diener, 2008), there are a number of pathological factors of migraine that could either trigger the onset of a stroke or render the neurovascular system more sensitive to infarct. Migraine triggers a specific pattern of cortical spreading depression (a phenomenon first described in 1944 as a response to brain injury (Leao, 1944)) that originates in the occipital lobe and spreads through the cortex (Welch, Cao, Aurora, Wiggins, & Vikingstad, 1998). The depolarisation caused by this phenomenon induces, among other things, a reduction in cerebral blood flow (Friberg, Olesen, Lassen, Olsen, & Karle, 1994), which increases the risk of migrainous infarction.

Migraine also appears to have the potential to induce hypercoagulability, by increasing the circulating levels of platelet-activating factor (PAF) (Sarchielli, et al., 2004) and (vWF) (Cesar, Garcia-Avello, Vecino, Sastre, & Alvarez-Cermeno, 1995). In addition to its possible capability to directly trigger stroke, there is also a growing body of evidence that the physical stress of a migraine increases the risk of developing non-migrainous stroke, possibly by causing long-term permanent endothelial disruption (Tietjen, 2007) (which would be supported by Cesar et al.'s findings (Cesar, et al., 1995), as vWF is a biomarker for endothelial damage). In addition, many of the conditions underlying cardiovascular disease are co-morbid with migraine (Yoon, et al., 2005), suggesting that migraine and risk for ischemia may be manifestations of the same pathology.

A recent study by Chuang et al. (Chuang, Liu, Pan, & Lin, 2007) shows a correlation between a relatively uncommon birth defect of the circle of Willis and an increased risk for ischemic stroke caused by anterior cerebral artery (ACA) occlusion. The birth defect, hypoplasia of the A1 segment of the ACA, was shown to correlate to both increased risk for ipsilateral hemispheric borderline stroke, and, unexpectedly, an increase in the presence of small-artery atherosclerosis in the ipsilateral striatum, indicating that weaknesses in afferent arteries may heavily contribute to the ischemic weakness of their distal counterparts.

2.2 – Pathology

A stroke is composed of two distinct regions, the ischemic core and the penumbra, which present highly different pathologies, time courses, and prospects for

therapeutic intervention. The ischemic core is comprised of the region most directly affected by loss of circulation and so, consequently, experiences the most drastic impact in terms of tissue damage. A reduction in cerebral blood flow to below 0.2 mL/g/min is enough to cause highly metabolically active neural tissue to deplete its oxygen and metabolite reserves in under a minute, at which point mitochondrial respiration ceases (Warlow, 2001). Anaerobic respiration cannot sustain the level of ATP production necessary for neuronal function, so the cells rapidly acquire an ATP deficit. This results in the disruption of major cellular functions, most notably membrane transport. Neural tissues maintain an exquisitely regulated balance of ions and membrane potential, which is critical for cellular function and survival. Disruption of ion transport pumps causes cellular depolarisation and Ca^{2+} influx, which triggers a burst of excitatory glutamate neurotransmission (Mergenthaler, Dirnagl, & Meisel, 2004). The burst of glutamate is sufficient to activate AMPA and NMDA receptors, triggering further membrane depolarisation and calcium influx. Severe ATP deficits also disable glutamate re-uptake mechanisms, creating a two-fold overload of excitatory neurotransmitter (both in its overabundance and its persistence) (Lo, Dalkara, & Moskowitz, 2003). This feeds back into overstimulation of the AMPA receptor which causes efflux of Na^+ and Cl^- ions into the intracellular space, creating an osmotic gradient which forces water into the cell. In conditions of extreme ATP deficits, the excitotoxic feedback loop is capable of generating an osmotic gradient sufficient to induce lysis in cells. This rapid, uncontrolled cell death, necrosis, is the hallmark of the ischemic core, and is generally considered to be

beyond the reach of acute therapy, as the cells rupture and die within minutes of onset.

In regions directly adjacent to the core, however, a slightly different sequence of events unfolds, the severity of which is dependent on the distance from the primary infarct (Moustafa & Baron, 2008). Due to residual circulation in unaffected blood vessels, this surrounding region, termed the penumbra, does not experience acute cellular oedema and lysis, due to the preservation of some activity by membrane transport pumps (Hossmann, 2006). Instead, it undergoes a characteristic sequence of events known as the ischemic cascade (Hinkle & Bowman, 2003). The initial sequence of events of the ischemic cascade is qualitatively similar to that of the necrotic pathway, but the dysregulation of ionic balance never achieves the critical point necessary to induce osmotic lysis. Instead, the milder hypoxic climate of the penumbra causes these cells to slowly degrade, showing many similarities to apoptosis¹. Penumbral cells continue to accumulate abnormally high levels of Ca²⁺, which triggers two distinct processes – mitochondrial apoptosis control, and free radical accumulation. In addition, recent evidence suggests that post-ischemic apoptosis is also mediated by FasL mediated death receptor signalling (Broughton, Reutens, & Sobey, 2009).

¹ Hypoxic stress still brings with it some features of necrosis, leading Lo et al. (Lo, et al., 2003) to term the cell death process in the penumbra ‘aponecrosis’. For purposes of this review, the apoptotic-like behaviour of penumbral cells will be referred to as ‘apoptosis’ in order to make a clearer distinction between the ‘slow death’ experienced by the penumbra and the rapid lysis in the core. It should be noted, however, that the speed of stroke-induced cell death (and its similarity to either necrosis or apoptosis) is determined by its distance from the central infarct, and its resultant position on a hypoxic gradient.

2.2.1 – Mechanisms of Cell Damage in the Penumbra

Intracellular calcium levels activate an intrinsic apoptotic pathway by activation of calpains. In addition to caspases-8 signalling caused by the extrinsic apoptotic pathway (detail below), these signals result in the cleavage and activation of BCL-interacting domain (BID, or tBID in its truncated, active form), a member of the BCL-2 family of proapoptotic proteins (Sugawara, et al., 2004). tBID binds and targets the outer mitochondrial membrane, activating other proapoptotic proteins such as Bak, Bax, Bad, and Bcl-XS (Sugawara, et al., 2004). These signals are presumed to induce release of apoptogenic factors from the intermembrane space through an event known as the mitochondrial permeability transition (mPT)(Zoratti & Szabo, 1995). The mPT is a Ca^{2+} and oxidant-mediated opening of a pore in the inner mitochondrial membrane leading to severe disruption of mitochondrial function and massive release of free radicals, divalent cations, and any number of enzymes that contribute to cell death, including endonucleases, ATPases, and phospholipases (Mergenthaler, et al., 2004; Zoratti & Szabo, 1995), all of which contribute to the ongoing damage of the hypoxic environment. In addition to the above, the mPT also induces release of mitochondrially-sequestered Cytochrome *c* (Elmore, 2007), which initiates the caspase-dependent apoptotic pathway. Cytochrome *c* and its co-factors, procaspase-9 and apoptotic protein-activating factor-1 (Apaf-1) assemble into a structure termed the ‘apoptosome’ (Love, 2003), causing procaspase-9 to activate caspase-9 (Elmore, 2007), which subsequently activates caspase-3, causing its translocation to the nucleus and initiation of the final stages of apoptosis. One of the primary targets of caspase-3 is poly(ADP-ribose) polymerase (PARP) – which, once

inactivated, contributes to the accumulation of genomic damage and eventual apoptotic cell death (Broughton, et al., 2009). A study by Endres et al. (Endres, Wang, Namura, Waeber, & Moskowitz, 1997) shows that pharmacological re-activation of PARP can reduce the size of the penumbra, indicating that PARP-mediated apoptosis plays a role in ischemic neuronal apoptosis and final infarct size.

In conjunction with the ionic imbalances, enzymatic activity and physical degradation of the neuron, hypoxic by-products (such as anaerobic respiration, the mPT, and calcium accumulation) also induce the accumulation of free radicals, which are capable of causing massive, irreversible cellular lesions (Durukan & Tatlisumak, 2007). Free radicals can cause damage to virtually any cellular component, including but not limited to genomic DNA, the nuclear and cellular membranes, and other organelles (Rao, 2009). The un-targeted nature of this type of damage can be especially negative, as particular free radical lesions (such as a ruptured cell membrane) may cause cells to simply lyse instead of apoptose, which has the potential of causing a necrotic chain reaction. Necrosis is a relatively uncontrolled form of cell death, during which a variety of enzymes and neurotransmitters are released into the intracellular space. The risk of a chain reaction occurring develops because these enzymes are capable of triggering membrane damage and excitotoxicity in neighbouring cells, potentially killing them and releasing their complement of enzymes into the intracellular medium. Free radicals also are a major source of damage in reperfusion injury, as discussed below.

Extracellular signals also play a role in the third type of death signalling, the FasL mediated extrinsic apoptotic pathway (Broughton, et al., 2009). In addition to

cell damage, ischemia also triggers a variety of changes in gene expression, including the hyperactivity of the Forkhead1 transcription factor (Sugawara, et al., 2004), which produces high quantities of the extracellular Fas ligand (FasL), a protein that begins accumulating in the extracellular space. FasL will bind to the Fas membrane receptor, recruiting Fas-Associated Death Domain (FADD), which possesses an N-terminal motif capable of activating procaspase-8 (Love, 2003). This entire complex is referred to as the death-inducing signalling complex (DISC). DISC acts by recruiting and cleaving caspase-8, which activates it. Caspase-8 is capable of triggering apoptosis through two different downstream targets – it can cleave BID to initiate intrinsic apoptotic signalling, or it can directly activate caspase-3 to trigger nuclear apoptosis (Cho & Choi, 2002).

Unfortunately for the patient, once the stroke begins, time is of the essence. The apoptotic pathway takes anywhere from one to three hours (Cronin, et al., 2008) to fully kill the cells in the penumbra, and during this time, reperfusion (restoration of blood flow) can trigger cell survival. This is the time when treatment with TPA and other thrombolytic drugs can have the greatest impact, as over 50% of the volume of a final stroke lesion originates from the penumbra (Mergenthaler, et al., 2004). However, there are three more sources of potential damage that the penumbra must endure before the patient is truly out of danger – the inflammatory response, the threat of hemorrhagic transformation, and reperfusion injury.

2.2.2 – Inflammation and the Immune Response

Ischemic stroke, like nearly all injuries, triggers a response from the immune system. Within a few hours of stroke, there is increased immune activity near the infarct, including infiltration of circulating neutrophils and monocytes, and activation of microglia, astrocytes and endothelial cells in the damaged region (Muir, Tyrrell, Sattar, & Warburton, 2007). Though this is a standard response to injury, there is evidence that inflammatory responses may exacerbate the damage caused by the initial stroke. Tissue damage initiates immediate up-regulation of interleukin 1 (IL-1) expression (Touzani, Boutin, Chuquet, & Rothwell, 1999) as well as C-reactive protein (CRP) activity, two inflammatory cytokines which are capable of stimulating cell death through the MAP kinase and NF κ B pathways. Though its direct role in neuronal damage has yet to be determined, there are a number of potential mechanisms through which IL-1 could induce neurodegeneration, including its role in proapoptotic signalling, generation of prostaglandins, expression of adhesion molecules, increased expression of acute phase proteins, stimulation of immune cell invasion, and activation of complement. All of these mechanisms have been implicated in neuronal damage (Touzani, et al., 1999). Simple recruitment of additional immune cells presents a major problem in the brain – the infiltration of large numbers of these cells can physically block the narrow blood vessels of the brain, preventing reperfusion even if the initial arterial blockage has been removed (Barone, et al., 1992). In addition, the recruitment of phagocytotic immune cells can cause further cell death; these cells engulf and destroy ‘damaged’ neurons as part of their typical immune behaviour.

Contrary to the deleterious role played by up-regulation of pro-inflammatory cytokines like IL-1, the immune response to stroke also induces modulation of anti-inflammatory cytokines such as TGF- β 1 and IL-10 (Mergenthaler, et al., 2004). Experimental over-expression of anti-inflammatory factors has been shown to have a neuroprotective effect (Strle, et al., 2001), indicating that the immune response plays a very complex role in the ongoing damage to the penumbra. Furthermore, a recent study by Suzuki et al. (Suzuki, Tanaka, & Suzuki, 2009) implicates IL-6, a pro-inflammatory cytokine, as both a contributor to inflammatory damage in the acute phase of ischemia, and as a neurotrophic / neuroprotective mediator in later phases. This suggests that the complexity inherent in an immune response may provide an interesting arena for therapeutic intervention during both the therapeutic and post-ischemic time points, which bears examination in other studies.

2.2.3 – The Blood - Brain Barrier

Infarction can disrupt the integrity of the blood-brain barrier by causing damage to the cellular matrix components responsible for its structural stability. Induction of matrix metalloproteases (MMPs) in the 1-3h post-ischemic window, especially MMP2 and MMP9, has been shown to cause damage to basal lamina and the astrocytes and endothelial cells comprising the BBB matrix (Lapchak, Chapman, & Zivin, 2000; Mergenthaler, et al., 2004). Gene knock-out of MMP9 has also been shown as a potential treatment for reducing infarct volume (Asahi, et al., 2001). Proteolytic damage of the BBB is dangerous for two reasons – first, it causes increased invasion and activation of immune cells (as the brain is, in general, a

relatively immune-privileged area), and second, because the damage to the matrix risks vascular damage, which primes the penumbra for potential hemorrhagic transformation.

2.2.4 – Reperfusion Injury

One of the major risks associated with thrombolytic treatments (or, in fact, non-pharmaceutical removal of arterial blockage) is the risk of a brain hemorrhage (Molina, 2006). As discussed above, an ischemic stroke carries with it the risk of vascular weakening. Once a blockage is removed, the vascular system of the penumbra is submitted to a tremendous amount of physical stress as circulation returns, carrying with it a potential to rupture blood vessels and induce a secondary hemorrhagic stroke (Rosell, Foerch, Murata, & Lo, 2008). Aside from the risk for physical damage, restoration of blood flow to the penumbra can cause complications at a cellular level, as the restoration of full metabolic function carries a “heavy cost”, involving generation of free radicals, specifically reactive oxygen species (ROS) (Chan, 1996; Granger, 1988; Wong & Crack, 2008). Specifically, the overconsumption of ATP during hypoxia results in the accumulation of catabolites such as hypoxanthine and xanthine, which, upon re-ignition of cellular metabolism by reperfusion, are rapidly metabolised by xanthine oxidase, resulting in a massive spike of superoxide and hydrogen peroxide levels (Granger, 1988). It is important to note that the danger in this situation is not simply the accumulation of ROS (which is, after all, a standard by-product of cellular metabolism), but also, the rate at which these products accumulate during reperfusion. The flood of ROS during reperfusion

causes damage by overwhelming the cells' natural ROS mitigation mechanisms (enzymes such as superoxide dismutase (SOD), when over-expressed, have been shown to reduce final infarct volume in animal models of ischemic stroke (Wong & Crack, 2008), an indicator that additional damage handling capability is neuroprotective.)

In addition to direct oxidative damage, the accumulation of free radicals during reperfusion injury is also implicated in the magnification of the inflammatory process. A recent study by Nishi et al. casts some light on the role of oxidative stress in modulating immune response (Nishi, Maier, Hayashi, Saito, & Chan, 2005). In brief, they studied the expression of two chemokines using DNA microarray technology: MIP-1a and MCP-1, proteins that modulate the function of macrophages and monocytes, respectively. Using a strain of SOD-overexpressing mice, they observed changes in MIP-1a and MCP-1 expression in response to the level of oxidative stress in the reperfused penumbra. The results suggested that MCP-1 is down-regulated by increased activity of SOD, creating a neuroprotective effect, implying that the converse (i.e., increased chemokine activity in response to an increase in ROS activity) is a contributor to the inflammatory damage caused by reperfusion.

3 – The Study of Stroke

Given the complexities of the pathology of stroke, data obtained from clinical patients is limited. For obvious reasons, histological studies are only possible on patients who do not recover from their strokes, and the random nature of the location of stroke makes standardisation of observational data, such as that garnered through MRI and PET scans, quite difficult. In response, a number of animal models of ischemic stroke have been developed, a brief review of which follows.

The first consideration to make when choosing an animal model of stroke is, by necessity, which animal to use. As is the case with all experimental animal models, a balance must be struck between physiological similarity to humans and ethical and financial considerations. For this reason, the vast majority of stroke studies are performed in rats and mice (Durukan & Tatlisumak, 2007; Gonzalez & Kolb, 2003). The choice of rodent generally depends on the type of study. Due to their ease of handling and care, and their remarkably similar vascular anatomy, most physiological and behavioural studies are performed on rats; studies with a genetic component are performed on mice. There are also a small but significant number of studies examining higher primates, due to their greater physiological similarity to humans (Durukan & Tatlisumak, 2007). In general, once an experimental technique has been demonstrated as effective in a rodent model, it is then ported into a primate system before finally being cleared for human study ("Recommendations for standards regarding preclinical neuroprotective and restorative drug development," 1999).

Second, the huge clinical variability of human stroke necessitates a wide variety of model systems in order to simulate ischemic events of varying duration (from transient models, which feature 60-120 minutes of ischemia and are followed by reperfusion, to permanent models, which result in complete devascularisation and a permanent ischemic lesion), intensity (from highly localised focal lesions to hemispheric disruption), and location (Durukan & Tatlisumak, 2007). In addition to different pathologies, there are also a number of different methods of inducing stroke, which, for simplicity's sake, are divided into two categories, those not requiring craniectomy, and those that do.

3.1 – Experimental Models of Stroke

3.1.1 – Methods not Requiring Craniectomy

The first of these methods, induced thromboembolism, is also one of the oldest, and seeks to mimic the cause of a vast majority of human strokes (Durukan & Tatlisumak, 2007). This method of stroke induction uses either fragments of a pre-existing autologous thrombus (Kaneko, Nakamura, & Ogawa, 1985), or fragments of a human blood clot (S. M. Papadopoulos, Chandler, Salamat, Topol, & Sackellares, 1987) to induce clotting activity in the cerebral arteries of the experimental subject. Generally, the 'seed thrombus' is injected into the carotid artery. It will stimulate the development of clotting at a downstream point, or simply physically block an artery. One of the major advantages of this method is its similarity to the pathology of a human embolic stroke – its source is in fact a blood clot. This is especially useful for examining the effects of thrombolytic and neuroprotective drugs, but can be

problematic due to lack of experimenter control. As the seed thrombus is injected into a major artery, it is very difficult to control the final location of the induced stroke (rendering it a suboptimal method for behavioural studies). In addition, clots of this nature are unpredictably vulnerable to spontaneous autolysis, making the duration of ischemia difficult to control (rendering the standardization of data across multiple animals difficult). Though some of these difficulties are controllable using artificial means to induce an embolism (see (Durukan & Tatlisumak, 2007; Gonzalez & Kolb, 2003) for review), the resultant thrombus is still vulnerable to any number of factors that vary from individual to individual.

In an attempt to maintain an accurate simulation of arterial blockage as the source of neural ischemia, but obtain greater control of the duration and localization of the resulting infarct, a number of non-thrombotic methods of inducing arterial blockage have been developed. The most commonly used of these (and, in fact, the most common stroke model), is occlusion of the middle cerebral artery (MCAO) (Yanamoto, et al., 2003). Using a minimally-invasive surgical technique, a small suture is inserted into the carotid artery, and can simply be advanced to the point where it blocks the middle cerebral artery, resulting in ischemia at all points downstream. Once the desired ischemic duration has been reached, the suture can be simply withdrawn from the carotid, allowing for reperfusion of the affected tissue. A variety of techniques have been used to modulate the pathology of MCAO, including alterations of suture diameter (Yanamoto, et al., 2003), and the chemical composition of the sutures themselves (Laing, Jakubowski, & Laing, 1993). The major disadvantages of this method are the potential for mechanical damage of the artery,

resulting in subarachnoid hemorrhage, and induction of systemic hyperthermia (Durukan & Tatlisumak, 2007; F. Li, Omae, & Fisher, 1999). Furthermore, though some selectivity of localization is possible, MCAO still results in very large infarctions, which renders the technique suboptimal for the study of focal ischemia.

A notable craniectomy-less model of stroke involves injection of endothelin-1, a potent vasoconstrictor, into the MCA (Robinson, Macrae, Todd, Reid, & McCulloch, 1990). This avoids many issues present in MCAO-type methods, as the surgical invasiveness is limited to a relatively simple injection. Another major advantage of endothelin-1 is that it can be injected directly onto the cortex in order to generate focal lesions (Fuxe, et al., 1997), which allows for the study of stroke in specific brain areas. This is particularly useful when studying behaviour, and behavioural recovery. Disadvantages of this method include the dose-dependent response of vasoconstriction to the injection, and the fact that the injected endothelin-1 is exhausted and metabolised, which limits the effectiveness of this method for inducing strokes of a controlled duration (Durukan & Tatlisumak, 2007). Also of note is the increased invasiveness of this method when used to generate focal lesions, as it requires direct surgical access to the cortex.

Another experimental model for localised stroke induction, photothrombosis, involves injection of a photoreactive dye into the bloodstream, and its subsequent activation by a laser set to a specific frequency (Watson, Dietrich, Busto, Wachtel, & Ginsberg, 1985). The active form of the dye generates singlet oxygen, causing endothelial damage and platelet activation, triggering an intense focal ischemia. This photochemical method allows the experimental induction of identical lesions in

multiple animals, but due to the intensity of the lesion generated, creates nearly no penumbra (as any area targeted by the laser experiences full oxygen deprivation and complete necrosis within minutes). Though manipulation of the intensity and duration of the laser burst has been shown to alter the physiology of the lesion (Durukan & Tatlisumak, 2007), the study of the penumbra (and specifically any drug therapies that target it) is best done in other models.

3.1.2 – Methods involving Craniectomy

Craniectomy-type model systems involve opening the skull of the animal and using a variety of means, physical or chemical, to induce vascular damage in selected brain regions. Though the main disadvantage of these methods, namely the complications associated with general anaesthesia and open-brain surgery, is significant, these methods allow a great deal of experimenter control in terms of the location, extent, and duration of ischemia. Two main subcategories of surgical stroke models exist – surgical occlusion of the cerebral arteries, and direct interference with the neurovascular system (Durukan & Tatlisumak, 2007). Surgical occlusion of the MCA is the most common model of this type. MCA occlusion is accomplished either by physical obstruction of the vessel (using either ligatures, micro-clamps or suturing (Buchan, Xue, & Slivka, 1992; Shigeno, Teasdale, McCulloch, & Graham, 1985)) or electrocauterization (Hellstrom, Wanhainen, Valtysson, Persson, & Hillered, 1994), the former of which allows for reversal of ischemia and the study of reperfusion. The extent of the lesion is dependent on the location of MCA occlusion (Tamura, Graham,

McCulloch, & Teasdale, 1981), as discussed above. This allows surgical MCAO to be quite precise in the generation of lesions.

One final method of note is devascularisation of the motor cortex. Though this method involves craniotomy, and the resultant dangers of atmospheric exposure and potential disruption of the blood-brain barrier, it is extremely reliable as a model for permanent focal ischemia. Physical ablation of the pia matter results in permanent devascularisation of the cortex, causing both a large ischemic core, as well as a consistent penumbra surrounding this core. Furthermore, a detailed comparison of multiple stroke methods (Gonzalez & Kolb, 2003) reveals that the devascularisation method generates increased dendritic plasticity when compared to MCAO. Though the causes of this differing morphological aetiology are yet to be determined, this finding suggests that the method of stroke induction plays a major role in the brain's response to injury.

4 – Post-Stroke Gene Expression and Neural Plasticity

Following a stroke to the motor cortex, the brain undergoes a series of plastic responses in an attempt to recover from the resultant damage. These plastic responses include axonal sprouting and synaptic plasticity (Carmichael, 2006), as well as a limited degree of neurogenesis (Jin, et al., 2001) and migration of neural progenitors to the peri-infarct region (Kokaia, Thored, Arvidsson, & Lindvall, 2006). This enhanced plasticity allows the brain to re-arrange the sensorimotor maps of the damaged area and compensate, to a degree, for the function lost during the stroke (Carmichael, 2006). It has long been known that neural plasticity is a direct result of changes in the protein complement of a neuron (Sutton & Schuman, 2006), and that this change is driven by altered gene expression in response to synaptic activity (Cavallaro, Schreurs, Zhao, D'Agata, & Alkon, 2001). Recent evidence suggests that these altered levels of gene expression are under the control of at least two epigenetic mechanisms; DNA methylation and histone acetylation (Miller, Campbell, & Sweatt, 2008).

4.1 - Epigenetics

Epigenetic changes, sequence independent heritable alterations in gene expression, include three major processes (Egger, Liang, Aparicio, & Jones, 2004). The first of these involves methylation of DNA, most commonly at cytosine residues. Addition of methyl groups to carbon 5 of cytosine in DNA acts as a direct blocker of transcription, which leads directly to the silencing of the methylated regions. The

second process, histone modification, is directed at controlling the structural organisation of DNA. Histones, small nuclear proteins which use their predominantly positively charged functional groups to bind and ‘wrap up’ the negatively charged DNA molecule, are the target of numerous modification enzymes. These enzymes can attach and remove functional groups in order to change the fundamental chemical properties of histones, and, correspondingly, their ability to bind DNA. The more tightly the histone binds the DNA, the lower the expression of the genes contained in this region. The third and most recently discovered process, non-coding RNA interaction, will be covered in greater detail in section 4.2. Cells are capable of regulating their translational activity by the use of small RNA molecules, which are capable of binding mRNA transcripts and influencing their activity downstream of their exit from the nucleus. RNA epigenetic regulation operates as a second level of control – DNA methylation and histone modification both can directly affect the amount of mRNA produced, while non-coding RNA controls what happens to the mRNA after it is produced (Fabbri, Ivan, Cimmino, Negrini, & Calin, 2007).

Interestingly, recent evidence suggests that all three of these processes are capable of directing each other’s activity (Egger, et al., 2004). It also appears that these processes operate most often to silence, rather than activate, genes. Considering that each cell must specialise with respect to function, though it contains all of the sequence information necessary to form an entire organism, this is not surprising. Epigenetic regulation is more accurately defined as the interplay of multiple independent regulators of gene silencing, to specialise and differentiate cells into specific phenotypes.

4.1.1 – DNA Methylation

Cytosine DNA methylation was the first identified epigenetic regulation mechanism, and has thus been the most closely studied. It is a crucial mechanism for normal cellular function, and has been shown to play a major functional role in development, cell proliferation, and maintenance of genome stability (Baylin, 2005; Baylin & Ohm, 2006; Jaenisch & Bird, 2003). Most DNA methylation occurs at CpG dinucleotides, which consist of a C next to a G in the nucleotide sequence, causing the complementary strand to consist of a G next to a C. 60-80% of CpG dinucleotides are methylated (Weber & Schubeler, 2007). A large majority of genes contain CpG islands in their promoter regions, and are therefore sensitive to methylation changes. The symmetry of these CpGs is crucial in the maintenance of methylation patterns through multiple generations of cells, as explained below. Healthy cells have relatively unmethylated CpG islands in front of their active genes, while silent genes, repetitive sequences and transposons are heavily methylated (Luczak & Jagodzinski, 2006).

The activity of a region of DNA depends directly on the ability of DNA-binding proteins, such as transcription factors and polymerases, to bind to it. DNA binding domains are capable of detecting variations in sequence which are responsible for the specificity of their parent proteins by interacting with the major groove of the DNA (Wolfe, Necludova, & Pabo, 2000). Once a transcription factor binds its target DNA sequence, it is capable of initiating assembly of the RNA polymerase holoenzyme, causing gene products downstream of the promoter to be transcribed. However, should the major groove of DNA become cluttered and

spatially inaccessible, both transcription factors and polymerases have difficulty binding the DNA and performing their required tasks of initiation and elongation, respectively. Addition of a methyl group to the 5' carbon on the cytosine molecule results in a protrusion of this methyl group into the major groove of the DNA molecule (Newell-Price, Clark, & King, 2000). Should a region of DNA become heavily methylated in this manner, these methyl groups will significantly block access to the sequence, resulting in a silent gene (Weber & Schubeler, 2007).

The second major factor whereby CpG methylation is responsible for downregulation of gene expression is due to the behaviours of the methyl CpG binding domain (MBD) family of proteins (Klose & Bird, 2006). These proteins, including MeCP2, MBD1, MBD2, and MBD3, selectively interact with methylated DNA and play a pivotal role in methylation-mediated chromatin remodelling and gene silencing (Jaenisch & Bird, 2003; Newell-Price, et al., 2000; Robertson, 2002). For a description of chromatin remodelling through histone alterations, see section 4.1.2.

Methylation takes place through the activity of enzymes known as DNA methyltransferases, enzymes capable of covalently adding methyl groups to unmethylated DNA bases (Robertson, 2005). It can be subdivided into two categories; *de novo* methylation, which describes the addition of novel methyl groups to unmethylated DNA, and maintenance methylation, which adds methyl groups to hemimethylated DNA during replication. In mammals, three DNA methyltransferases are primarily responsible for establishing and maintaining CpG methylation patterns, with DNMT1 being the primary maintenance methyltransferase

and DNMT3a and DNMT3b in charge of *de novo* methylation (Goll & Bestor, 2005). When DNA is replicated, the parental strand maintains its methylation pattern, which is read by DNMT1 due to its localisation at the replication fork. If a methyl-CpG is detected, DNMT1 will add a methyl group to its symmetrical complimentary CpG (Goll & Bestor, 2005; Weber & Schubeler, 2007). Due to this symmetry, the methylation pattern can be maintained in both copies of the new DNA. The *de novo* methyltransferases, on the other hand, are capable of modifying unmethylated CpG pairs and are important in tissue differentiation as well as the establishment of new hypermethylated CpG islands in cancer (Esteller, 2007). This seems, at first, paradoxical, since cancer's primary phenotype is that of a loss of differentiation, which is brought about by methylation. An explanation of this paradox, as well as its clinical implications, will be covered below.

4.1.2 – Histone Modification

A second level of epigenetic regulation is provided by alteration of the histone proteins, which regulate the packaging and organisation of the DNA. Through the action of the histone proteins, a single linear strand of DNA can be condensed into the highly structured mitotic chromosome. Histones show high affinity to DNA since they are positively charged, while DNA is negatively charged. Post-translational modifications of histones are capable of altering both their charge, and their capability for non-electrostatic intramolecular interaction, and therefore their affinity for DNA, which has an impact on the transcriptional activity of the DNA. If DNA is tightly wrapped around a histone, it becomes more difficult for transcription factors to

access it, which, as discussed above, results in a net decrease in the activity of the gene involved. Histone modification is unique among the epigenetic regulatory mechanisms, as it is the only modification capable of actually amplifying the activity of a specific region of DNA, since modifications to histones can either increase or decrease their net positive charge (Jenuwein & Allis, 2001). This variability of histone/DNA interaction is reflected in the large number of possible modifications to histones, including acetylation, phosphorylation, methylation, ubiquitination, and their inverse processes (Reamon-Buettner & Borlak, 2007). Discussion of every form of histone modification is outside the scope of this work, but the major subtypes of histone modification, methylation, acetylation and their inverse processes bear examination.

Subunit H3 and H4 contain several highly conserved lysine residues at their N-terminal tails. These specific residues are the primary targets of the acetylation mechanism, which uses enzymes known as HATs (histone acetyltransferases) to catalyse the transfer of an acetyl group from acetyl coenzyme A to the histone lysine (Nakao, 2001). Addition of the negatively charged acetyl group reduces the positive charge of the histone, and consequently reduces its affinity to DNA. This relatively loosely wound and transcriptionally active DNA is known as euchromatin. Deacetylation, the reverse process, is carried out by HDACs (histone deacetylases), which transfer acetyl groups by hydrolysing NAD to produce acetyl-ADP-ribose (North & Verdin, 2004). Due to the relative ease with which it can open and close the chromatin, histone acetylation is a very attractive target for further studies of pharmacological intervention. Methylation of lysine 9 of histone H3 is associated

with chromatin compaction and gene silencing, while methylation of lysines 4 and 27 of histone H3 results in transcription activation and chromatin relaxation.

Additionally, histone residues can be mono-, di- and tri-methylated, which results in an enormously complicated histone code (Cheung & Lau, 2005; Weidman, Dolinoy, Murphy, & Jirtle, 2007). In addition, DNA methylation and histone modification have been shown to be causally linked through the activity of methyl-CpG binding proteins such as MeCP2 (Jones, et al., 1998).

4.2 – Short RNA Epigenetic Modulation

The role of the third major type of epigenetic regulation is short RNA mediated alterations of translation. The role that this form of regulation plays in neural plasticity has not been fully explored. One of the main goals of the current study was to examine the roles that micro-RNA, an endogenously expressed form of short RNA responsible for the suppression of translation in mammalian cells, may play in the plasticity that accompanies stroke recovery.

4.2.1 – A brief History of microRNA

In 1993, Rosalind Lee, Rhonda Feinbaum and Victor Ambros discovered a strange gene in *C. elegans* that would create an entire field of study, and trigger the exploration of a new field of novel epigenetic regulation. The *lin-4* gene, known to regulate the timing of developmental events in the worm, was found to produce two RNA molecules, but no subsequent protein products (R. C. Lee, Feinbaum, & Ambros, 1993). One of these products, 61nt in length, was predicted to be a stem-

loop precursor molecule for the other, which was 21nt in length (R. C. Lee, et al., 1993; Wightman, Ha, & Ruvkun, 1993). Intriguingly, the 21nt molecule was found to have anti-sense complementarity to the 3' untranslated region (3'UTR) of a gene known as *lin-14*, which had been previously shown to be regulated by the *lin-4* gene product through an unknown form of 3'UTR interaction (Wightman, Burglin, Gatto, Arasu, & Ruvkun, 1991). The study also showed that *lin-4* mediated regulation of *lin-14* reduced the circulating levels of *lin-14* protein, but not its corresponding mRNA (R. C. Lee, et al., 1993). These studies, taken together, suggested that the short RNA product of *lin-4* bound the 3'UTR of *lin-14* and prevented the production of its protein.

These discoveries were initially described as an oddity of the worm genome, and left unexplored until the discovery of another regulatory short RNA in *C. elegans*, *let-7* (Reinhart, et al., 2000). Unlike *lin-4*, *let-7* is a highly conserved gene in multiple phylogenies, and shows a similar expression and regulatory pattern across all of them (Pasquinelli, et al., 2000). Shortly thereafter, a large number of *let-7* like gene products were discovered, and the entire class of molecules were named microRNA (miRNA). These molecules have since been implicated in a multitude of cellular processes, including development, proliferation, differentiation, neuronal patterning, and carcinogenesis (D. P. Bartel, 2004; B. Zhang, Wang, & Pan, 2007). Since this revelation, there have been literally hundreds of miRNAs identified and isolated in numerous species, including nearly 700 identified in humans as of early 2009 (G. L. Papadopoulos, Reczko, Simossis, Sethupathy, & Hatzigeorgiou, 2009),

with the latest computational models predicting roughly 1000 human miRNAs in total (Berezikov, et al., 2005).

4.2.2 – MicroRNA Genomics

The majority of miRNAs are transcribed from genomic regions quite distant from other annotated gene products (Lau, Lim, Weinstein, & Bartel, 2001), indicating that they exist as independent transcription units (Berezikov, et al., 2005; Ying & Lin, 2009). A significant minority of miRNAs, however, are located within the introns of other genes (D. P. Bartel, 2004; Ying & Lin, 2009), and though they share a similar method of action as independent miRNA genes, their biogenesis is different, as it involves components of the spliceosome (Ying & Lin, 2009). This has led to some degree of speculation that intronic miRNA and independent miRNA may have slightly different regulatory roles (Lin, Miller, & Ying, 2006). The genomic arrangement of miRNA varies, with a number of independent miRNA genes being located in two different types of clusters, either based on sequence homology (Lagos-Quintana, Rauhut, Lendeckel, & Tuschl, 2001) or product function (Lau, et al., 2001). Intronic miRNAs, of course, are clustered with the products of the genes from which they are produced, leading to the speculation that such protein-miRNA pairings form the basis of regulatory mechanisms (D. P. Bartel, 2004). A number of miRNAs are also associated with other prominent gene clusters, notably the Hox gene cluster (Aravin, et al., 2003; Lagos-Quintana, Rauhut, Meyer, Borkhardt, & Tuschl, 2003), suggesting that co-regulation of miRNA and protein expression plays a major role in the developmental process.

4.2.3 – miRNA Biogenesis

miRNAs go through a complex process to progress from their genomic form to their active cytoplasmic form (Figure 1). Independent miRNA genes are generally transcribed by Pol II (Esquela-Kerscher & Slack, 2006; Lau, et al., 2001), resulting in a primary transcript of varying length (depending on the structure of the miRNA gene – polycistronic miRNAs can have a primary transcript kilobases long (D. P. Bartel, 2004)). There is additional evidence suggesting that some miRNA genes are also transcribed by Pol III, as an artificial miRNA construct driven by the Pol III promoter can produce functional mature miRNAs (C. Z. Chen, Li, Lodish, & Bartel, 2004). Intronic miRNA is likewise transcribed by Pol II, as a consequence of the transcription of its home gene. Once the gene is fully transcribed, the spliceosome excises the introns from the primary transcript (Lin, et al., 2006; Ying & Lin, 2009). The major differences between the two types of primary transcript are the presence of a 5' cap and a polyA tail in the independently expressed miRNA genes, and the lack thereof in intronic miRNA. Following transcription, the primary transcript, known as the pri-miR is processed by an RNase III enzyme Drosha, and a dsRNA binding protein Pasha (DCGR8) (Esquela-Kerscher & Slack, 2006). The Drosha enzyme cuts the flanking sequences away from the base of the 70-80nt stem-loop structure (Y. Lee, et al., 2003), resulting in a pre-miR (the 61nt structure found in the original 1993 study (R. C. Lee, et al., 1993)). Drosha cuts the pre-miR leaving a characteristic pattern, a 5' phosphate and a 2nt long 3' overhang (Y. Lee, et al., 2003), a pattern that may play a major role in the later maturation of the miRNA sequence by determining which strand will be processed as a mature miRNA (D. P. Bartel, 2004). Pre-miRs

are subsequently exported from the nucleus by a complex of Exportin-5 and RAN-GTP (Bohnsack, Czaplinski, & Gorlich, 2004; Yi, Qin, Macara, & Cullen, 2003), and undergo another processing step in which the active miRNA (bound to its complementary strand, known as the miRNA:miRNA* duplex (D. P. Bartel, 2004)) is excised from the pre-miR by the Dicer RNase III enzyme (Ketting, et al., 2001). Dicer was previously known as the RNase responsible for the generation of short interfering RNA (siRNA), and appears to mediate the cleavage of pre-miRs in a similar fashion (Bernstein, Caudy, Hammond, & Hannon, 2001). Dicer cleaves the loop from a pre-miR, producing a 19-22nt long dsRNA miRNA:miRNA* structure.

From this point, the biological activity of miRNA mimics the pathways already identified in RNA interference, where short dsRNA sequences are used to target post-transcriptional gene silencing (PTGS) (D. P. Bartel, 2004). In RNAi, long cytoplasmic dsRNAs are also processed by Dicer, which cleaves them into 19-22nt long fragments. Though the exact process remains unclear, what is known is that these 19-22nt fragments are loaded onto a member of the Argonaute family, processed, and eventually incorporated into a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC) as single-stranded RNA molecules, known as guide strands. The choice of the guide strand is currently thought to be determined by the differences in thermodynamic stability of the 5' ends of the two strands of the dsRNA duplex (Siomi & Siomi, 2009), causing the strand whose 5' end is located at the less stable end of the duplex to be chosen as the guide strand (Schwarz, et al., 2003). Though the enzyme responsible for the selection and unwinding of the guide strand remains unidentified, this asymmetry rule, coupled

with the eventual discarding of the miRNA*, has led to speculation that a helicase-type enzyme (given the temporary name *unwindase*) samples each end of the duplex multiple times, probing its thermodynamic stability, and then fully unwinds the duplex and removes the unused complementary strand. Assembly of the RISC proceeds either subsequently to or simultaneously with the processing of the guide strand, though currently its exact constituent members are not yet known (and, in fact, are thought to vary, forming several different subtypes of RISC (D. P. Bartel, 2004)). Despite this, a number of common constituent members have been identified, including multiple members of the argonaute family (AGO1, AGO2), fragile X proteins (FRAX) (Soifer, Rossi, & Saetrom, 2007), p54 (Esquela-Kerscher & Slack, 2006), and Tudor-SN (van den Berg, Mols, & Han, 2008).

Once assembled, the RISC uses the guide strand to scan the transcriptome and induce post-transcriptional silencing by one of two methods, mRNA cleavage or translational repression (D. P. Bartel, 2004). It has recently been determined that Ago2 is the nuclease responsible for RISC-mediated cleavage of mRNAs (van den Berg, et al., 2008). The decision to cleave or not to cleave is not determined by the source of the short RNA in the RISC. Instead, the complex initiates cleavage if there is sufficient homology between the guide strand and the targeted mRNA. This cleavage occurs in the mRNA location corresponding to nucleotides 10 and 11 of the guide strand, in both mi-and-siRNA directed cleavage (Elbashir, Lendeckel, & Tuschl, 2001; Hutvagner & Zamore, 2002). Following Ago2 mediated cleavage, the RISC un-docks from the target mRNA, and can use the same guide strand to locate new mRNA targets.

Should the guide strand lack sufficient complementarity to an mRNA target, the RISC induces translational silencing instead of direct mRNA cleavage. The mechanism of translational repression has been most closely studied in *C. elegans lin-4*, where a number of possibilities have been proposed. First, the complex of the 3' end of the mRNA containing the RISC may coil around and physically interfere with ribosomes, stalling them on the transcript. Alternately, the action of the RISC may in fact target newly synthesised polypeptides for degradation, though this seems less likely as a mechanism (D. P. Bartel, 2004). A more recent study suggests that the RISC physically interfaces with the 5' cap of the mRNA, preventing ribosomes from loading (Pillai, et al., 2005). This was demonstrated by the fact that M⁷G-cap-independent translation of a luciferase reporter construct was not suppressed by *let-7* activity. There is still some controversy regarding the mechanism of RISC-mediated translational suppression, giving rise to the need for further study of the biochemistry and stoichiometry of RISC assembly and behaviour (van den Berg, et al., 2008).

Once translation has been suppressed, the question arises as to the fate of the suppressed mRNA. The first insight into this was the co-localization of translationally repressed mRNAs and a de-capping protein Dcp1a (Pillai, et al., 2005). The Pillai group postulated the existence of cytoplasmic foci, termed p-bodies, which contained the apparatus for degradation of suppressed mRNA, a hypothesis which was further strengthened by the subsequent discovery of Ago2 co-localization to the same foci (Sen & Blau, 2005). The final piece of the puzzle came in the same year, showing that active miRNAs likewise translocate to the same area (Liu, Valencia-Sanchez, Hannon, & Parker, 2005). Inside these p-bodies, mRNAs

are simultaneously uncapped and digested, which frees the components of the RISC to resume scanning the transcriptome (van den Berg, et al., 2008).

4.2.4 – Target Recognition

The difficulty in predicting the behaviour of miRNAs lies in their capability of acting through imperfect complementarity. Unlike siRNAs and plant miRNAs, which generally induce mRNA cleavage due to perfect guide strand / target strand complementarity, the vast majority of animal miRNA regulation appears to utilize the translational repression pathway (B. Bartel & Bartel, 2003; D. P. Bartel, 2009). This has led to a variety of computational and experimental methods attempting to determine the nature of miRNA – mRNA targeting. Historically, the first iteration of miRNA target prediction was based primarily on the degree of complementarity (Lewis, Shih, Jones-Rhoades, Bartel, & Burge, 2003), a method which generated a high number of false positives. Fortunately, due to recent biochemical and bioinformatic analyses, it was determined that the complementarity of the 5' end of the miRNA is a 'seed sequence' (Doench & Sharp, 2004; Parker, Roe, & Barford, 2006). In order for miRNA targeting to occur effectively, nucleotides 2-7 of the 5' end of the guide strand must demonstrate perfect or near-perfect complementarity to the target sequence in the mRNA 3'UTR (Smalheiser & Torvik, 2006). Inclusion of a strict "seed sequence" principle greatly reduced the amount of false positives produced by computational models (D. P. Bartel, 2009). Interestingly, a great deal of miRNA activity can be predicted simply by seed sequence alignment (Lewis, Burge, & Bartel, 2005). These models have also begun to take into account conservation of

sequences as a predictive tool. Given that miRNA regulation appears to be a major regulatory pathway, it can be postulated that miRNA/mRNA targeting is under some degree of selective pressure. Since mature miRNA sequences have been shown to be conserved across multiple phyla, one can safely assume that the target sequences are likewise conserved. By increasing the stringency of cross-species comparisons, modern computational models are rapidly increasing in accuracy and reliability for the prediction of miRNA targeting (D. P. Bartel, 2009).

In light of the available data, roughly six flavours of miRNA target pairing have been identified. The 7mer-A1 site possesses a perfect seed match at positions 2-7, and an adenine at residue 1, and represents the standard model for seed sequence binding. Variants of this binding pattern, such as the 7mer-m8 site (lacking the adenine, but possessing an additional base pair at nt8) and the 8-mer site (adenine at position 1, perfect match from 2-8), are also quite common. These three sites account for roughly 60% of all predicted miR-target interaction in humans (D. P. Bartel, 2009; Friedman, Farh, Burge, & Bartel, 2009). The remaining 40%, known as atypical binding sites, are comprised of 6-mer (adenine at position 1, seed match from 2-6) and offset 6-mer sites (no adenine, seed match from 3-8). These marginal sites typically show reduced miRNA efficacy (D. P. Bartel, 2009). A final type of target pairing is very rare, and consists of a mismatch in the seed region, which is compensated for by additional base pairing in the 3' region of the miRNA, a targeting method that again shows reduced efficacy when compared to the canonical sites. In addition to any of the above seed region pairings, many miRNAs also possess supplementary 3' complementarity sites, which were thought to enhance miRNA

action. This may or may not be the case, as there is currently a lack of experimental support for frequent 3' supplementary action (D. P. Bartel, 2009; Brennecke, Stark, Russell, & Cohen, 2005; Doench & Sharp, 2004), and furthermore, all of the energetic models developed to describe this action lack predictive power (Baek, et al., 2008; D. P. Bartel, 2009).

The initial hypothesis regarding the importance of supplementary 3' pairing was developed owing to an effect that is quite common in molecular biology research – the initial discovery drives the tone of future research. The binding of *let-7* to *lin-41* in *C. elegans* is dependent on supplementary 3' interaction (Vella, Choi, Lin, Reinert, & Slack, 2004), which led researchers to believe that this type of binding exemplified miRNA-target interaction (Vella, Reinert, & Slack, 2004). The relative rarity of this type of binding (1% of all predicted interaction (D. P. Bartel, 2009)) was only determined at a later date (Friedman, et al., 2009), which then led to further evolution of algorithms for target prediction. The relatively low frequency of 3' supplementary sites in the genome may be due to the increased informational complexity of the system, which is far more difficult to conserve in an evolutionary sense (D. P. Bartel, 2009).

If miRNA binding appears to be almost solely dependent on the sequence of the seed region, what, then, is the purpose of the other 11-13 nucleotides of the miR, and how does the physical arrangement of the miRNA in the RISC contribute to its function? One possible model (D. P. Bartel, 2004; Mallory, et al., 2004) suggests that the seed region of the miRNA is loaded into the Argonaute protein, and arranged into an A-form helix from nucleotides 2-7, which enhances both the specificity and

affinity of Watson-Crick base pairing in the seed region. This avoids the spatial interference that would arise from arranging the entire miRNA in a helical formation (D. P. Bartel, 2009). The increased affinity for the seed region predicted by this model was shown in two recent studies (Ameres, Martinez, & Schroeder, 2007; Haley & Zamore, 2004), both of which demonstrate a significant increase in hybridization kinetics if the RISC becomes involved. This suggests that miR-target interaction is catalysed by the above conformational changes induced in the miRNA by Ago.

Regarding the behaviours of the distal 3' end of the miRNA, a model has been proposed by a number of researchers, termed the 'seed-nucleation' model. Based on evidence from a number of earlier studies (Ameres, et al., 2007; D. P. Bartel, 2004; Haley & Zamore, 2004; Mallory, et al., 2004), this model proposes a two-stage process whereby the seed region, first, pairs to the target strand, associating it with the RISC, and second, induces the 3' tail of the miRNA to coil around the remainder of the mRNA using base pairing rules, creating the dsRNA structure that Ago2 is capable of cleaving (in the case of perfect complementarity). It is postulated that the association between the 3' end of the miRNA and the Ago protein protects it from degradation from cytoplasmic RNAses. This would necessitate the release of the miRNA from the Ago protein in order to allow it to bind the target strand. Bartel suggests that beginning at the seed region, successive base pairing induces sequential release of the miRNA nucleotides from the Ago protein, allowing the miRNA to slowly bind the target strand in a helical formation (D. P. Bartel, 2009). This model would also account for the apparent lack of kinetic advantage provided by 3' miRNA binding, as the energy gains from the bonding of the central nucleotides would be

offset by the energy losses caused by the disengagement of the miRNA from Ago and its resultant conformational changes.

4.2.5 – Analysis of miRNA Expression

With over 1000 miRNAs active in humans, it is necessary, and relevant, to analyse the behaviour of the entire miRNAome, as well as the interactions between miRs and their targets (given that one miRNA can have multiple targets, and one gene can be targeted by multiple miRs (D. P. Bartel, 2004)). A number of techniques have been adapted to the miRNA field to accomplish this, including Northern blotting, reverse transcriptase polymerase chain reaction (RT-PCR), miRNA microarrays, and ISH (W. Li & Ruan, 2009).

Northern blot consists of the separation of miRNAs from total cellular RNA by polyacrylamide gel electrophoresis, transfer of this RNA to a membrane, and qualitative and quantitative detection of a target sequence by hybridization of radiolabelled probes. This technique is useful, as it can be used to measure the expression level of several miRNAs relatively inexpensively (Sempere et al, in a recent study, examined the expression levels of 119 different miRNAs in various mouse tissues (Sempere, et al., 2004)), though the portion of total cellular RNA comprised of miRNA is quite low. Several protocols have been developed to increase the affinity of probes to miRNA and increase the signal-to-noise ratio of Northern detection of miRNA, including the use of locked nucleic acid (LNA) probes (W. Li & Ruan, 2009), which are synthetic oligonucleotides that show enhanced affinity to RNA.

Another method in common use, RT-PCR, makes use of reverse transcriptase and the polymerase chain reaction to amplify and quantify specific RNA sequences of interest. A major difficulty of this method is the similarity in size between traditional PCR probes and mature miRNA sequences (W. Li & Ruan, 2009). This problem has been overcome by the development of stem-loop primers (C. Chen, et al., 2005) which bind the miRNA, and subsequently, can be extended by the PCR reaction. They amplify themselves, and their relative levels of amplification after a given number of PCR cycles can be used to quantify the original levels of miRNA. Stem-loop primers have such high specificity and efficiency that they can be used to detect miRNAs arising from a single cell (C. Chen, et al., 2005).

The third major method for miRNA analysis, in-situ hybridization, permits spatial distribution of miRNAs in microscopic preparations to be visualized (W. Li & Ruan, 2009). This is especially useful for the examination of roles that the miRNA may play in heterogeneous tissues (for example, the activity of a given miRNA in a neuron may have a vastly different etiological consequence than the activity of that same miRNA in a glial cell). ISH suffers from much the same problem as Northern blotting, due to the relatively low affinity between standard probes and short RNAs. This issue has been addressed by adaptation of LNA probe technology to the method, allowing for very precise detection of miRNA distribution (Wienholds, et al., 2005).

Perhaps one of the most powerful tools for analysis of miRNA expression at the level of the miRNAome is the microarray. This method, though it lacks the reputation of Northern blotting, the extreme sensitivity of RT-PCR, and the spatial sensitivity of ISH, combines extremely high throughput and rapid data analysis, while

still maintaining excellent levels of quantitative sensitivity (W. Li & Ruan, 2009). Microarrays function on the principle of nucleic acid hybridization between target molecules and corresponding complementary probes. These probes, designed in advance from sequence databases, in this case, corresponding to the mature sequence of miRNAs, are cross-linked onto glass slides, forming the array. Isolated samples are then treated with a fluorescent dye, and hybridized to the array, with the miRNAs of interest binding specifically to their complimentary probes. Luminescent analysis of the array indicates the presence or absence of a particular anti-probe in a given sample, as well as an estimation of the relative quantity of any given miRNA, as more abundant miRNAs will generate a greater fluorescent signal as they concentrate on the probe. Statistical analysis of two different samples applied to duplicate arrays allows for a side-by-side comparison of the miRNAomes of two different conditions, indicating increased or decreased expression levels of miRNAs of interest.

5 – Statement of Purpose

This study aims to examine the role that microRNA plays in the progression of stroke recovery, at a distal time point (4 months post-lesion). Given the high degree of plasticity exhibited by recovering brains, and the necessity for tightly-regulated protein expression to control the informational capacity of a neuron, it can be predicted that miRNA regulation will play a role in the micromanagement of this process. Furthermore, it can be predicted that epigenetic factors, including alterations in DNA methylation, histone acetylation, and short RNA modulation, will play a major role in the phenotypic changes associated with many experimental drug therapies. One such therapy, discovered by Bryan Kolb et al in 2007, involves simultaneous infusion of epithelial growth factor (EGF) and erythropoietin (EPO) into the contralateral ventricle following a motor cortex stroke (Kolb, et al., 2007). This drug therapy produces highly promising behavioural results, with test animals demonstrating a significant improvement in recovery compared to untreated animals. Even more impressive than the behavioural results was the induction of neurogenesis caused by this treatment – causing the lesion to fill in with newly-generated tissue. The authors also determined that the fresh tissue was not directly responsible for the behavioural improvement. Instead, it appeared to be performing auxiliary processing that assisted the unaffected tissue to reorganise and reproduce the animal's lost behavioural repertoire.

6 – Materials and Methods

6.1 - Animals

Male Long-Evans rats (N= 20), 120-150 days old at the beginning of the studies, were used as subjects. The rats were housed and maintained according to the regulations of the Canadian Council on Animal Care. Rats with motor cortex stroke were treated with EGF + EPO (n = 8) and compared with untreated animals (n = 8) and normal controls (n = 4).

6.2 - Surgery

Under isoflurane anaesthesia (3%), a rectangular hole was drilled into the frontal and parietal bones running from +3 to -4mm anterior/posterior to the Bregma and running laterally from 1.5 to 4.5mm from midline. The focal, permanent devascularisation (stroke) has been described in detail elsewhere (Gonzalez, Whishaw, & Kolb, 2003). Briefly, the dura was removed and a sterile saline-soaked cotton swab was used to wipe the pia and attached blood vessels from the cortical surface. Surgery was performed on the left hemisphere for half of the test animals, and on the right hemisphere for the other half. Infusion cannulae (23 gauge) were implanted in the lateral ventricle (LV) ipsilateral to the preferred paw using skull coordinates 0.8mm anterior–posterior from the Bregma, 1.4mm lateral from the midline, and 3.5mm ventral from the skull surface. The cannulae were connected to filled, pre-tested, s.c. placed osmotic minipumps (Alzet, California, model 1007D) via coiled, flexible polyethylene tubing. Cannulae were filled with either vehicle

(artificial cerebral spinal fluid plus 0.1% bovine serum albumin) or with aCSF and EGF (10 mg/mL) + EPO (1365 IU/mL). There was no mortality as a consequence of the surgeries. For 24 h after the devascularisation, the animals were lethargic and sensitive to touch, after which they returned to their pre-surgical behaviour.

6.3 – Tissue Collection

Following a four-month recovery period, test animals were euthanized with an intraperitoneal injection of Euthansol (Schering-Plough Animal Health, Pointe-Claire, Québec). Animals were decapitated and their neural tissue was collected by surgical removal of either the motor cortex (in the case of sham animals), the peri-infarct region (in the case of untreated animals with strokes), or the regenerated tissue plug (in the case of EGF/EPO treated animals, which was subsequently flash-frozen with liquid nitrogen. Furthermore, in all animals, a region of the motor cortex contralateral to the lesion was collected for comparison. All samples were subsequently stored at -80°C.

6.4 – miRNA microarray expression analysis

Total RNA was extracted from harvested brain tissues using TRIzol Reagent (Invitrogen, Burlington, Ontario) according to the manufacturer's instructions. miRNA microarray analysis was performed by LC Sciences (Houston, TX). Ten micrograms of total RNA were size-fractionated (<200 nucleotides) by using a *mirVana* kit (Ambion, Austin, TX). Poly-A tails were added to the RNA sequences at the 3' ends using a poly(A) polymerase, and nucleotide tags were then ligated to the

poly-A tails. The tagged RNAs were hybridized to dual-channel microarray μ ParaFlo microfluidics chips (LC Sciences) containing 439 miRNA probes to rat and mouse miRNAs, and then labelled with tag-specific dendrimer Cy3 and Cy5 fluorescent dyes. Dye switching was performed to eliminate the dye bias. The melting temperature of the detection probes was balanced by incorporation of varying numbers of modified nucleotides with increased binding affinities.

Hybridization images were collected using a GenePix 4000B laser scanner (Molecular Devices, Sunnyvale, CA) and digitized using Array-Pro image analysis software (Media Cybernetics, Silver Spring, MD). The maximum signal level of background probes was 180. A miRNA detection signal threshold was defined as twice the maximum background signal. Normalization was performed with a cyclic LOWESS (locally-weighted regression) method to remove system-related variations (Bolstad, Irizarry, Astrand, & Speed, 2003). Data adjustments included data-filtering, log₂ transformation, and gene centering and normalization. T-test analysis was conducted between control and E2-treated sample groups. miRNAs with p values < 0.05 were selected for cluster analysis. The clustering analysis was performed with a hierarchical method and with average linkage and Euclidean distance metrics (Eisen, Spellman, Brown, & Botstein, 1998).

miRNAs chosen for subsequent analysis were selected based on an arbitrary mean minimum cut-off value of 500 units of fluorescence from the microarray data, in order to remove data points that, while statistically significant, may lack biological impacts.

6.5 – Confirmation of a putative miRNA target

To examine whether or not *noggin* is, in fact, functionally targeted by miR-152, a 3'-untranslated region (UTR) segment of the *nog* gene corresponding to a region of 250 bp (nucleotides 948 to 1198 of the total transcript) of *nog* (accession no. NM_012990) was amplified by PCR from rat brain-derived cDNA using primers that included a *Xba*I and *Eco*RI tails on the 5' and 3' strands, respectively. PCR products were restricted with both *Xba*I and *Eco*RI restriction endonucleases and then gel purified. *nog*-3'-UTR was ligated into pGL3 firefly luciferase vectors (Promega) by using the *Xba*I site located immediately downstream of the stop codon of luciferase.

HEK293 cells were transfected with the firefly luciferase UTR-report vector, control *Renilla* luciferase pRL-TK vector (Promega), and precursor miR-152 for the *nog*-3'-UTR construct using LipofectAMINE 2000 reagent according to the manufacturer's protocol (Invitrogen). Twenty-four hours after transfection, cells were lysed with a 1× passive lysis buffer and the activity of both *Renilla* and firefly luciferases was assayed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Data were normalized to (-) controls and equalized to the expression of *renilla* luciferase, and presented in RLU (relative luciferase units)

6.6 – Western Blot analysis of Protein Expression

Brain tissue lysates were prepared by homogenization of 50 mg of tissue in 500 µl of lysis buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 mg/ml each aprotinin,

leupeptin, pepstatin; 1 mM Na₃VO₄, 1 mM NaF), sonication, and incubation at 4°C for 30 min. This was followed by centrifugation at 10,000 x g at 4°C for 20 min. Extracts containing equal quantities of proteins were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to PVDF membranes. Membranes were probed with primary antibodies against Noggin (1:1000; Santa Cruz Biotechnology) MeCP2 (1:1000; Abcam), DNA methyltransferase 3a (DNMT3a) (1:1000; Abcam), DNA methyltransferase 3b (DNM3b) (216S, 1:500, Cell Signalling), and Bone Morphogenic Protein 4 (BMP4) (1:500; Santa Cruz Biotechnology). Alkaline phosphatase-coupled donkey anti-rabbit secondary antibodies were used for visualization. Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) and the ECL Plus immunoblotting detection system (GE Healthcare). Chemiluminescence was detected by Biomax MR films (Eastman Kodak, New Haven, CT). Signals were quantified using NIH ImageJ 1.63 Software and normalized to actin. The protein levels in the tissues of exposed animals were normalized relative to controls. Two technical replicates were used for each immunoblotting.

7 - Results

7.1 – miRNA microarray expression analysis

7.1.1 – General Trends

Experimental stroke induced numerous changes in the miRNAome, in both the lesioned and unlesioned cortex when compared to shams. Furthermore, EGF/EPO therapy as described by Kolb et al (Kolb, et al., 2007) caused its own distinct pattern of miRNAome activity, influencing a larger number of miRNAs to change their activity than the non-treated conditions.

Specifically, in the lesioned cortex of untreated animals in comparison to shams, 9 miRNAs were down-regulated while 9 different miRNAs showed up-regulation (Figure 3a). In comparison, examination of the regenerated tissue plug with respect to shams revealed the up-regulation of 18 miRNAs, and the downregulation of 35 (Figure 3b).

Comparisons directly between similar tissue types revealed a second subset of changes, with 3 miRNAs showing an increase and 11 showing a decrease in expression in the EGF/EPO treated lesion relative to the untreated lesion (Figure 4a).

Examination of non-lesioned contralateral cortex revealed a similar pattern, with 8 miRNAs overexpressed and 12 miRNAs underexpressed in the treated brains relative to the untreated (Figure 4b).

A third comparison was made within-brain, of both treated and untreated animals. In the untreated condition, the lesioned cortex showed up-regulation of 4 miRNAs when compared to its corresponding contralateral cortex (Figure 5a). Upon

EGF/EPO treatment, this disparity between hemispheres increased to 14 up-regulated miRs and 16 down-regulated miRs (Figure 5b).

Among these lists, a subset of miRNAs was selected based on an arbitrary cut-off value of 500 units of fluorescence (i.e. any miRNA showing less than 500 units of fluorescence in both tissue types under comparison was disregarded due to questionable biological impact of extremely low levels of circulating miRNA) and examined further.

7.1.2 – Specific miRNAs of probable biological significance

The following miRNAs showed fold changes greater than 2.0

miR-199a showed increased expression levels in the non-lesioned motor cortex of untreated stroke animals when compared with unlesioned shams (fold change = 6.5, $p = 0.03$), and decreased expression levels in the non-lesioned motor cortex of treated animals when compared with the non-lesioned motor cortex of untreated animals (fold change = 26.7, $p = 0.0007$).

miR-199a* showed a highly significant increase in expression levels in the lesioned motor cortex of EGF/EPO treated animals when compared to the non-lesioned contralateral cortex (fold change = 4.3, $p = 0.005$).

miR-199b showed increased expression levels in the non-lesioned motor cortex of untreated stroke animals when compared with unlesioned shams (fold change = 5.9, $p = 0.03$), and decreased expression levels in the non-lesioned motor cortex of treated animals when compared with the non-lesioned motor cortex of untreated animals (fold change = 28.3, $p = 0.002$). The microarray detected no

significant difference between unlesioned shams and the non-lesioned cortex of treated animals.

miR-138 showed a highly significant elevation in expression level in the lesioned cortex of EGF/EPO treated animals when compared to unlesioned shams (fold change = 2.6, $p = 0.009$). miR-138 was also showed highly significant elevation in the nonlesioned cortex of EGF/EPO treated animals when compared to unlesioned shams (fold change = 1.9, $p = 0.002$).

miR-214 showed a highly significant increase in expression in the lesioned cortex of EGF/EPO treated animals when compared to their non-lesioned cortex (fold change = 2.5 $p = 0.004$), and a significant decrease in non-lesioned cortex of stroked animals when compared to unlesioned shams (fold change = 3.0, $p = 0.05$).

miR-152 showed a highly significant increase in expression levels in the EGF/EPO treated stroke lesions when compared to contralateral, non-lesioned tissue (fold change = 2.5, $p = 0.003$). In untreated brains, however, miR-152 showed a smaller but still significant trend in the opposite direction, with a slight decrease in the peri-infarct when compared to contralateral nonlesioned tissue (fold change = 1.6, $p = 0.04$).

miR-21 was significantly up-regulated in both the non-lesioned (fold change = 1.3, $p = 0.05$) and lesioned (fold change = 2.2, $p = 0.02$) cortex of EGF/EPO treated animals when compared to sham. Furthermore, when comparing directly between the two hemispheres, miR-21 also showed a detectable increase in expression from the lesioned hemisphere to the non-lesioned hemisphere (fold change = 1.7, $p = 0.05$).

miR-145 showed a highly significant increase in expression in the lesioned cortex of EGF/EPO treated animals when compared to their non-lesioned cortex (fold change = 2.0, $p = 0.005$). It was significantly down-regulated in non-lesioned treated cortex when compared to unlesioned shams (fold change = 1.8, $p = 0.02$). The microarray detected no significant difference in expression levels of miR-145 between unlesioned shams and treated lesioned groups.

7.1.3 – Specific miRNAs of possible biological significance

The following miRNAs showed a fold change less than 2, while achieving statistical significance by T-test.

miR-129-5p showed a highly significant decrease in expression in EGF/EPO treated animals, both in the non-lesioned cortex (fold change = 1.5, $p = 0.002$), and the lesioned cortex (fold change = 1.9, $p = 0.0007$), compared to shams. This miRNA also showed a significant decrease in the non-lesioned cortex of EGF/EPO treated animals when compared to lesioned cortex (fold change = 1.4, $p = 0.01$).

miR-132 showed a significant increase in expression in the lesioned hemisphere of EGF/EPO treated animals when compared to the non-lesioned hemisphere (fold change = 1.5, $p = 0.03$) and unlesioned shams (fold change = 1.7, $p = 0.01$). In untreated, lesioned animals, however, miR-132 showed a highly significant decrease in activity when compared to shams (fold change = 1.5, $p = 0.002$).

miR-127 showed a significant decrease in expression in both the non-lesioned cortex (fold change = 1.4, $p = 0.02$) and the lesioned cortex (fold change = 1.6, $p =$

0.03) of EGF/EPO treated animals when compared to shams. miR-127 showed no significant changes in expression level in other experimental treatments.

miR-107 showed a small but significant decrease in expression in the non-lesioned hemisphere of EGF/EPO treated animals when compared to shams (fold change = 1.3, $p = 0.05$). There were no significant alterations of miR-107 elsewhere.

miR-124a showed a small but significant elevation of expression in the lesioned hemisphere of untreated animals when compared to shams (fold change = 1.2, $p = 0.02$), and no significant changes in other conditions.

miR-9 is significantly up-regulated in the non-lesioned cortex of EGF/EPO treated animals when compared to the non-lesioned cortex of untreated animals (fold change = 1.2, $p = 0.02$). Additionally, miR-9 shows an increase in expression in the non-lesioned cortex of treated animals when compared to the lesion/treated side as well (fold change = 1.2, $p = 0.02$)

7.2– Confirmation of a putative miRNA target

Figure 2 shows that miR-152 inhibited the luciferase activity from the construct with the Noggin-3'-UTR segment in a concentration-dependent manner. There was no change in the luciferase reporter activity when the cells were co-transfected with negative control (scrambled oligonucleotides). No luciferase expression changes were seen when the cells were transfected with the plasmid lacking the Noggin-3'-UTR fragment.

7.3– Analysis of Protein Expression

Given the ability of miRNAs to affect the cellular phenotype by dramatically altering the translational activity of mRNA, it becomes highly relevant to observe the activity of the proteome in response to any alterations in miRNA activity. With that relationship in mind, this study examined the activity of two subsets of proteins through Western Blot analysis – Noggin and BMP4, the former of which is a putative target of miR-152. The second group of proteins analysed in this manner were three key DNA methylation proteins, DNMT3a, DNMT3b and MeCP2. This was done to examine the state of a second epigenetic regulatory pathway in response to EGF/EPO therapy, as the activity of all types of epigenetic regulators are intrinsically linked (Guil & Esteller, 2009).

7.3.1 – Noggin and BMP4

Noggin was not significantly down-regulated in the lesioned cortex of rats with a stroke (92% relative to control), but was significantly reduced in lesioned cortex of EGF/EPO treated animals (83% relative to control). BMP4 levels showed a significant decrease in both lesioned (85% relative to control) and treated-lesioned conditions (42% relative to control) (Figure 6)

7.3.2 – MeCP2 and de novo DNA Methyltransferases

The expression levels of several key methylation proteins were affected both by stroke and stroke coupled with EGF/EPO therapy. MeCP2, methyl CpG binding

protein, was significantly under-expressed in both lesioned (79% control activity) and EGF/EPO treated lesioned cortex (68% control activity). Both treated and untreated non-lesioned cortex showed no significant change from shams. (Significant effect of LESION, no significant effect of TREATMENT) (Figure 7). DNMT3a was significantly up-regulated in EGF/EPO treated lesioned cortex (134% control), and showed non-significant variation in all other conditions. DNMT3b was significantly down-regulated in untreated animals, both in the non-lesioned cortex (80% control) and the lesioned cortex (72% control). In treated animals, this was mirrored in the lesioned cortex (83% control), but in the non-lesioned cortex, DNMT3b was over-expressed (118% control). (Figure 8)

8 – Discussion

The results of this study, in general, demonstrate the existence of a large epigenetic component to the regulation of neural tissue in the months following a stroke, and also, that the therapeutic benefit of EGF/EPO drug therapy appears to induce an additional epigenetic effect above and beyond that of a stroke. Clearly, this study shows that in the brain, an organ heavily dependent on precisely controlled gene expression for normal function (learning and memory, synaptic outgrowth and plasticity, regulation of its high metabolic demands), abnormal function likewise taps into the mechanisms of epigenetic regulation. This study indicates a number of potential effects of both stroke and EGF/EPO therapy, as evidenced both by the miRNA microarray and the subsequent Western Blot analysis of several key proteins of interest, a significant finding of the current work.

8.1 – miRNA microarray expression analysis

8.1.1 – General Trends

The behaviour of the miRNAome in this study supports the prediction that EGF/EPO therapy taps into and enhances the pre-existing epigenetic mechanism already at work in the recovering brain. However, quite intriguingly, it appears that a great deal of the miRNA changes at work in the untreated brain are either de-activated or superseded by the treatment, as not all of the miRNAs on the ‘untreated brain’ lists are represented on the corresponding treated brain list. This in itself presents questions regarding the mechanisms of EGF/EPO based miRNAome

regulation to be examined in future work. Additionally, when considering the effects of EGF/EPO therapy, it is important to remember the high likelihood that the molecular effects of this therapy on brain tissue are not limited to miRNA / DNA methylation changes, as EGF (Higashiyama, et al., 2008) and EPO (Arcasoy, 2008) are both known to directly induce gene expression through transcription factor mediated pathways.

Figure 3a shows a comparison between lesioned tissue and untreated shams in both conditions. Of note is the difference in magnitude of the number of up-regulated vs. down-regulated miRs. Addition of EGF/EPO causes the number of up-regulated miRs to change from 9 to 18, a two-fold increase, whereas the number of down-regulated miRs changes from 9 to 35 – nearly a four-fold increase. This suggests that one of the mechanisms of EGF/EPO therapy in the lesioned hemisphere is a net global suppression of miRNA activity. Given that the mechanism of miRNA regulation is a suppression of gene expression in general, this is consistent with the prediction that EGF/EPO therapy is activating or enabling a wave of gene expression leading to the growth of new tissues.

The behaviour of the miRNAome in the direct comparison of treated and untreated brain regions (Figure 4) follows this trend, with a net down-regulatory effect present in both treated and untreated. However, it should also be noted that the expression patterns between the lesioned and unlesioned hemispheres were quite different, involving effectively two different subsets of the miRNAome. Additionally, EGF/EPO therapy appears to have induced a greater degree of up-regulation in the nonlesioned hemisphere. This could be a potential indication of

slightly ‘tighter’ control over gene expression in the nonlesioned cortex, possibly because this tissue is effectively uninjured – too much de-repression of expression in this region could lead to uncontrolled cell division and growth, which could bring about a deleterious phenotype.

The within-brain comparison showed the most dramatic difference between untreated and treated brains (Figure 5), a trend which highlights a large difference in neural EGF/EPO response depending on the injury status of the tissue. This result is support for the conclusion that the cellular environment and molecular status (in this case, injury signals) of the brain plays a major role in the epigenetic effects of a given drug therapy. What exactly is inducing the changed response between injured and non-injured tissue remains unknown, though due to the self-regulating ability of many miRNA clusters, it is possible that the miRNA profile of a given brain region directly influences its miRNAome response to drug therapy.

8.1.2 – Specific miRNAs

Of the miRNAs identified by the microarray, those having pre-existing confirmed mRNA targets are examined in greater detail here. A number of these miRNAs showed very small fold changes that, while determined to be statistically significant by the microarray, may be of minimal biological impact. The following section ranks miRNAs by the magnitude of their respective fold changes. A microRNA with a fold change of <2 is indicative of a possible Type I error, though speculation regarding its role in the biological context of EGF/EPO and untreated stroke remains relevant.

8.1.2 – Specific miRNAs of probable biological significance

*miR-199a and 199a**

miR-199a and 199a* are generated from the complimentary strands of the same precursor molecule (Kim, et al., 2008) though the data from this study suggests that either elements of the neural environment or the phenotypic changes brought about by EGF/EPO therapy and neurogenic recovery influence the behaviour of the RISC complex while processing the pre-miR. Even though both miRs are under the influence of the same nuclear factors, including transcription levels, pri-miR processing and nuclear export, the cytoplasmic levels of the two miRs vary across experimental conditions. A study by Kim et al (Kim, et al., 2008) presents an in-depth analysis of the behaviour of miR-199a* in various tissues and cancer cell lines, and experimentally confirms its down-regulation of the MET proto-oncogene and its downstream signalling component ERK2. Though their study focuses on the behaviour of miR-199a* as a possible tumour suppressor gene (and, in fact, shows that in an uninjured brain, miR-199a* is transcriptionally repressed), its behaviour in the context of an injured, EGF/EPO treated brain indicates that it could play a role in the regulation of EGF/EPO induced neurogenesis. The increase of miR-199a* in the newly-generated tissue corresponds to a decrease in the levels of active MET, which is a proliferative factor. This finding suggests that the altered regulation of miR-199a* could be acting as a “braking” signal to the neurogenesis triggered by EGF/EPO therapy four months prior.

miR-199b

Currently, miR-199b is not confirmed to exist in rats; however, the detection of a readable mmu-miR-199b signal by the LCS microarray shows that the mature sequence of miR-199b is present in the cytoplasm of rat brain cells. Human miR-199b has been confirmed to target LAMC2 (laminin gamma 2), an extracellular matrix glycoprotein, which plays a major role in cell migration and differentiation (Tanno, et al., 2006). Laminins have long been known to play a critical role in promoting the outgrowth of neurites from adult neurons (Cowen & Gavazzi, 1998) in concert with growth factors. The two distinct patterns of miR-199b regulation shown at four months post-lesion indicate that stroke and EGF/EPO therapy both have independent influence on the regulation of cellular adhesion molecules. Stroke appears to trigger an increase in miR-199b activity in the non-lesioned cortex; EGF/EPO therapy returns miR-199b levels to baseline. Specifically, the 28-fold decrease of miR-199b expression in treated animals compared to their untreated counterparts suggests that the treated animals demonstrate de-repression of LAMC2 expression, promoting neurite outgrowth and plasticity in the non-lesioned cortex. This could partially account for the finding by Kolb et al (Kolb, et al., 2007) which suggested that the newly generated cortical tissue's role in functional recovery may be the promotion of reorganization and modification of pre-existing neural circuitry.

miR-138

The observed changes in the expression pattern of miR-138 indicate that its effects are tied to the global changes induced by the combination of EGF and EPO, as no significant differences were observed in untreated animals. It is interesting to note

that miR-138 was elevated in both hemispheres of treated animals' brains, but to a higher extent in the lesioned cortex, which suggests that the lesion effect may modulate the magnitude of miR-138 alteration following its induction.

miR-138 has been confirmed to target telomerase reverse transcriptase (TERT) (Mitomo, et al., 2008), the catalytic subunit of the telomerase holoenzyme, which is responsible for maintenance of chromosomal telomeres. TERT activity is associated with the maintenance of neural progenitor and stem cells in the brain, with depletions of TERT causing the development of abnormal brain function and behaviour in mice (J. Lee, et al., 2009). The inverse correlation between TERT and stem cells suggests that an opposite miRNA expression pattern exists (i.e. lower levels of miR-138 causing de-repression of TERT activity), but it appears that at four months post-lesion and treatment, microRNA signalling is moving to repress TERT levels. This may indicate that at the four-month timepoint, miR-138 is contributing to the array of antiproliferative signals in the newly-generated tissue, and in the brain at large. It is also possible that increased miR-138 activity in the treated brain is operating as a protective mechanism to attenuate the hyperproliferation of activated neural stem cells. This outcome would prevent tumorigenesis, as high activity of TERT is a hallmark of the development of glioblastoma (Casalbore, et al., 2009).

miR-214

miR-214 is well-known in molecular oncology, targeting PTEN phosphatase (Yang, et al., 2008). PTEN acts as an inhibitor of the PKB/Akt cell survival pathway (Song, Ouyang, & Bao, 2005). The up-regulation of miR-214 in the peri-infarct region of treated animals suggests that the microRNAome is playing a key role in the

modulation of cell survival in the new neural tissue, in this case, by promoting antiapoptotic signalling to ensure the survival of the newly generated tissue.

8.1.2k – miR-21

miR-21 is a particularly interesting microRNA to study in the context of the brain, as it is one of the most intensely studied of the onco-miRs (i.e., microRNAs that play a role in cancer). It responded very strongly to EGF/EPO therapy during this study, and was up-regulated in both motor cortices of treated animals. The array-based detection of an additional significant difference in expression, with the lesioned cortex having a 1.66 fold increase on top of the increase seen in the nonlesioned cortex, is important. It indicates that some element of the injury-specific effect of EGF/EPO likely operates on the miR-21 regulatory network, causing its highly elevated levels of expression in lesioned, treated tissue.

miR-21 has been experimentally confirmed to target multiple mRNA transcripts, tropomyosin 1 T (PM1) (Zhu, Si, Wu, & Mo, 2007), serpin peptidase inhibitor, clade B (ovalbumin), member 5 (SERPINB5) (Zhu, et al., 2008), PTEN phosphatase (Meng, et al., 2006; Meng, et al., 2007), and programmed cell death 4 (PCDC4) (Asangani, et al., 2008; Frankel, et al., 2008; Lu, et al., 2008; Zhu, et al., 2008). The role of many of these genes as tumour suppressors makes miR-21 of great interest in the study of cellular proliferation, since increases in miR-21 elevates the rate of cell division. In addition, and relevant to the neurological context of this work, miR-21 targets Sprouty2 (SPRY2), an inhibitor of branching morphogenesis and neurite outgrowth (Sayed, et al., 2008).

TPM1 is an actin-binding protein known to stabilise microfilaments (Zhu, et al., 2007), which becomes down-regulated in cancerous conditions, leading to the generalised lack of morphology characteristic of neoplastic cells. In a neural context, alterations of cellular form are highly significant, though the role that a myosin-dependent protein plays in specific neural plasticity may not be relevant. SERPINB5, which is also known as mapsin, is an oncologically-relevant serine proteinase inhibitor (Cao, et al., 2007). Although to date, the exact biochemistry and biological function of this protein are unknown, expression levels of mapsin serve as a useful prognostic barometer in determining the severity of tumours (Zhu, et al., 2008). They are also implicated in the suppression of tumour growth, invasion and metastasis, the induction of tumour redifferentiation, and enhancement of cell sensitivity to apoptosis (Lockett, Yin, Li, Meng, & Sheng, 2006). PTEN phosphatase controls a critical cell cycle checkpoint (Chu & Tarnawski, 2004), and plays a major role in the development of neuroma and glioma (Endersby & Baker, 2008). The existing body of work surrounding PTEN as an antiproliferative agent in the brain would make it an excellent target for further study in EGF/EPO based effects on stroke recovery.

Perhaps the most important phenotypic consideration of miR-21 induction and the consequent down-regulation of all of its downstream antiproliferative regulatory targets is the indication that this epigenetic change appears to be sustaining a pro-proliferative cellular environment in both the lesioned and non-lesioned hemispheres.

Apart from its obvious impacts on proliferation in the brain, miR-21 activity appears to be a potent regulator of plasticity as well, by repressing a repressor of

cellular outgrowths, Sprouty2. Regulation of SPRY2 could be playing two roles in the post-stroke brain, as it induces two separate phenotypic effects, depending on the age of the neurons it is expressed in. In immature neurons, SPRY2 overexpression will inhibit the plasticity and development of these neurons by preventing the growth of new neural processes (Gross, et al., 2007). High levels of miR-21 based repression of this mediator in the lesioned cortex would permit the neural progenitors to develop more rapidly and more fully. Conversely, in mature, differentiated neurons, high levels of SPRY2 correlate with neuronal apoptosis; low levels of SPRY2 correlate with neuronal survival (Gross, et al., 2007). In both hemispheres of the post-lesioned brain, repression of SPRY2 would contribute to pro-survival signalling.

The magnitude and breadth of miR-21 induction in EGF/EPO therapy indicate a potential side effect of this drug treatment – any enhancement of proliferative activity should be immediately examined for its oncogenic potential. Growth factor-based therapies may prove dangerous in this regard, though they give rise to an intriguing conundrum: in the case of stroke, where the typical patient is elderly, is the behavioural and lifestyle improvement brought about by drug therapy worth the potential risk for future cancer? Reconciling this “dilemma” depends on the timing of any potential carcinogenic induction – if growth factor therapy is shown to increase the risk of brain cancer 20 years post-treatment, it could serve as a very useful tool to increase the quality of life of an elderly stroke patient. More rapid progression of potential cancers would be an important ethical issue to address when analysing the potential cost/benefit analysis of EGF/EPO stroke therapy.

miR-145

The two different expression patterns of miR-145 taken in context suggest that the phenotypic changes induced by EGF/EPO contribute to the recovery of ‘normal’ levels of this microRNA in the lesioned hemisphere. This implies that devascularisation followed by EGF/EPO treatment induces a depletion of miR-145 in the unlesioned cortex, or that stroke triggers a global depletion of miR-145, which is reactivated in the peri-infarct region by EGF/EPO therapy.

miR-145 has been shown to target two different transcripts for down-regulation – FLJ21308 (also known as Poly(ADP-ribose) polymerase 8 (PARP8)) (Kiriakidou, et al., 2004) and IRS1 (insulin receptor substrate-1) (Shi, et al., 2007). PARP8 is a member of the PARP superfamily of proteins, a diverse group of enzymes capable of poly(ADP-ribosyl)ation of nuclear proteins by virtue of the conserved PARP domain (Ame, Spenlehauer, & de Murcia, 2004). Though the current structure and enzymatic role of PARP8 has not been determined to date, the more closely studied members of this family facilitate and contribute to DNA repair processes and, consequently, cell survival. Interestingly, reduction of PARP activity by selective inhibition has been shown to provide some neuroprotective effects in models of mouse ischemia (Burkle, 2001), as a sharp increase in PARP activity appears to be a precursor to ischemic cell death. However, the role of PARP in ischemia appears to be dependent on a variety of other factors, as a second study demonstrated that limited PARP activity in concert with maintenance of cellular levels of NAD⁺ in fact prevented reperfusion damage in the rat hippocampus

(Nagayama, et al., 2000). Without further research into the specific function and mechanism of PARP8-mediated poly(ADP-ribosyl)ation, it is difficult to determine which side of the PARP-ischemia equation it contributes to.

IRS1 is the major substrate insulin-like growth factor receptor (IGF-IR), and plays a major role in mitogenic signalling (Baserga, 2000), cell growth and cell differentiation (Shi, et al., 2007). In a neuronal context, it appears that IRS-1 activity plays a major role in antiapoptotic signalling, by activation of PI3K, which in turn activates PKC and Akt/protein kinase B (PKB) (Zhao & Alkon, 2001).

Consequently, it is possible that the mir-145 induced suppression of IRS1 plays a distinct role in the brain following treatment. Similarly to the behaviour of miR-199a*, miR-145 signalling appears to be suppressing proliferative and antiapoptotic signalling. This suggests that multiple aspects of the miRNAome are operating in concert to arrest the growth triggered by EGF/EPO four months prior.

8.1.3 – Specific miRNAs of possible biological significance

miR-129-5p

miR-129-5p has two confirmed mRNA targets, having been shown to decrease the activity of both EIF2C3 (eukaryotic translation initiation factor 2C, 3) and CAMTA1 (calmodulin binding transcription activator 1) (Liao, et al., 2008).

This miRNA is induced in the contralateral nonlesioned cortex in both treated and untreated animals, indicating that the decrease in miR-129-5p levels is a feature of the neural environment induced contralateral to a motor cortex stroke. As a result, it may play a role in the phenotypic changes associated with increases in neural plasticity

and re-distribution of neural function observed in the contralateral hemisphere. The additional down-regulation of miR-129-5p in the lesioned, treated cortex is a possible direct effect of EGF/EPO therapy.

EIF2C3 is a member of the Argonaute family of proteins (Sasaki, Shiohama, Minoshima, & Shimizu, 2003), which, as discussed previously, are critical components of the RISC / Dicer complex (Liao, et al., 2008). De-repression of this protein in the non-lesioned cortex would result in the modulation of the behaviour of all Dicer-mediated short RNA activity, which may be a contributing factor in the plastic changes seen in the recovering brain. Though the specific role of EIF2C3 in short RNA processing are not known at this time, the miR-129-5p / EIF2C3 pathway has been implicated in the differentiation of human hematopoietic stem cells (Liao, et al., 2008). This link may be relevant in a neural context due to the recent finding by Kozłowska et al (Kozłowska, et al., 2007). These authors generated a lineage of neural-like stem cells (dubbed HUCB-NSC) from hematopoietic umbilical cord progenitors. It is possible that the modulation of short RNA signalling in the contralateral cortex plays a role in neuronal cell fate, and during modulation of any potential stem cells that are endogenously present in the non-injured cortex.

CAMTA1, though relatively unstudied, has been shown to play a role in the induction of cell differentiation and cell cycle regulation (Nakatani, et al., 2004). Its potential de-repression by decreases in miR-129-5p activity in the contralateral cortex could play an additional role in compensatory plasticity by modulating the differentiation of any active neural progenitors in the contralateral cortex. By de-repressing EIF2C3 and CAMTA1, miR-129-5p may be promoting a pro-neuronal and

pro-survival phenotype for the growth of new tissue in the lesioned cortex, and potentially supporting alterations in neural plasticity in the non-lesioned one.

miR-132

miR-132 is known to be a target of CREB (the cAMP response element binding protein), and it is also known to target p250GAP, a GTPase activating protein (Vo, et al., 2005). The expression pattern of miR-132 indicates that EGF/EPO therapy induces increased expression of miR-132 in the lesioned cortex following a stroke, in contrast to the decreased levels of miR-132 found within the lesions of untreated animals. This drug-dependent response makes miR-132 an ideal candidate for further study as a downstream effect of EGF/EPO therapy.

CREB is considered a prototypical transcription factor in the brain, capable of responding to a variety of hormonal, electrical, and growth factor based signalling (Lonze & Ginty, 2002). In a recent study, Vo et al identified a variety of CREB-regulated targets implicated in neuronal plasticity, miR-132 among them (Vo, et al., 2005). They selected miR-132 for further study due to its high abundance in neurons and its responsiveness to neurotrophic signalling. These authors discovered that overexpression of miR-132 induced sprouting of neuronal processes, and that inactivation of the miRNA reversed this process. The study demonstrated that miR-132 targets p250GAP for suppression, and consequently, that it increases neural plasticity, by inhibiting p250GAP / Rac / cdc42 regulated control of neurite outgrowth (Nakazawa, et al., 2003; Vo, et al., 2005).

If EGF/EPO therapy induces increased plasticity in the peri-infarct region, this immediately raises questions about the role of p250GAP in the post-ischemic brain.

It is known that enhanced signalling in the peri-infarct region triggers excitotoxicity and the ischemic cascade. In this case, it is possible that decreased miR-132 signalling and subsequent enhancement of p250GAP behaviour acts as a neuroprotective buffer in the normal brain, by decreasing neural plasticity during phases when the peri-infarct region is vulnerable to excitotoxic damage (Di Filippo, et al., 2008). Though beyond the scope of this work, an in-depth analysis of the temporal behaviour of miR-132 and p250GAP would reveal the nature of this regulation, and answer several key questions. Does EGF/EPO enhance the neuroprotective benefit of p250GAP signalling at more acute timepoints? When does the enhanced plasticity brought about by p250GAP suppression come into effect? Does this effect play a role in the function of the freshly-regenerated tissue (i.e., enhancing the activity of surrounding regions instead of directly replacing lost functions)?

miR-127

miR-127 is down-regulated in both cortices as consequence of EGF/EPO therapy, regardless of the status of tissue injury. miR-127 is transcribed from the anti-sense strand of the *Rtl1/Peg11* gene, which encodes a polycistronic cluster of five pre-miRs (Davis, et al., 2005). This gene is preferentially expressed in embryo, placenta, brain and skeletal muscle. It appears to be highly regulated during embryonic imprinting, due to its ability to *trans* regulate (i.e. the paternal anti-Peg11 miRNA cluster is capable of targeting and down-regulating the products of the maternal *Rtl1/Peg11* gene). Unfortunately, data on the activities of the *Rtl/Peg11* locus outside of imprinting do not exist. Further exploration of its behaviour may

provide answers about the extent to which EGF/EPO mediated neurogenesis mimics neural development.

miR-127 also targets BCL6, which is best known as a proto-oncogene capable of down-regulating the p53 tumour suppressor (Saito, et al., 2006). A reduction of the levels of miR-127, in this case, could contribute to proliferative signalling in the post-EGF/EPO brain.

miR-107

miR-107 only shows a small alteration in expression levels, which only reach significance in the non-lesioned cortex of EGF/EPO treated animals. This finding is evidence that EGF/EPO drug therapy induces specific epigenetic effects in uninjured tissue, in addition to any induction effect it has on injured and proliferating tissue. It also suggests that the phenotypic effects of this therapy extend beyond the infarction itself. It has been confirmed that miR-107 targets two mRNA transcripts – the NF1-A transcription factor (Garzon, et al., 2007), and BACE1 (beta-secretase 1) (Wang, et al., 2008). NF1-A is involved in a complex regulatory feedback loop involving miR-223, miR-107, and C/EBPalpha, which directs granulopoiesis (Fazi, et al., 2005) in both a developmental and a carcinogenic context. The role it plays in the brain is unclear to date. BACE1, on the other hand, has been implicated in Alzheimer's-related neurodegeneration (Wang, et al., 2008), with levels of miR-107 increasing over the course of the disease progression and causing a decrease in levels of BACE1. The very slight decrease of miR-107 levels in the non-lesioned treated cortex should therefore be examined very closely during any future clinical trials of this drug therapy. Any measureable or detectable alteration or change in the level of this

protein could be indicative of side effects related to AD-related dementia. Indeed, this result supports the use of miRNA microarrays as potential predictors of pharmacological side effects before any drug therapies are moved from animal to human tests.

miR-124a

miR-124a is a constitutively expressed neural microRNA, generating some of the highest levels of raw fluorescent signal of any of the miRs revealed by the microarray analysis during this study. These results are consistent with the literature, with studies indicating that high levels of miR-124a promote the expression of a neuronal mRNA phenotype (Conaco, Otto, Han, & Mandel, 2006). This promotion of a neuronal phenotype involves the repression of, as the authors put it, ‘hundreds of nonneuronal transcripts’, to the extent that experimental induction of miR-124a causes HeLa cells to produce a strong neuronal profile. The elevation of miR-124a seen in untreated peri-infarct tissue could be a result of increased pro-neuronal signalling that attempts to guide endogenous stem cells (as reported in (Jin, et al., 2001)) toward a neuronal fate. It could also be a potential means of maintaining the brain’s nonproliferative state. The alteration in miR-124a levels is not apparent in treated animals, which suggests that the neuronal differentiation forced by erythropoietin is independent of miR-124a-induced proneuronal signalling. This may indicate that the role of miR-124a is more strongly related to maintenance of the neuronal phenotype *in vivo*, though this requires further study.

miR-124a, in addition to its strong proneuronal effect, has a number of confirmed mRNA targets, which further illustrates its role in both neuronal

differentiation and neural plasticity in general (Karginov, et al., 2007). One such target, VAMP3 (vesicle-associated membrane protein 3), is a component of the synaptic vesicle SNARE complex which specifically localises to endocytic compartments (Hu, Rickman, Carroll, & Davletov, 2004). An increase in miR-124a could be linked to a decrease in synaptic vesicle plasticity, as a potential cause or effect of the decreased efficacy of neural signalling in the damaged tissue. Reductions in synaptic neurotransmitter re-uptake capability could contribute to a plasticity decrease in this manner.

miR-9

miR-9 appears to be specifically induced in the non-lesioned cortex of EGF/EPO treated animals. It shows significant increases in this tissue when compared both to untreated tissue and the lesioned area in the same animals. This suggests that a tissue-specific induction of miR-9 occurs in uninjured cortex.

miR-9 is known to target two mRNAs – granuphilin/SLP4 (Plaisance, et al., 2006) and BACE1 (Hebert, et al., 2008). The behaviour of granuphilin in the brain is only beginning to be understood, but it appears to play a role in the management of vesicle storage and exocytosis (Fukuda, 2003; Plaisance, et al., 2006). miR-9 mediated down-regulation of granuphilin in the non-lesioned cortex could, in this case, be influencing synaptic plasticity as a part of the global induction of plasticity and rearrangement of neural function that follows a stroke.

Similar regulation of BACE1 in this context is especially interesting, given the possible modulation of BACE1 in the nonlesioned EGF/EPO treated hemisphere, as already identified in this study (see section 8.1.2i). An increase in miR-109 levels

would tend to decrease the levels of BACE1, which lends more support to the idea that it has potential to induce AD-like symptoms in the recovering patient. Since the behaviour of BACE1 is modified by two miRNAs of interest, it would be an excellent candidate for further study, particularly given its role in neurodegenerative processes.

8.2 – Alterations in DNA Methylation Machinery

Alterations in DNA methylation are directly linked with changes in neural phenotype, including synaptic regulation (Monteggia & Kavalali, 2009) and memory formation (Miller, et al., 2008). During this study, both of the *de novo* methyltransferases, DNMT3a and DNMT3b, were examined to highlight any potential changes in methylation status. Furthermore, MeCP2, a methyl CpG binding protein highly enriched in brain tissue, was examined to determine its role in methylation-dependent alteration of gene expression, as well as possible roles in neural plasticity.

8.2.1 – MeCP2

MeCP2 plays a variety of roles in the brain that made it an important target to examine in this study. The functional product of MeCP2 is a DNA binding protein which associates with methylated cytosines in the genome, and is thought to induce transcriptional repression of methylated targets. Recent evidence shows that the role of MeCP2 may in fact be more complex, with studies indicating that it may act as either an activator or repressor in the hypothalamus (Chahrour, et al., 2008), and that it closely associates with several highly active promoter regions (Yasui, et al., 2007).

MeCP2 is highly expressed in the mammalian central nervous system. It is preferentially expressed in neurons as compared to glia (Monteggia & Kavalali, 2009), and has a neuronal morphology-dependent pattern of expression, showing a relative increase in protein level from immature to post-mitotic neurons (Kishi & Macklis, 2004; Matarazzo, et al., 2004). The work of Kishi et al (Kishi & Macklis, 2004) showed that MeCP2 plays a major role in determining a neuronal fate, both in development and in the differentiation of neural precursor stem cells. This evidence, in concert with the decrease in MeCP2 expression in both treated and untreated animals' peri-infarct regions that was detected during this study, suggests that both the peri-infarct region and the regenerated cortical tissue are comprised to some degree of neural progenitors and stem cells. This possibility is also consistent with the original finding by Kolb et al (Kolb, et al., 2007), in which the regenerated tissue was not organised into fully mature cortical tissue. It is possible that the decreased levels of MeCP2 noted above are a result of increased numbers of neural precursors and partially-differentiated stem cells in the peri-infarct region or the regenerated tissue.

Moreover, the role of MeCP2 as a regulator of transcription clearly generates a significant phenotypic impact on the recovering brain. Mutations and deletions in MeCP2 are thought to be the causal agent of Rett syndrome, one of the leading causes of mental retardation in females (R. Z. Chen, Akbarian, Tudor, & Jaenisch, 2001). This disorder, in which patients exhibit stereotypical symptoms of lower brain weight and reduced neuronal cell size but no obvious structural defects or signs of neurodegeneration, appears to be tied to both the developmental effects of MeCP2,

and its role in maintaining a mature phenotype in neurons. A depletion of MeCP2 in adult neurons is sufficient to generate a Rett-like phenotype (R. Z. Chen, et al., 2001), and its restoration reverses the phenotype (Luikenhuis, Giacometti, Beard, & Jaenisch, 2004). A number of recent studies have also implicated MeCP2 in synaptogenesis (Cohen, et al., 2003), and, more generally, as a key regulator of activity-dependent neuronal gene expression (Luikenhuis, et al., 2004).

What does this mean in the brains of animals recovering from a stroke? From the above findings, it appears that depletion of MeCP2 in post-mitotic neurons induces a functional deficit in neuronal activity and plasticity. This functional deficit could partially explain the loss of behavioural capacity resulting from stroke in untreated animals, though detailed behavioural analysis would be required to determine the functional capacity of peri-infarct tissue four months post-lesion. Interestingly, the recent finding by Li et al (Y. Li, Lu, Keogh, Yu, & Wei, 2007) suggests that the similar levels of MeCP2 depletion result from two different causes. Given that erythropoietin is independently capable of stimulating angiogenesis and neuroprotection in the ischemic region, it is possible that the lower levels of MeCP2 observed in treated animals are more closely tied to the immature anatomical phenotype of the regenerated tissue plug, but in untreated animals, more closely related to the aftermath of the ischemic damage.

8.2.2 – DNMT3a and DNMT3b

The only significant change to DNMT3a levels during this study occurred in the peri-infarct region following EGF/EPO therapy, indicating that this phenotypic

change was linked with neurogenesis and neuronal differentiation. Unlike DNMT1 mentioned above, DNMT3a is a *de novo* methyltransferase – it is capable of methylating unmethylated CpG dinucleotides. Activity of *de novo* methyltransferases is responsible for the establishment of new methylation patterns, which are capable of changing the phenotypic state of a given tissue (Hermann, Gowher, & Jeltsch, 2004).

The expression pattern of DNMT3a and DNMT3b in EGF/EPO treated animals is, at first glance, somewhat contradictory, with a simultaneous increase and decrease, respectively, in expression level. However, a recent finding by Watanabe et al (Watanabe, Uchiyama, & Hanaoka, 2006) suggests that this finding is consistent with previous results about the behaviour of methyltransferases in a neural system. These authors reported a temporally-mediated transition between levels of the two enzymes in neural progenitor cells, with undeveloped cells showing a higher level of DNMT3b activity which is gradually replaced by DNMT3a as the cells developed. This transition is highly consistent with the results of this study as well as the original work by Kolb et al (Kolb, et al., 2007), where the over-expression of DNMT3a and under-expression of DNMT3b in the repaired peri-infarct tissue could be guiding the cells to a more fully differentiated phenotype.

Though a role for *de novo* methylation in the brain has yet to be firmly established, one possibility presented by Hermann et al (Hermann, et al., 2004) is the potential for *de novo* methylation to contribute to formation and preservation of a ‘snap shot’ of a gene’s current methylation state by modification of an equilibrium state with demethylation mechanisms. Transient repression may be able to ‘mark’ certain regions of the genome for later application of more persistent methylation

(Thomassin, Flavin, Espinas, & Grange, 2001). This would involve the possible creation of an epigenetic memory trace. Given the very obvious phenotypic changes induced by EGF/EPO, it is possible that alterations of methyltransferase activity (especially the presumed increase in methylation caused by over-expression of DNMT3a) could be directly involved in either activating neural stem cells, directing them towards neuronal fates due to EPO-mediated signalling cascades, or causing these new neurons to 'lock in' their gene expression patterns.

In light of the ties between DNA methylation machinery, *de novo* methyltransferase activity, and methyl-CpG binding activity, it appears that the neurogenesis initiated by EGF/EPO drug therapy is modifying the epigenome of both the neural stem cells originating in the sub-ventricular zone (SVZ) and the new neurons which migrate into the former lesion site. It is possible, at the four-month point, that the new tissue, though functional, is held either in a state of neural immaturity, or is still approaching a fully post-mitotic state. The former is supported by the lack of cortical organisation found in the tissue plug. The latter is supported by the finding of decreased levels of MeCP2 (indicative of immature neurons) and the shift from a DNMT3b-dominated *de novo* methylation phenotype to a DNMT3a one. To date, the genetic and epigenetic profiles of EGF/EPO directed neurogenic tissue have not been fully established, nor have their temporal properties been elucidated. Future studies may reveal the exact nature of the alterations of the methyl-ome, leading to a greater understanding of the process of growth-factor induced neurogenesis at an epigenetic level.

8.3 – Confirming a putative miRNA target – miR-152 and Noggin

There are no regulatory targets that have been confirmed for miR-152 to date. The trends observed for this microRNA – a relative decrease in the peri-infarct region of untreated animals and a relative increase in the peri-infarct region of treated animals, were sufficient reason to select miR-152 as a prospective miRNA for some additional study. In order to determine the biological activity of miR-152, in silico analysis was carried out using the Sanger miRBase target prediction algorithm (Griffiths-Jones, 2006). Genes of interest were selected from the list of putative targets. A putative binding site of miR-152 was found on the 3'UTR of noggin, an extracellular antagonist of bone morphogenic proteins (Valenzuela, et al., 1995). To confirm the activity of mir-152 on the noggin transcript, the 3' untranslated region (3'UTR) of noggin was cloned, and ligated to the 3' end of a luciferase reporter gene.

The observed decrease of the luciferase/nogginUTR reporter construct shows that miR-152 targets the UTR of noggin in cells, and subsequently down-regulates its activity. Initially discovered and characterised in *Xenopus*, noggin has been found to be expressed and active in the adult mammalian brain (Valenzuela, et al., 1995), where it has been implicated in adult neurogenesis (Colak, et al., 2008), as well as in memory formation and regulation in the hippocampus (Fan, Cai, Yang, Xu, & Zhang, 2003). Noggin acts as a specific antagonist of bone morphogenic proteins (BMPs) by interfering with their ability to bind to the bone morphogenic protein receptor (BMPR). These BMPs are extracellular ligands, which transduce a signal through the BMPR to phosphorylate SMAD1, 5 and 8 (Colak, et al., 2008), which then influence

transcription through a cAMP mediated pathway. It has been shown that adult neurogenesis and differentiation require the activity of bone morphogenic proteins. Thus, it is possible that a portion of EGF/EPO induced neurogenic activity could be caused by the translational suppression of noggin due to elevated levels of miR-152 in the months following a stroke.

It is clear from the results that a hypoxic lesion induces a long-term, if not permanent, reduction in cellular BMP4 levels. Previous studies have shown that hypoxia results in a short-term increase in the circulating level of bone morphogenic proteins (C. Zhang, et al., 2006). This finding, coupled with the other results of this study, suggest that the immediate response of the peri-infarct region is an overproduction of BMP, which is then reduced as the cell death and tissue damage resulting from the initial hypoxic insult reach an equilibrium point. After the initial hypoxic insult, BMP levels then decrease. It should be noted, although detected levels of BMP4 are still decreased in the EGF/EPO peri-infarct region, that noggin mediated suppression does not reduce levels of BMP. Instead, it reduces BMP activity. Noggin-mediated effects on BMP activity, as a result, would be undetectable by Western Blot testing (analysis?).

A model can be proposed where miR-152 based suppression of Noggin functions to counteract the natural decrease in BMP activity following a stroke. Undoubtedly, the greater phenotypic change induced by EGF/EPO therapy is far more complex, since EGF/EPO therapy also appears to induce a dramatic reduction in BMP4 levels at 4 months. By suppressing noggin, mir-152 could act as a de-repressor of BMP activity, forcing a greater percentage of neural precursors towards a

neuronal rather than oligodendritic fate. Alternately, increased BMP2/BMP4 activity in hypoxic cells could be inducing astrocyte differentiation in SVZ progenitor cells (Xin, Li, Chen, & Chopp, 2006). This EGF/EPO induced modulation of mir-152 could alter the cellular environment of the peri-infarct region, which directs the differentiation of neural precursor cells to endpoints that promote recovery; either neuronal endpoints, which may be integrating into pre-existing circuitry, or astrocytes, which play a key neuroprotective role in the immediate response to brain trauma (Bazan, Marcheselli, & Cole-Edwards, 2005). This possibility raises what could be a key point of investigation in future studies, since astroglial activity is only neuroprotective shortly following initial neural trauma. In the long-term, astrocytes form glial scarring, which inhibits CNS regeneration, and is a possible explanation for the reduction of BMP4 levels at this distal time point. A detailed study of astrocytes in the days and weeks immediately following an infarction, as well as their modulation by EGF and EPO, should generate a far clearer image regarding their role in stroke recovery.

9 – Conclusions, General Trends, Future Outlook

This study admittedly only scratches the surface regarding the role of miRNA, and, more broadly, epigenetic regulation in the post-ischemic brain. It is clear from the findings detailed above that the analysis of epigenetic regulation in the brain is an incredibly complicated field, given both the importance of exquisitely timed regulation of gene expression, and the self-regulating capability of miRNA. Despite this complexity, this study reveals a very important and diverse array of changes in epigenetic regulation following a stroke, indicating a great deal of potential for future development of epigenetic mechanisms to pharmacologically influence the brain. Due to the ability of miRNA precursors to bypass the blood-brain barrier, their potential as a delivery method for neural gene therapy cannot be ignored.

miRNA regulation and alterations of DNA methylation are clearly only single components of a complicated, dynamic process, considering the complexity and novelty of EGF/EPO mediated induction of neurogenesis. Four months after the stroke and subsequent therapy, miRNAs are acting to promote both survival mechanisms (such as miR-214, miR-129-5p, miR-127, miR-21, and miR-152) and cell death mechanisms (miR-199b, miR145, miR-138, and miR-107). This could represent an analogue of the ‘pruning’ mechanisms undergone by the infant brain during the developmental cycle, wherein a developing brain initially grows an overabundance of neurons, and then induces the death of **over half** of them, which constitutes a necessary developmental step. Additionally, this study reveals what

may very well be a note of caution both in the pursuit of EGF/EPO based drug therapies and pharmacological intervention in the brain in general. Specific to this therapy, the regulatory impact on potential neurodegenerative mechanisms will necessitate careful monitoring of patients undergoing such a drug therapy, as well as careful consideration of the risk/benefit ratio. More generally, by revealing a connection between drug therapy, epigenetic regulation and potential disease processes, this study confirms the validity of using epigenetic methods both to analyse the functional mechanisms of experimental drug therapies and direct future research into what the potential side effects of gene therapy may be.

10 – Works Cited

- Adams, H. P., Jr. (2009). Secondary prevention of atherothrombotic events after ischemic stroke. *Mayo Clin Proc*, 84(1), 43-51.
- Ame, J. C., Spenlehauer, C., & de Murcia, G. (2004). The PARP superfamily. *Bioessays*, 26(8), 882-893.
- Ameres, S. L., Martinez, J., & Schroeder, R. (2007). Molecular basis for target RNA recognition and cleavage by human RISC. *Cell*, 130(1), 101-112.
- Aravin, A. A., Lagos-Quintana, M., Yalcin, A., Zavolan, M., Marks, D., Snyder, B., et al. (2003). The small RNA profile during *Drosophila melanogaster* development. *Dev Cell*, 5(2), 337-350.
- Arcasoy, M. O. (2008). The non-haematopoietic biological effects of erythropoietin. *Br J Haematol*, 141(1), 14-31.
- Asahi, M., Wang, X., Mori, T., Sumii, T., Jung, J. C., Moskowitz, M. A., et al. (2001). Effects of matrix metalloproteinase-9 gene knock-out on the proteolysis of blood-brain barrier and white matter components after cerebral ischemia. *J Neurosci*, 21(19), 7724-7732.
- Asangani, I. A., Rasheed, S. A., Nikolova, D. A., Leupold, J. H., Colburn, N. H., Post, S., et al. (2008). MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pcdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene*, 27(15), 2128-2136.
- Auer, R. N., & Sutherland, G. R. (2005). Primary intracerebral hemorrhage: pathophysiology. *Can J Neurol Sci*, 32 Suppl 2, S3-12.
- Baek, D., Villen, J., Shin, C., Camargo, F. D., Gygi, S. P., & Bartel, D. P. (2008). The impact of microRNAs on protein output. *Nature*, 455(7209), 64-71.
- Barone, F. C., Schmidt, D. B., Hillegass, L. M., Price, W. J., White, R. F., Feuerstein, G. Z., et al. (1992). Reperfusion increases neutrophils and leukotriene B4 receptor binding in rat focal ischemia. *Stroke*, 23(9), 1337-1347; discussion 1347-1338.
- Bartel, B., & Bartel, D. P. (2003). MicroRNAs: at the root of plant development? *Plant Physiol*, 132(2), 709-717.
- Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, 116(2), 281-297.
- Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell*, 136(2), 215-233.
- Baserga, R. (2000). The contradictions of the insulin-like growth factor 1 receptor. *Oncogene*, 19(49), 5574-5581.
- Baylin, S. B. (2005). DNA methylation and gene silencing in cancer. *Nat Clin Pract Oncol*, 2 Suppl 1, S4-11.
- Baylin, S. B., & Ohm, J. E. (2006). Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? *Nat Rev Cancer*, 6(2), 107-116.
- Bazan, N. G., Marcheselli, V. L., & Cole-Edwards, K. (2005). Brain response to injury and neurodegeneration: endogenous neuroprotective signaling. *Ann NY Acad Sci*, 1053, 137-147.
- Berezikov, E., Guryev, V., van de Belt, J., Wienholds, E., Plasterk, R. H., & Cuppen, E. (2005). Phylogenetic shadowing and computational identification of human microRNA genes. *Cell*, 120(1), 21-24.
- Bernstein, E., Caudy, A. A., Hammond, S. M., & Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*, 409(6818), 363-366.
- Bohnsack, M. T., Czaplinski, K., & Gorlich, D. (2004). Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA*, 10(2), 185-191.
- Bolstad, B. M., Irizarry, R. A., Astrand, M., & Speed, T. P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics*, 19(2), 185-193.
- Brennecke, J., Stark, A., Russell, R. B., & Cohen, S. M. (2005). Principles of microRNA-target recognition. *PLoS Biol*, 3(3), e85.
- Broughton, B. R., Reutens, D. C., & Sobey, C. G. (2009). Apoptotic mechanisms after cerebral ischemia. *Stroke*, 40(5), e331-339.
- Buchan, A. M., Xue, D., & Slivka, A. (1992). A new model of temporary focal neocortical ischemia in the rat. *Stroke*, 23(2), 273-279.

- Burkle, A. (2001). Physiology and pathophysiology of poly(ADP-ribosyl)ation. *Bioessays*, 23(9), 795-806.
- Cao, D., Zhang, Q., Wu, L. S., Salaria, S. N., Winter, J. W., Hruban, R. H., et al. (2007). Prognostic significance of maspin in pancreatic ductal adenocarcinoma: tissue microarray analysis of 223 surgically resected cases. *Mod Pathol*, 20(5), 570-578.
- Carmichael, S. T. (2006). Cellular and molecular mechanisms of neural repair after stroke: making waves. *Ann Neurol*, 59(5), 735-742.
- Casalbore, P., Budoni, M., Ricci-Vitiani, L., Cenciarelli, C., Petrucci, G., Milazzo, L., et al. (2009). Tumorigenic potential of olfactory bulb-derived human adult neural stem cells associates with activation of TERT and NOTCH1. *PLoS One*, 4(2), e4434.
- CAST: randomised placebo-controlled trial of early aspirin use in 20,000 patients with acute ischaemic stroke. CAST (Chinese Acute Stroke Trial) Collaborative Group (1997). *Lancet*, 349(9066), 1641-1649.
- Cavallaro, S., Schreurs, B. G., Zhao, W., D'Agata, V., & Alkon, D. L. (2001). Gene expression profiles during long-term memory consolidation. *Eur J Neurosci*, 13(9), 1809-1815.
- Cesar, J. M., Garcia-Avello, A., Vecino, A. M., Sastre, J. L., & Alvarez-Cermeno, J. C. (1995). Increased levels of plasma von Willebrand factor in migraine crisis. *Acta Neurol Scand*, 91(5), 412-413.
- Chahrour, M., Jung, S. Y., Shaw, C., Zhou, X., Wong, S. T., Qin, J., et al. (2008). MeCP2, a key contributor to neurological disease, activates and represses transcription. *Science*, 320(5880), 1224-1229.
- Chan, P. H. (1996). Role of oxidants in ischemic brain damage. *Stroke*, 27(6), 1124-1129.
- Chen, C., Ridzon, D. A., Broomer, A. J., Zhou, Z., Lee, D. H., Nguyen, J. T., et al. (2005). Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res*, 33(20), e179.
- Chen, C. Z., Li, L., Lodish, H. F., & Bartel, D. P. (2004). MicroRNAs modulate hematopoietic lineage differentiation. *Science*, 303(5654), 83-86.
- Chen, R. Z., Akbarian, S., Tudor, M., & Jaenisch, R. (2001). Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. *Nat Genet*, 27(3), 327-331.
- Cheung, P., & Lau, P. (2005). Epigenetic regulation by histone methylation and histone variants. *Mol Endocrinol*, 19(3), 563-573.
- Cho, S. G., & Choi, E. J. (2002). Apoptotic signaling pathways: caspases and stress-activated protein kinases. *J Biochem Mol Biol*, 35(1), 24-27.
- Chu, E. C., & Tarnawski, A. S. (2004). PTEN regulatory functions in tumor suppression and cell biology. *Med Sci Monit*, 10(10), RA235-241.
- Chuang, Y. M., Liu, C. Y., Pan, P. J., & Lin, C. P. (2007). Anterior cerebral artery A1 segment hypoplasia may contribute to A1 hypoplasia syndrome. *Eur Neurol*, 57(4), 208-211.
- Cohen, D. R., Matarazzo, V., Palmer, A. M., Tu, Y., Jeon, O. H., Pevsner, J., et al. (2003). Expression of MeCP2 in olfactory receptor neurons is developmentally regulated and occurs before synaptogenesis. *Mol Cell Neurosci*, 22(4), 417-429.
- Colak, D., Mori, T., Brill, M. S., Pfeifer, A., Falk, S., Deng, C., et al. (2008). Adult neurogenesis requires Smad4-mediated bone morphogenic protein signaling in stem cells. *J Neurosci*, 28(2), 434-446.
- Conaco, C., Otto, S., Han, J. J., & Mandel, G. (2006). Reciprocal actions of REST and a microRNA promote neuronal identity. *Proc Natl Acad Sci U S A*, 103(7), 2422-2427.
- Cowen, T., & Gavazzi, I. (1998). Plasticity in adult and ageing sympathetic neurons. *Prog Neurobiol*, 54(3), 249-288.
- Cronin, C. A., Weisman, C. J., & Llinas, R. H. (2008). Stroke treatment: beyond the three-hour window and in the pregnant patient. *Ann N Y Acad Sci*, 1142, 159-178.
- Davis, E., Caiment, F., Tordoir, X., Cavaille, J., Ferguson-Smith, A., Cockett, N., et al. (2005). RNAi-mediated allelic trans-interaction at the imprinted Rtl1/Peg11 locus. *Curr Biol*, 15(8), 743-749.
- Di Filippo, M., Tozzi, A., Costa, C., Belcastro, V., Tantucci, M., Picconi, B., et al. (2008). Plasticity and repair in the post-ischemic brain. *Neuropharmacology*, 55(3), 353-362.
- Doench, J. G., & Sharp, P. A. (2004). Specificity of microRNA target selection in translational repression. *Genes Dev*, 18(5), 504-511.

- Donnan, G. A., Fisher, M., Macleod, M., & Davis, S. M. (2008). Stroke. *Lancet*, 371(9624), 1612-1623.
- Durukan, A., & Tatlisumak, T. (2007). Acute ischemic stroke: overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia. *Pharmacol Biochem Behav*, 87(1), 179-197.
- Egger, G., Liang, G., Aparicio, A., & Jones, P. A. (2004). Epigenetics in human disease and prospects for epigenetic therapy. *Nature*, 429(6990), 457-463.
- Eisen, M. B., Spellman, P. T., Brown, P. O., & Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A*, 95(25), 14863-14868.
- Elbashir, S. M., Lendeckel, W., & Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev*, 15(2), 188-200.
- Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol*, 35(4), 495-516.
- Endersby, R., & Baker, S. J. (2008). PTEN signaling in brain: neuropathology and tumorigenesis. *Oncogene*, 27(41), 5416-5430.
- Endres, M., Wang, Z. Q., Namura, S., Waeber, C., & Moskowitz, M. A. (1997). Ischemic brain injury is mediated by the activation of poly(ADP-ribose)polymerase. *J Cereb Blood Flow Metab*, 17(11), 1143-1151.
- Esquela-Kerscher, A., & Slack, F. J. (2006). Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer*, 6(4), 259-269.
- Esteller, M. (2007). Epigenetic gene silencing in cancer: the DNA hypermethylome. *Hum Mol Genet*, 16 Spec No 1, R50-59.
- Fabbri, M., Ivan, M., Cimmino, A., Negrini, M., & Calin, G. A. (2007). Regulatory mechanisms of microRNAs involvement in cancer. *Expert Opin Biol Ther*, 7(7), 1009-1019.
- Fan, X. T., Cai, W. Q., Yang, Z., Xu, H. W., & Zhang, J. H. (2003). Effect of antisense oligonucleotide of noggin on spatial learning and memory of rats. *Acta Pharmacol Sin*, 24(5), 394-397.
- Fazi, F., Rosa, A., Fatica, A., Gelmetti, V., De Marchis, M. L., Nervi, C., et al. (2005). A microcircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell*, 123(5), 819-831.
- Feigin, V. L. (2005). Stroke epidemiology in the developing world. *Lancet*, 365(9478), 2160-2161.
- Frankel, L. B., Christoffersen, N. R., Jacobsen, A., Lindow, M., Krogh, A., & Lund, A. H. (2008). Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. *J Biol Chem*, 283(2), 1026-1033.
- Friberg, L., Olesen, J., Lassen, N. A., Olsen, T. S., & Karle, A. (1994). Cerebral oxygen extraction, oxygen consumption, and regional cerebral blood flow during the aura phase of migraine. *Stroke*, 25(5), 974-979.
- Friedman, R. C., Farh, K. K., Burge, C. B., & Bartel, D. P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*, 19(1), 92-105.
- Fukuda, M. (2003). Slp4-a/granuphilin-a inhibits dense-core vesicle exocytosis through interaction with the GDP-bound form of Rab27A in PC12 cells. *J Biol Chem*, 278(17), 15390-15396.
- Fuxe, K., Bjelke, B., Andbjer, B., Grahn, H., Rimondini, R., & Agnati, L. F. (1997). Endothelin-1 induced lesions of the frontoparietal cortex of the rat. A possible model of focal cortical ischemia. *Neuroreport*, 8(11), 2623-2629.
- Garzon, R., Pichiorri, F., Palumbo, T., Visentini, M., Aqeilan, R., Cimmino, A., et al. (2007). MicroRNA gene expression during retinoic acid-induced differentiation of human acute promyelocytic leukemia. *Oncogene*, 26(28), 4148-4157.
- Goll, M. G., & Bestor, T. H. (2005). Eukaryotic cytosine methyltransferases. *Annu Rev Biochem*, 74, 481-514.
- Gonzalez, C. L., & Kolb, B. (2003). A comparison of different models of stroke on behaviour and brain morphology. *Eur J Neurosci*, 18(7), 1950-1962.
- Gonzalez, C. L., Whishaw, I. Q., & Kolb, B. (2003). Complete sparing of spatial learning following posterior and posterior plus anterior cingulate cortex lesions at 10 days of age in the rat. *Neuroscience*, 122(2), 563-571.
- Granger, D. N. (1988). Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury. *Am J Physiol*, 255(6 Pt 2), H1269-1275.
- Griffiths-Jones, S. (2006). miRBase: the microRNA sequence database. *Methods Mol Biol*, 342, 129-138.

- Gross, I., Armant, O., Benosman, S., de Aguilar, J. L., Freund, J. N., Kedinger, M., et al. (2007). Sprouty2 inhibits BDNF-induced signaling and modulates neuronal differentiation and survival. *Cell Death Differ*, 14(10), 1802-1812.
- Grysiewicz, R. A., Thomas, K., & Pandey, D. K. (2008). Epidemiology of ischemic and hemorrhagic stroke: incidence, prevalence, mortality, and risk factors. *Neurol Clin*, 26(4), 871-895, vii.
- Guil, S., & Esteller, M. (2009). DNA methylomes, histone codes and miRNAs: tying it all together. *Int J Biochem Cell Biol*, 41(1), 87-95.
- Haley, B., & Zamore, P. D. (2004). Kinetic analysis of the RNAi enzyme complex. *Nat Struct Mol Biol*, 11(7), 599-606.
- Hebert, S. S., Horre, K., Nicolai, L., Papadopoulou, A. S., Mandemakers, W., Silahatoglu, A. N., et al. (2008). Loss of microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/beta-secretase expression. *Proc Natl Acad Sci U S A*, 105(17), 6415-6420.
- Hellstrom, H. O., Wanhainen, A., Valtysson, J., Persson, L., & Hillered, L. (1994). Effect of tirilazad mesylate given after permanent middle cerebral artery occlusion in rat. *Acta Neurochir (Wien)*, 129(3-4), 188-192.
- Hermann, A., Gowher, H., & Jeltsch, A. (2004). Biochemistry and biology of mammalian DNA methyltransferases. *Cell Mol Life Sci*, 61(19-20), 2571-2587.
- Higashiyama, S., Iwabuki, H., Morimoto, C., Hieda, M., Inoue, H., & Matsushita, N. (2008). Membrane-anchored growth factors, the epidermal growth factor family: beyond receptor ligands. *Cancer Sci*, 99(2), 214-220.
- Hinkle, J. L., & Bowman, L. (2003). Neuroprotection for ischemic stroke. *J Neurosci Nurs*, 35(2), 114-118.
- Hossmann, K. A. (2006). Pathophysiology and therapy of experimental stroke. *Cell Mol Neurobiol*, 26(7-8), 1057-1083.
- Hu, K., Rickman, C., Carroll, J., & Davletov, B. (2004). A common mechanism for the regulation of vesicular SNAREs on phospholipid membranes. *Biochem J*, 377(Pt 3), 781-785.
- Hutvagner, G., & Zamore, P. D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. *Science*, 297(5589), 2056-2060.
- Jaenisch, R., & Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet*, 33 Suppl, 245-254.
- Jenuwein, T., & Allis, C. D. (2001). Translating the histone code. *Science*, 293(5532), 1074-1080.
- Jin, K., Minami, M., Lan, J. Q., Mao, X. O., Batteur, S., Simon, R. P., et al. (2001). Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. *Proc Natl Acad Sci U S A*, 98(8), 4710-4715.
- Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., et al. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet*, 19(2), 187-191.
- Kaneko, D., Nakamura, N., & Ogawa, T. (1985). Cerebral infarction in rats using homologous blood emboli: development of a new experimental model. *Stroke*, 16(1), 76-84.
- Karginov, F. V., Conaco, C., Xuan, Z., Schmidt, B. H., Parker, J. S., Mandel, G., et al. (2007). A biochemical approach to identifying microRNA targets. *Proc Natl Acad Sci U S A*, 104(49), 19291-19296.
- Katsarava, Z., Rabe, K., & Diener, H. C. (2008). From migraine to stroke. *Intern Emerg Med*, 3 Suppl 1, S9-16.
- Ketting, R. F., Fischer, S. E., Bernstein, E., Sijen, T., Hannon, G. J., & Plasterk, R. H. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes Dev*, 15(20), 2654-2659.
- Kim, S., Lee, U. J., Kim, M. N., Lee, E. J., Kim, J. Y., Lee, M. Y., et al. (2008). MicroRNA miR-199a* regulates the MET proto-oncogene and the downstream extracellular signal-regulated kinase 2 (ERK2). *J Biol Chem*, 283(26), 18158-18166.
- Kiriakidou, M., Nelson, P. T., Kouranov, A., Fitziev, P., Bouyioukos, C., Mourelatos, Z., et al. (2004). A combined computational-experimental approach predicts human microRNA targets. *Genes Dev*, 18(10), 1165-1178.

- Kishi, N., & Macklis, J. D. (2004). MECP2 is progressively expressed in post-migratory neurons and is involved in neuronal maturation rather than cell fate decisions. *Mol Cell Neurosci*, *27*(3), 306-321.
- Klose, R. J., & Bird, A. P. (2006). Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci*, *31*(2), 89-97.
- Kokaia, Z., Thored, P., Arvidsson, A., & Lindvall, O. (2006). Regulation of stroke-induced neurogenesis in adult brain--recent scientific progress. *Cereb Cortex*, *16 Suppl 1*, i162-167.
- Kolb, B., Morshead, C., Gonzalez, C., Kim, M., Gregg, C., Shingo, T., et al. (2007). Growth factor-stimulated generation of new cortical tissue and functional recovery after stroke damage to the motor cortex of rats. *J Cereb Blood Flow Metab*, *27*(5), 983-997.
- Kozłowska, H., Jablonka, J., Janowski, M., Jurga, M., Kossut, M., & Domanska-Janik, K. (2007). Transplantation of a novel human cord blood-derived neural-like stem cell line in a rat model of cortical infarct. *Stem Cells Dev*, *16*(3), 481-488.
- Lagos-Quintana, M., Rauhut, R., Lendeckel, W., & Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. *Science*, *294*(5543), 853-858.
- Lagos-Quintana, M., Rauhut, R., Meyer, J., Borkhardt, A., & Tuschl, T. (2003). New microRNAs from mouse and human. *RNA*, *9*(2), 175-179.
- Laing, R. J., Jakubowski, J., & Laing, R. W. (1993). Middle cerebral artery occlusion without craniectomy in rats. Which method works best? *Stroke*, *24*(2), 294-297; discussion 297-298.
- Langhorne, P., Williams, B. O., Gilchrist, W., & Howie, K. (1993). Do stroke units save lives? *Lancet*, *342*(8868), 395-398.
- Lapchak, P. A., Chapman, D. F., & Zivin, J. A. (2000). Metalloproteinase inhibition reduces thrombolytic (tissue plasminogen activator)-induced hemorrhage after thromboembolic stroke. *Stroke*, *31*(12), 3034-3040.
- Lau, N. C., Lim, L. P., Weinstein, E. G., & Bartel, D. P. (2001). An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science*, *294*(5543), 858-862.
- Leao, A. (1944). Spreading depression of activity in the cerebral cortex. *J Neurophysiol*, *7*, 359-390.
- Lee, J., Jo, Y. S., Sung, Y. H., Hwang, I. K., Kim, H., Kim, S. Y., et al. (2009). Telomerase Deficiency Affects Normal Brain Functions in Mice. *Neurochem Res*.
- Lee, R. C., Feinbaum, R. L., & Ambros, V. (1993). The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*, *75*(5), 843-854.
- Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., et al. (2003). The nuclear RNase III *Drosha* initiates microRNA processing. *Nature*, *425*(6956), 415-419.
- Lewis, B. P., Burge, C. B., & Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, *120*(1), 15-20.
- Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P., & Burge, C. B. (2003). Prediction of mammalian microRNA targets. *Cell*, *115*(7), 787-798.
- Li, F., Omae, T., & Fisher, M. (1999). Spontaneous hyperthermia and its mechanism in the intraluminal suture middle cerebral artery occlusion model of rats. *Stroke*, *30*(11), 2464-2470; discussion 2470-2461.
- Li, W., & Ruan, K. (2009). MicroRNA detection by microarray. *Anal Bioanal Chem*, *394*(4), 1117-1124.
- Li, Y., Lu, Z., Keogh, C. L., Yu, S. P., & Wei, L. (2007). Erythropoietin-induced neurovascular protection, angiogenesis, and cerebral blood flow restoration after focal ischemia in mice. *J Cereb Blood Flow Metab*, *27*(5), 1043-1054.
- Liao, R., Sun, J., Zhang, L., Lou, G., Chen, M., Zhou, D., et al. (2008). MicroRNAs play a role in the development of human hematopoietic stem cells. *J Cell Biochem*, *104*(3), 805-817.
- Lin, S. L., Miller, J. D., & Ying, S. Y. (2006). Intronic MicroRNA (miRNA). *J Biomed Biotechnol*, *2006*(4), 26818.
- Liu, J., Valencia-Sanchez, M. A., Hannon, G. J., & Parker, R. (2005). MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat Cell Biol*, *7*(7), 719-723.
- Lo, E. H., Dalkara, T., & Moskowitz, M. A. (2003). Mechanisms, challenges and opportunities in stroke. *Nat Rev Neurosci*, *4*(5), 399-415.
- Lockett, J., Yin, S., Li, X., Meng, Y., & Sheng, S. (2006). Tumor suppressive maspin and epithelial homeostasis. *J Cell Biochem*, *97*(4), 651-660.

- Lonze, B. E., & Ginty, D. D. (2002). Function and regulation of CREB family transcription factors in the nervous system. *Neuron*, 35(4), 605-623.
- Love, S. (2003). Apoptosis and brain ischaemia. *Prog Neuropsychopharmacol Biol Psychiatry*, 27(2), 267-282.
- Lu, Z., Liu, M., Stribinskis, V., Klinge, C. M., Ramos, K. S., Colburn, N. H., et al. (2008). MicroRNA-21 promotes cell transformation by targeting the programmed cell death 4 gene. *Oncogene*, 27(31), 4373-4379.
- Luczak, M. W., & Jagodzinski, P. P. (2006). The role of DNA methylation in cancer development. *Folia Histochem Cytobiol*, 44(3), 143-154.
- Luikenhuis, S., Giacometti, E., Beard, C. F., & Jaenisch, R. (2004). Expression of MeCP2 in postmitotic neurons rescues Rett syndrome in mice. *Proc Natl Acad Sci U S A*, 101(16), 6033-6038.
- Mallory, A. C., Reinhart, B. J., Jones-Rhoades, M. W., Tang, G., Zamore, P. D., Barton, M. K., et al. (2004). MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5' region. *EMBO J*, 23(16), 3356-3364.
- Matarazzo, V., Cohen, D., Palmer, A. M., Simpson, P. J., Khokhar, B., Pan, S. J., et al. (2004). The transcriptional repressor Mecp2 regulates terminal neuronal differentiation. *Mol Cell Neurosci*, 27(1), 44-58.
- Meng, F., Henson, R., Lang, M., Wehbe, H., Maheshwari, S., Mendell, J. T., et al. (2006). Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. *Gastroenterology*, 130(7), 2113-2129.
- Meng, F., Henson, R., Wehbe-Janeck, H., Ghoshal, K., Jacob, S. T., & Patel, T. (2007). MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology*, 133(2), 647-658.
- Mergenthaler, P., Dirnagl, U., & Meisel, A. (2004). Pathophysiology of stroke: lessons from animal models. *Metab Brain Dis*, 19(3-4), 151-167.
- Miller, C. A., Campbell, S. L., & Sweatt, J. D. (2008). DNA methylation and histone acetylation work in concert to regulate memory formation and synaptic plasticity. *Neurobiol Learn Mem*, 89(4), 599-603.
- Mitomo, S., Maesawa, C., Ogasawara, S., Iwaya, T., Shibasaki, M., Yashima-Abo, A., et al. (2008). Downregulation of miR-138 is associated with overexpression of human telomerase reverse transcriptase protein in human anaplastic thyroid carcinoma cell lines. *Cancer Science*, 99(2), 280-286.
- Molina, C. A. (2006). Hemorrhagic transformation: a foe that could be a friend? *Int J Stroke*, 1(4), 226-227.
- Monteggia, L. M., & Kavalali, E. T. (2009). Rett syndrome and the impact of MeCP2 associated transcriptional mechanisms on neurotransmission. *Biol Psychiatry*, 65(3), 204-210.
- Moustafa, R. R., & Baron, J. C. (2008). Pathophysiology of ischaemic stroke: insights from imaging, and implications for therapy and drug discovery. *Br J Pharmacol*, 153 Suppl 1, S44-54.
- Muir, K. W., Tyrrell, P., Sattar, N., & Warburton, E. (2007). Inflammation and ischaemic stroke. *Curr Opin Neurol*, 20(3), 334-342.
- Nagayama, T., Simon, R. P., Chen, D. X., Henshall, D. C., Pei, W., Stetler, R. A., et al. (2000). Activation of poly(ADP-Ribose) polymerase in the rat hippocampus may contribute to cellular recovery following sublethal transient global ischemia. *Journal of Neurochemistry*, 74(4), 1636-1645.
- Nakao, M. (2001). Epigenetics: interaction of DNA methylation and chromatin. *Gene*, 278(1-2), 25-31.
- Nakatani, K., Nishioka, J., Itakura, T., Nakanishi, Y., Horinouchi, J., Abe, Y., et al. (2004). Cell cycle-dependent transcriptional regulation of calmodulin-binding transcription activator 1 in neuroblastoma cells. *Int J Oncol*, 24(6), 1407-1412.
- Nakazawa, T., Watabe, A. M., Tezuka, T., Yoshida, Y., Yokoyama, K., Umemori, H., et al. (2003). p250GAP, a novel brain-enriched GTPase-activating protein for Rho family GTPases, is involved in the N-methyl-D-aspartate receptor signaling. *Mol Biol Cell*, 14(7), 2921-2934.
- Newell-Price, J., Clark, A. J., & King, P. (2000). DNA methylation and silencing of gene expression. *Trends Endocrinol Metab*, 11(4), 142-148.

- Nishi, T., Maier, C. M., Hayashi, T., Saito, A., & Chan, P. H. (2005). Superoxide dismutase 1 overexpression reduces MCP-1 and MIP-1 alpha expression after transient focal cerebral ischemia. *J Cereb Blood Flow Metab*, 25(10), 1312-1324.
- North, B. J., & Verdin, E. (2004). Sirtuins: Sir2-related NAD-dependent protein deacetylases. *Genome Biol*, 5(5), 224.
- Papadopoulos, G. L., Reczko, M., Simossis, V. A., Sethupathy, P., & Hatzigeorgiou, A. G. (2009). The database of experimentally supported targets: a functional update of TarBase. *Nucleic Acids Res*, 37(Database issue), D155-158.
- Papadopoulos, S. M., Chandler, W. F., Salamat, M. S., Topol, E. J., & Sackellares, J. C. (1987). Recombinant human tissue-type plasminogen activator therapy in acute thromboembolic stroke. *J Neurosurg*, 67(3), 394-398.
- Parker, J. S., Roe, S. M., & Barford, D. (2006). Molecular mechanism of target RNA transcript recognition by Argonaute-guide complexes. *Cold Spring Harb Symp Quant Biol*, 71, 45-50.
- Pasquinelli, A. E., Reinhart, B. J., Slack, F., Martindale, M. Q., Kuroda, M. I., Maller, B., et al. (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature*, 408(6808), 86-89.
- Pezzini, A., Del Zotto, E., Giossi, A., Volonghi, I., Grassi, M., & Padovani, A. (2009). The migraine-ischemic stroke connection: potential pathogenic mechanisms. *Curr Mol Med*, 9(2), 215-226.
- Pillai, R. S., Bhattacharyya, S. N., Artus, C. G., Zoller, T., Cougot, N., Basyuk, E., et al. (2005). Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science*, 309(5740), 1573-1576.
- Plaisance, V., Abderrahmani, A., Perret-Menoud, V., Jacquemin, P., Lemaigre, F., & Regazzi, R. (2006). MicroRNA-9 controls the expression of Granuphilin/Slp4 and the secretory response of insulin-producing cells. *J Biol Chem*, 281(37), 26932-26942.
- Rao, K. S. (2009). Free radical induced oxidative damage to DNA: relation to brain aging and neurological disorders. *Indian J Biochem Biophys*, 46(1), 9-15.
- Reamon-Buettner, S. M., & Borlak, J. (2007). A new paradigm in toxicology and teratology: altering gene activity in the absence of DNA sequence variation. *Reprod Toxicol*, 24(1), 20-30.
- Recommendations for standards regarding preclinical neuroprotective and restorative drug development (1999). *Stroke*, 30(12), 2752-2758.
- Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., et al. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature*, 403(6772), 901-906.
- Robertson, K. D. (2002). DNA methylation and chromatin - unraveling the tangled web. *Oncogene*, 21(35), 5361-5379.
- Robertson, K. D. (2005). DNA methylation and human disease. *Nat Rev Genet*, 6(8), 597-610.
- Robinson, M. J., Macrae, I. M., Todd, M., Reid, J. L., & McCulloch, J. (1990). Reduction of local cerebral blood flow to pathological levels by endothelin-1 applied to the middle cerebral artery in the rat. *Neurosci Lett*, 118(2), 269-272.
- Rosell, A., Foerch, C., Murata, Y., & Lo, E. H. (2008). Mechanisms and markers for hemorrhagic transformation after stroke. *Acta Neurochir Suppl*, 105, 173-178.
- Saito, Y., Liang, G., Egger, G., Friedman, J. M., Chuang, J. C., Coetzee, G. A., et al. (2006). Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. *Cancer Cell*, 9(6), 435-443.
- Sarchielli, P., Alberti, A., Coppola, F., Baldi, A., Gallai, B., Floridi, A., et al. (2004). Platelet-activating factor (PAF) in internal jugular venous blood of migraine without aura patients assessed during migraine attacks. *Cephalalgia*, 24(8), 623-630.
- Sasaki, T., Shiohama, A., Minoshima, S., & Shimizu, N. (2003). Identification of eight members of the Argonaute family in the human genome small star, filled. *Genomics*, 82(3), 323-330.
- Sayed, D., Rane, S., Lypowy, J., He, M., Chen, I. Y., Vashistha, H., et al. (2008). MicroRNA-21 targets Sprouty2 and promotes cellular outgrowths. *Mol Biol Cell*, 19(8), 3272-3282.
- Schwarz, D. S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., & Zamore, P. D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. *Cell*, 115(2), 199-208.
- Sempere, L. F., Freemantle, S., Pitha-Rowe, I., Moss, E., Dmitrovsky, E., & Ambros, V. (2004). Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed

- microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol*, 5(3), R13.
- Sen, G. L., & Blau, H. M. (2005). Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. *Nat Cell Biol*, 7(6), 633-636.
- Shi, B., Sepp-Lorenzino, L., Prisco, M., Linsley, P., deAngelis, T., & Baserga, R. (2007). Micro RNA 145 targets the insulin receptor substrate-1 and inhibits the growth of colon cancer cells. *J Biol Chem*, 282(45), 32582-32590.
- Shigeno, T., Teasdale, G. M., McCulloch, J., & Graham, D. I. (1985). Recirculation model following MCA occlusion in rats. Cerebral blood flow, cerebrovascular permeability, and brain edema. *J Neurosurg*, 63(2), 272-277.
- Siomi, H., & Siomi, M. C. (2009). On the road to reading the RNA-interference code. *Nature*, 457(7228), 396-404.
- Smalheiser, N. R., & Torvik, V. I. (2006). Complications in mammalian microRNA target prediction. *Methods Mol Biol*, 342, 115-127.
- Soifer, H. S., Rossi, J. J., & Saetrom, P. (2007). MicroRNAs in disease and potential therapeutic applications. *Mol Ther*, 15(12), 2070-2079.
- Song, G., Ouyang, G., & Bao, S. (2005). The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med*, 9(1), 59-71.
- Strle, K., Zhou, J. H., Shen, W. H., Broussard, S. R., Johnson, R. W., Freund, G. G., et al. (2001). Interleukin-10 in the brain. *Crit Rev Immunol*, 21(5), 427-449.
- Strong, K., Mathers, C., & Bonita, R. (2007). Preventing stroke: saving lives around the world. *Lancet Neurol*, 6(2), 182-187.
- Sugawara, T., Fujimura, M., Noshita, N., Kim, G. W., Saito, A., Hayashi, T., et al. (2004). Neuronal death/survival signaling pathways in cerebral ischemia. *NeuroRx*, 1(1), 17-25.
- Sutton, M. A., & Schuman, E. M. (2006). Dendritic protein synthesis, synaptic plasticity, and memory. *Cell*, 127(1), 49-58.
- Suzuki, S., Tanaka, K., & Suzuki, N. (2009). Ambivalent aspects of interleukin-6 in cerebral ischemia: inflammatory versus neurotrophic aspects. *J Cereb Blood Flow Metab*, 29(3), 464-479.
- Tamura, A., Graham, D. I., McCulloch, J., & Teasdale, G. M. (1981). Focal cerebral ischaemia in the rat: 1. Description of technique and early neuropathological consequences following middle cerebral artery occlusion. *J Cereb Blood Flow Metab*, 1(1), 53-60.
- Tanno, M., Hashimoto, S., Muramatsu, T., Matsuki, M., Yamada, S., & Shimono, M. (2006). Differential localization of laminin gamma and integrin beta in primary cultures of the rat gingival epithelium. *J Periodontal Res*, 41(1), 15-22.
- Thomassin, H., Flavin, M., Espinas, M. L., & Grange, T. (2001). Glucocorticoid-induced DNA demethylation and gene memory during development. *EMBO J*, 20(8), 1974-1983.
- Tietjen, E. G. (2007). Migraine and ischaemic heart disease and stroke: potential mechanisms and treatment implications. *Cephalalgia*, 27(8), 981-987.
- Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group (1995). *N Engl J Med*, 333(24), 1581-1587.
- Touzani, O., Boutin, H., Chuquet, J., & Rothwell, N. (1999). Potential mechanisms of interleukin-1 involvement in cerebral ischaemia. *J Neuroimmunol*, 100(1-2), 203-215.
- Valenzuela, D. M., Economides, A. N., Rojas, E., Lamb, T. M., Nunez, L., Jones, P., et al. (1995). Identification of mammalian noggin and its expression in the adult nervous system. *J Neurosci*, 15(9), 6077-6084.
- van den Berg, A., Mols, J., & Han, J. (2008). RISC-target interaction: cleavage and translational suppression. *Biochim Biophys Acta*, 1779(11), 668-677.
- Vella, M. C., Choi, E. Y., Lin, S. Y., Reinert, K., & Slack, F. J. (2004). The *C. elegans* microRNA let-7 binds to imperfect let-7 complementary sites from the lin-41 3'UTR. *Genes Dev*, 18(2), 132-137.
- Vella, M. C., Reinert, K., & Slack, F. J. (2004). Architecture of a validated microRNA::target interaction. *Chem Biol*, 11(12), 1619-1623.
- Vo, N., Klein, M. E., Varlamova, O., Keller, D. M., Yamamoto, T., Goodman, R. H., et al. (2005). A cAMP-response element binding protein-induced microRNA regulates neuronal morphogenesis. *Proc Natl Acad Sci U S A*, 102(45), 16426-16431.

- Wang, W. X., Rajeev, B. W., Stromberg, A. J., Ren, N., Tang, G., Huang, Q., et al. (2008). The expression of microRNA miR-107 decreases early in Alzheimer's disease and may accelerate disease progression through regulation of beta-site amyloid precursor protein-cleaving enzyme 1. *J Neurosci*, *28*(5), 1213-1223.
- Warlow, C. (2001). *Stroke : a practical guide to management* (2nd ed.). Malden, MA: Blackwell Science.
- Watanabe, D., Uchiyama, K., & Hanaoka, K. (2006). Transition of mouse de novo methyltransferases expression from Dnmt3b to Dnmt3a during neural progenitor cell development. *Neuroscience*, *142*(3), 727-737.
- Watson, B. D., Dietrich, W. D., Busto, R., Wachtel, M. S., & Ginsberg, M. D. (1985). Induction of reproducible brain infarction by photochemically initiated thrombosis. *Ann Neurol*, *17*(5), 497-504.
- Weber, M., & Schubeler, D. (2007). Genomic patterns of DNA methylation: targets and function of an epigenetic mark. *Curr Opin Cell Biol*, *19*(3), 273-280.
- Weidman, J. R., Dolinoy, D. C., Murphy, S. K., & Jirtle, R. L. (2007). Cancer susceptibility: epigenetic manifestation of environmental exposures. *Cancer J*, *13*(1), 9-16.
- Welch, K. M., Cao, Y., Aurora, S., Wiggins, G., & Vikingstad, E. M. (1998). MRI of the occipital cortex, red nucleus, and substantia nigra during visual aura of migraine. *Neurology*, *51*(5), 1465-1469.
- Wienholds, E., Kloosterman, W. P., Miska, E., Alvarez-Saavedra, E., Berezikov, E., de Bruijn, E., et al. (2005). MicroRNA expression in zebrafish embryonic development. *Science*, *309*(5732), 310-311.
- Wightman, B., Burglin, T. R., Gatto, J., Arasu, P., & Ruvkun, G. (1991). Negative regulatory sequences in the lin-14 3'-untranslated region are necessary to generate a temporal switch during *Caenorhabditis elegans* development. *Genes Dev*, *5*(10), 1813-1824.
- Wightman, B., Ha, I., & Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell*, *75*(5), 855-862.
- Wolfe, S. A., Nekludova, L., & Pabo, C. O. (2000). DNA recognition by Cys2His2 zinc finger proteins. *Annu Rev Biophys Biomol Struct*, *29*, 183-212.
- Wong, C. H., & Crack, P. J. (2008). Modulation of neuro-inflammation and vascular response by oxidative stress following cerebral ischemia-reperfusion injury. *Curr Med Chem*, *15*(1), 1-14.
- Xin, H., Li, Y., Chen, X., & Chopp, M. (2006). Bone marrow stromal cells induce BMP2/4 production in oxygen-glucose-deprived astrocytes, which promotes an astrocytic phenotype in adult subventricular progenitor cells. *J Neurosci Res*, *83*(8), 1485-1493.
- Yanamoto, H., Nagata, I., Niitsu, Y., Xue, J. H., Zhang, Z., & Kikuchi, H. (2003). Evaluation of MCAO stroke models in normotensive rats: standardized neocortical infarction by the 3VO technique. *Exp Neurol*, *182*(2), 261-274.
- Yang, H., Kong, W., He, L., Zhao, J. J., O'Donnell, J. D., Wang, J., et al. (2008). MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. *Cancer Res*, *68*(2), 425-433.
- Yasui, D. H., Peddada, S., Bieda, M. C., Vallerio, R. O., Hogart, A., Nagarajan, R. P., et al. (2007). Integrated epigenomic analyses of neuronal MeCP2 reveal a role for long-range interaction with active genes. *Proc Natl Acad Sci U S A*, *104*(49), 19416-19421.
- Yi, R., Qin, Y., Macara, I. G., & Cullen, B. R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev*, *17*(24), 3011-3016.
- Ying, S. Y., & Lin, S. L. (2009). Intron-mediated RNA interference and microRNA biogenesis. *Methods Mol Biol*, *487*, 387-413.
- Yoon, G., Baggaley, S., Bacchetti, P., Fu, Y. H., Digre, K. B., & Ptacek, L. J. (2005). Clinic-based study of family history of vascular risk factors and migraine. *J Headache Pain*, *6*(5), 412-416.
- Zhang, B., Wang, Q., & Pan, X. (2007). MicroRNAs and their regulatory roles in animals and plants. *J Cell Physiol*, *210*(2), 279-289.
- Zhang, C., Li, Y., Chen, J., Gao, Q., Zacharek, A., Kapke, A., et al. (2006). Bone marrow stromal cells upregulate expression of bone morphogenetic proteins 2 and 4, gap junction protein connexin-43 and synaptophysin after stroke in rats. *Neuroscience*, *141*(2), 687-695.
- Zhao, W. Q., & Alkon, D. L. (2001). Role of insulin and insulin receptor in learning and memory. *Mol Cell Endocrinol*, *177*(1-2), 125-134.

- Zhu, S., Si, M. L., Wu, H., & Mo, Y. Y. (2007). MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). *J Biol Chem*, 282(19), 14328-14336.
- Zhu, S., Wu, H., Wu, F., Nie, D., Sheng, S., & Mo, Y. Y. (2008). MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. *Cell Res*, 18(3), 350-359.
- Zoratti, M., & Szabo, I. (1995). The mitochondrial permeability transition. *Biochim Biophys Acta*, 1241(2), 139-176.

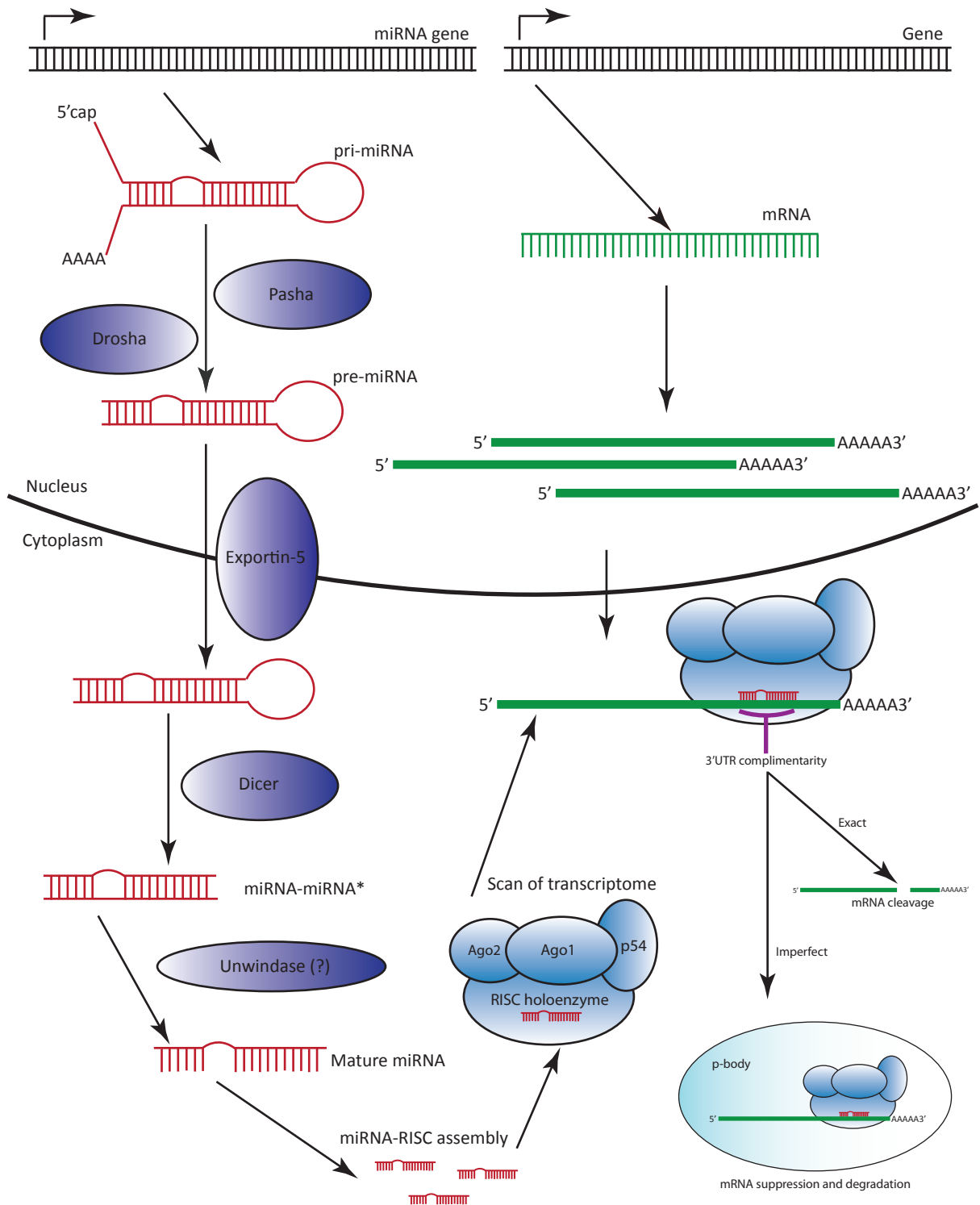


Figure 1. Schematic representation of the biogenesis and mode of action of microRNA. miRNA primary transcripts (pri-miRNAs) are transcribed from either miRNA genes or spliced from the introns of protein coding genes (not shown), whereupon they are processed by Pasha and Drosha to generate pre-miRNAs. Pre-miRNA is exported from the nucleus by the Exportin-5 enzyme, and processed by Dicer, forming the miR-anti-miR duplex. Through the action of an unidentified enzyme, temporarily named unwindase, and the members of the RNA-induced silencing complex (RISC), mature miRNAs are incorporated into the active RISC holoenzyme. Based on the complementarity of the miRNA to the 3'UTR of its target transcripts, the mRNA is either immediately cleaved and degraded, or transported to p-bodies for disposal.

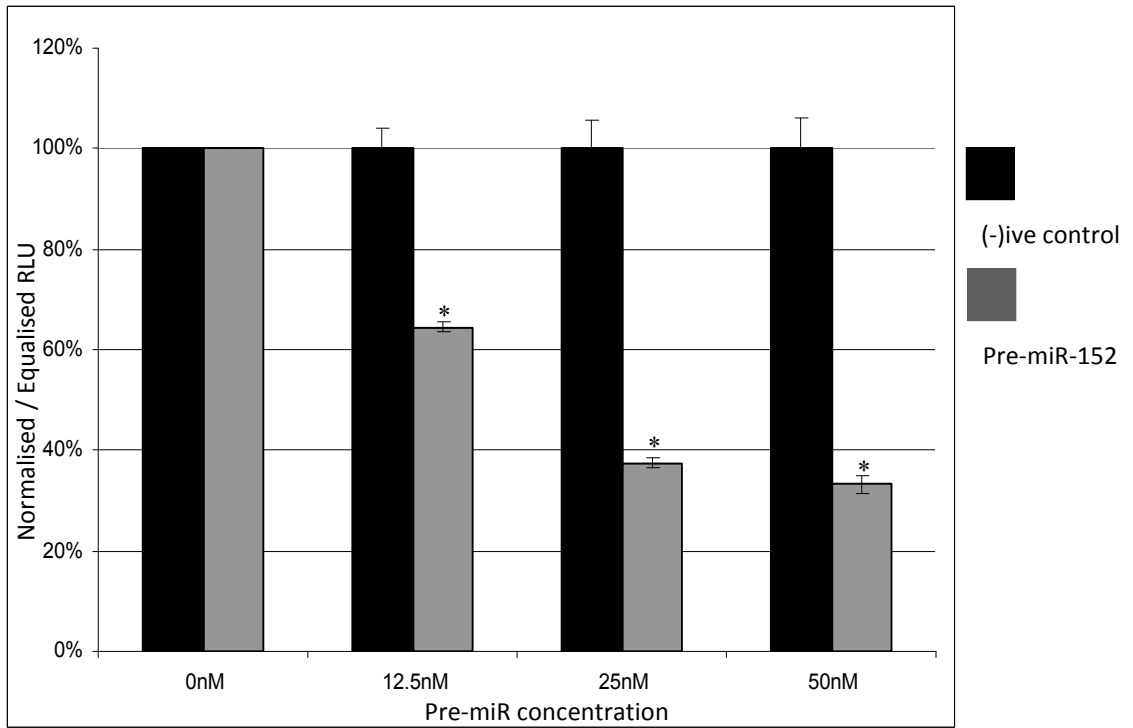


Figure 2. Normalised, equalised luminescence data, showing a decrease in the expression level of a luciferase-noggin3'UTR hybrid in HEK293 cells under the influence of increasing transfection concentrations of miR-152 precursors and negative control.

Legend:

L- lesioned side

NLct - sham controls

NL - nonlesioned contralateral

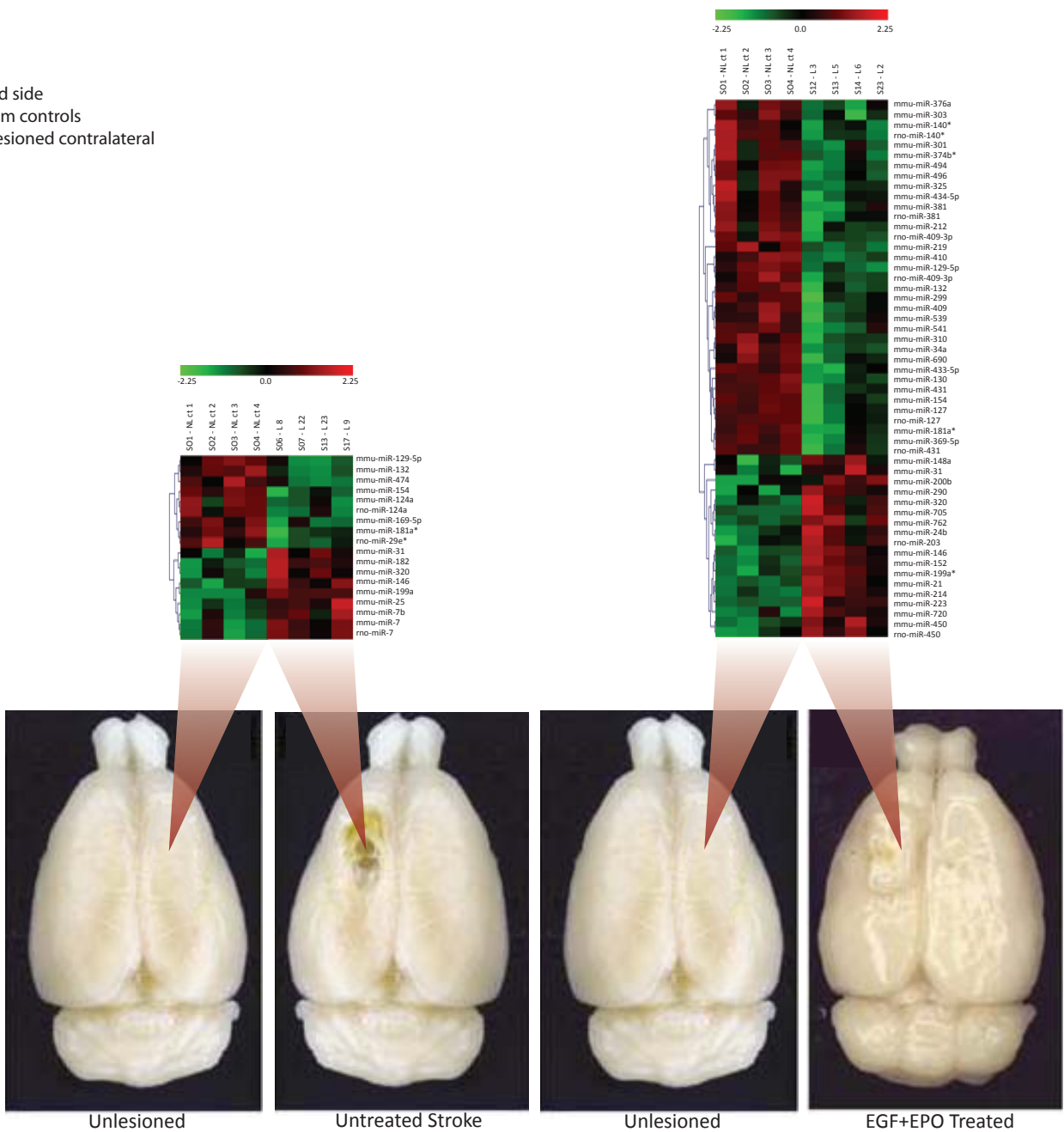
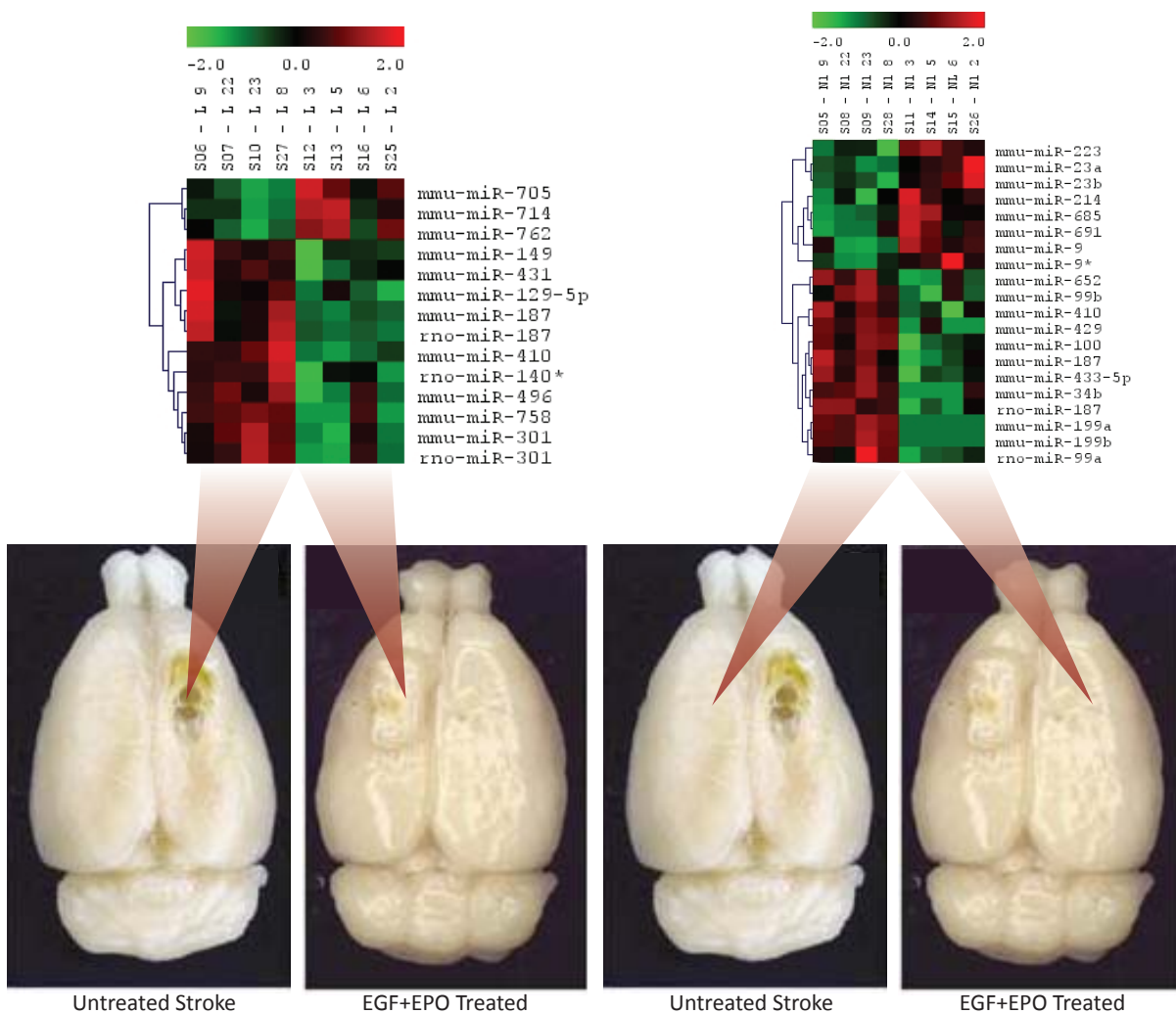


Figure 3. miRNA microarray analysis. Figure 3a (left) shows changes in miRNA expression in stroke tissue 4 months post-lesion compared to shams. Figure 3b (right) shows changes in miRNA expression in stroke tissue treated with EGF/EPO 4 months post-lesion compared to shams.

Legend:

L- lesioned side
NLct - sham controls
NL - nonlesioned contralateral



Legend:

L- lesioned side

NLct - sham controls

NL - nonlesioned contralateral

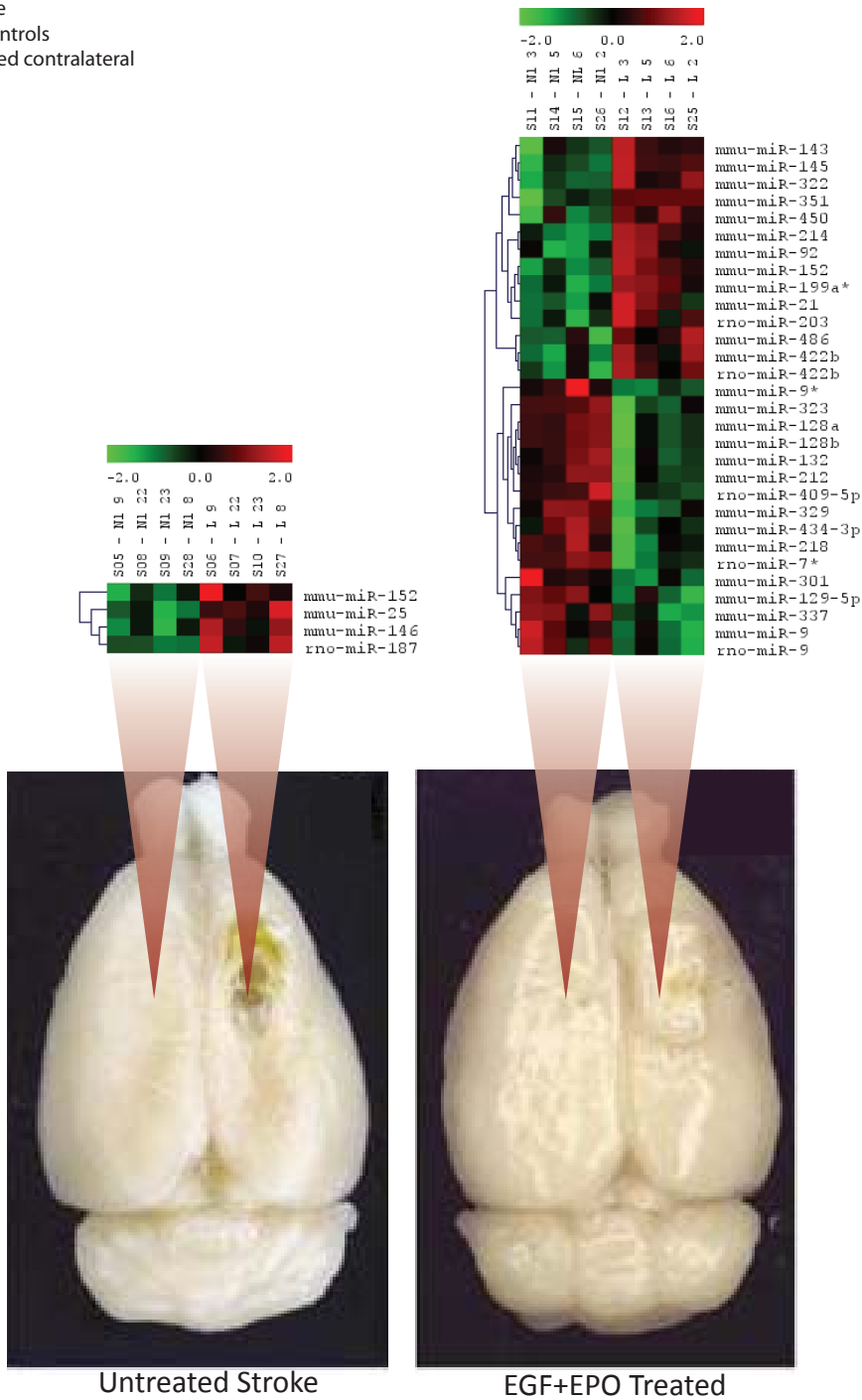
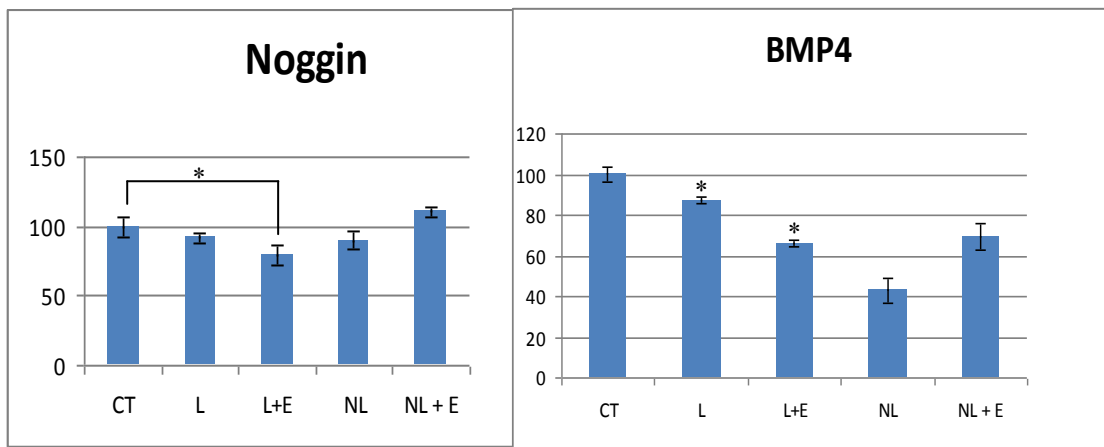
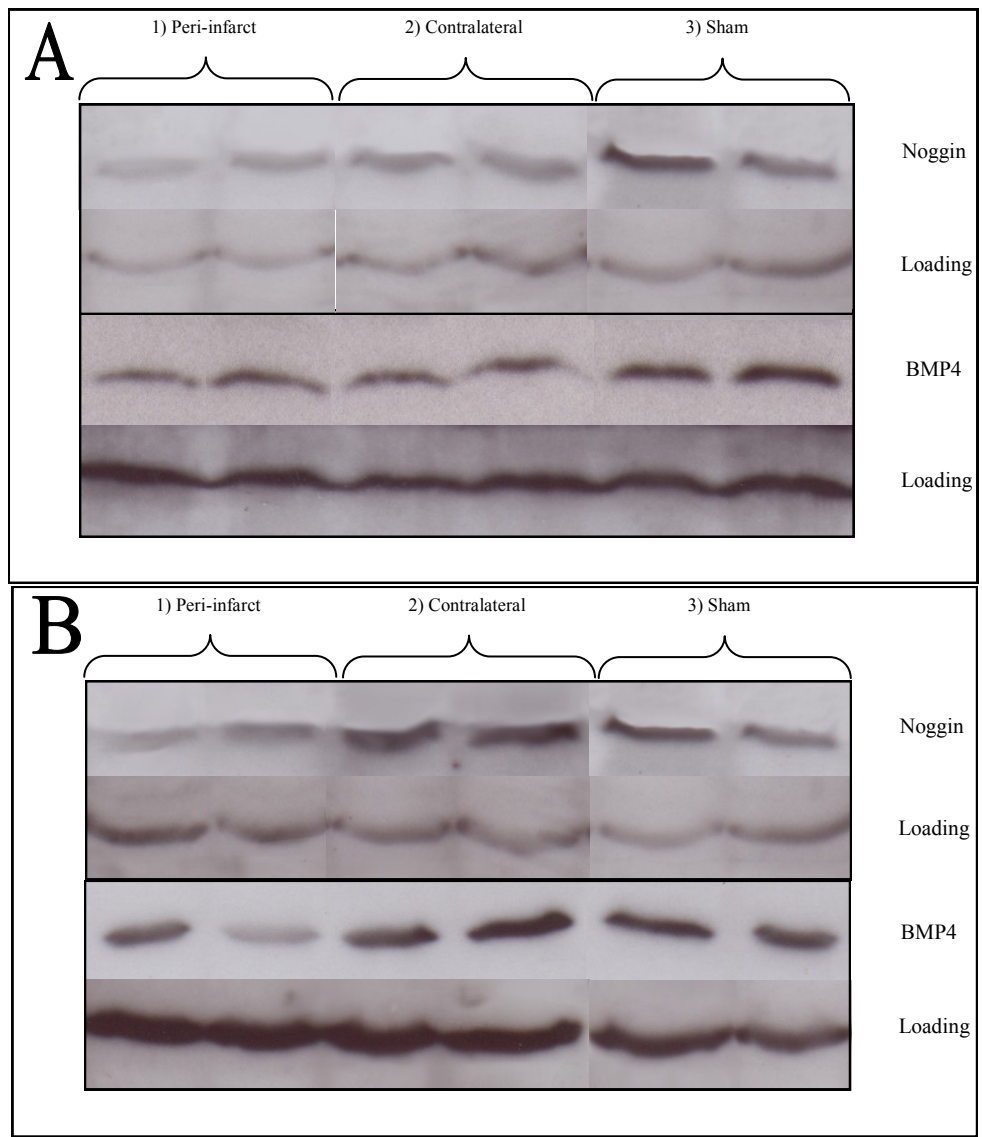


Figure 5. miRNA microarray analysis. Direct side-by-side comparison of differences in miRNA expression within a single experimental animal. Figure 5a (left) shows a comparison between the lesioned and nonlesioned hemisphere of an untreated animal, while figure 5b (right) shows a comparison between lesioned and nonlesioned tissue in an EGF/EPO treated animal.



Legend
 CT- Control
 L- Lesion
 NL- Nonlesion
 L+E- Lesion+ EGF/EPO
 NL+E- Nonlesion+ EGF/EPO

Figure 6. Representative Western immuno-blot images, showing untreated (A) and treated (B) conditions, indicative of levels of noggin and BMP4 in the peri-infarct region (1) compared to the contralateral region in the same animals (2), as well as shams (3). Graphs show noggin / BMP4 as a percentage of controls, +/- standard deviation.

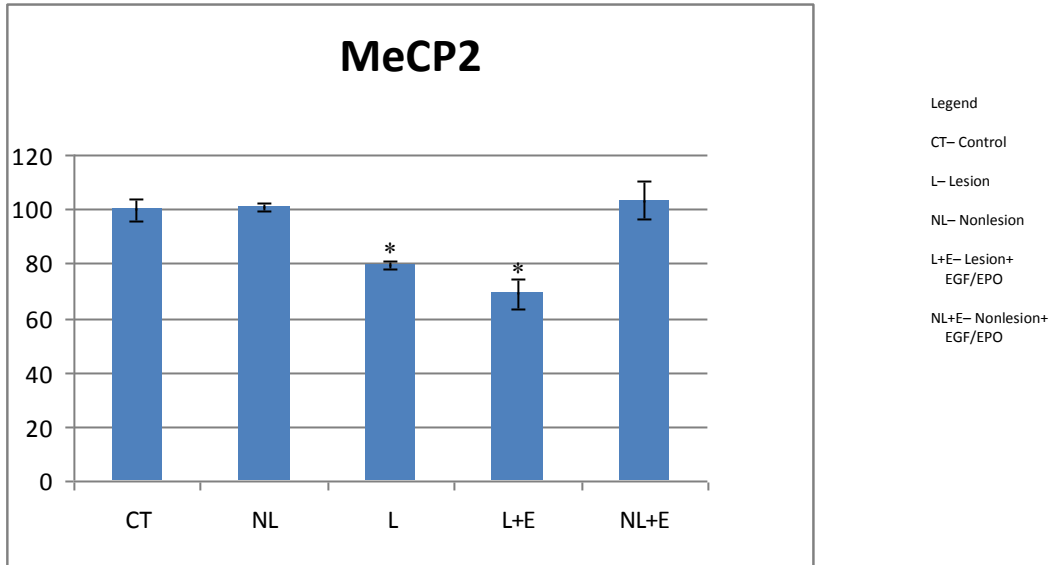


Figure 7 - Percentage variation in the levels of MeCP2 across multiple conditions with respect to shams (CT). Data shown is percentage of controls + / - standard deviation.

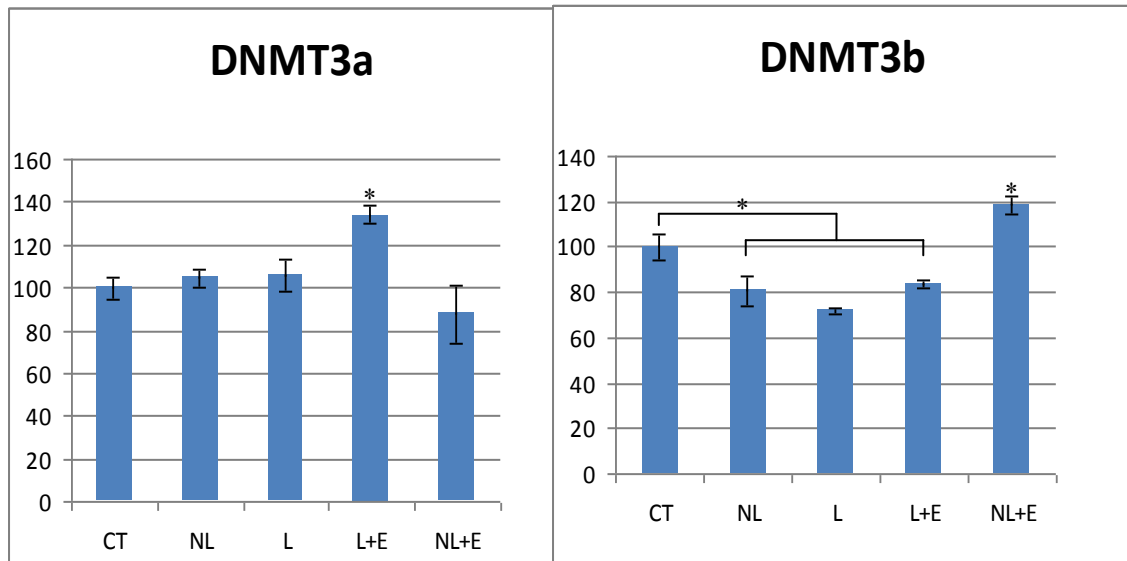


Figure 8 - Percentage variation in the levels of *de novo* methyltransferases DNMT3a and DNMT3b across multiple conditions with respect to shams (CT). Data shown is percentage of controls + / - standard deviation.