The Role of miRNAs in Stroke

Christopher Ronald Breen

BSc (Hons) MRes

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British Heart Foundation Glasgow Cardiovascular Research Centre, Institute of Cardiovascular and Medical Sciences, College of Medical, Veterinary and Life Sciences, University of Glasgow.

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Author's Declaration

I declare that this thesis has been written entirely by myself and is a record of work performed by myself. This thesis has not been submitted previously for a higher degree. The research was carried out in the Institute of Cardiovascular and Medical Sciences, University of Glasgow, under the supervision of Dr. Lorraine Work, Prof. Andrew H. Baker and Prof. I Mhairi Macrae.

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"If the fool would persist in his folly he would become wise" – Blake

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Table of Contents

X

List of Tables

List of Figures

List of Publications

"Role of microRNAs 99b, 181a, and 181b in the differentiation of human embryonic stem cells to vascular endothelial cells."

Kane NM, Howard L, Descamps B, Meloni M, McClure J, Lu R, McCahill A, Breen C, Mackenzie RM, Delles C, Mountford JC, Milligan G, Emanueli C, Baker AH.

Stem Cells. 2012 Apr;30(4):643-54. doi: 10.1002/stem.1026.

List of Abbreviations

AD	Alzheimer's Disease
Ago2	Argonaute RISC catalytic component 2
AIDS	Aquired Immune Deficiency Syndrome
AIF	Apoptosis Inducing Factor
AMI	Acute Myocardial Infarction
ANOVA	Analysis of Variance
AP	Alkaline Phosphate
ATP	Adenosine Triphosphate
BHF	British Heart Foundation
BI	Barthel Index
BID	Bcl2 interacting Domain
CCS	Causative Classification System
cDNA	Complementary DNA
COL11A1	Collagen Type-XI alpha 1
COL3A1	Collagen Type-III alpha 1
COPD	Chronic obstructive pulmonary disease
CT	Computed Tomography
CytC	Cytochrome C
DAMP	danger-associated molecular pattern molecules
DEPC	Diethyl Pyrocarbonate
DIAS	desmoteplase in acute stroke
DMSO	Dimethyl Sulphoxide
DWI	Diffusion Weighted Imaging
Dyrk2	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
ENACT	Expanding Nilotinib Access in Clinical Trials
ESC	Embryonic Stem Cells
exRNA	Exosomal RNA
FASLG	Fas ligand (TNF superfamily, member 6)
FLAIR	Fluid-attenuated inversion recovery
GFP	Green Fluorescent Protein
GMP	Good Manufacturing Practices
GRP-78	binding immunoglobulin protein
HDAC2	Histone Deacetylase 2
HDAC4	Histone Deacetylase 4
HGF	Hepatocyte Growth Factor
HIV	Human Immunodeficiency Virus
HSP70	70 kilodalton heat shock protein
HUVEC	Human Umbilical Vein Endothelial Cell

ICH	Intracerebral Hemorrhage
iPSC	Induced Pluripotent Stem Cells
KO	Knockout
LNA	Locked Nucleic Acid
lncRNA	Long Non-coding RNA
MCA	Middle Cerebral Artery
MCAO	Middle Cerebral Artery Occlusion
Mcl-1	Myeloid leukemia cell differentiation protein
miRNA	micro RNA
MMP2	Matrix Metalloproteinase 2
MMP9	Matrix Metalloproteinase 9
MOI	Multiplicity of Infection
MRI	Magnetic Resonance Imaging
mRS	Modified Rankin Scale
Multi-PART	Multicentre Preclinical Animal Research Team
NADPH	Nicotinamide adenine dinucleotide phosphate
NeuN	Hexaribonucleotide Binding Protein
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHPSS	Nonhuman Primate Stroke Scale
NIHSS	National Institutes of Health Stroke Scale
NINDS	National Institute of Neurological Disorders and Stroke
NXY-059	Disufenton sodium
P2X7	Purinoceptor 7
Pacs2	Phosphofurin acidic cluster sorting protein 2
PBS	Phosphate Buffered Saline
PCA	Prinicple Component Analysis
PCR	Polymerase Chain Reaction
Pdcd4	Programmed cell death protein 4
PFA	Paraformaldehyde
piRNA	piwi-interacting RNA
pMCAO	Permanent Middle Cerebral Artery Occlusion
PPARδ	Peroxisome proliferator-activated receptor delta
PSD-95	postsynaptic density protein 95
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
RNAi	RNA interference
ROS	Reactive Oxygen Species
SHRSP	Stroke Prone Spontaneously Hypertensive Rat
siRNA	Small Interfering RNA
SIS	Stroke Impact Scale

snoRNA	Small nucleolar RNA
SSC	Saline Sodium Citrate
SVZ	Subventricular Zone
TGF-β1	Transforming Growth Factor Beta 1
TLX	Nuclear receptor subfamily 2 group E member 1
tMCAO	Transient Middle Cerebral Artery Occlusion
TMEM49	Transmembrane protein 49
TNF	Tumor Necrosis Factor
TOAST	Trial of Org 10172 in acute stroke treatment classification
tPA	Tissue Plasminogen Activator
UCHL1	Ubiquitin carboxyl-terminal esterase L1
UTP	Uridine-5'-triphosphate
UV	Ultraviolet
VRF	Vetrinary Research Facility
WT	Wild Type

Summary

Stroke is currently one of the leading causes of death and disability worldwide. Despite recent advances in the treatment of stroke there is a major unmet clinical need for novel therapeutics for intervention. miRNAs are small coding RNAs which act to post-transcriptionally inhibit expression of genes. Emerging evidence has supported the view that miRNAs play an important role in the development and progression of ischaemic stroke, although understanding remains relatively poor. This research uses several models to investigate the effects of miRNAs in the context of stroke *in vivo* and *in vitro*, as well as assessment of patient serum samples in order to identify biomarkers for stroke. miR-29b was found to be significantly upregulated in SHRSP rat brain peri-infarct at 72h following stroke, and downregulated in ischaemic core at 24h and 72h following stroke, whilst miR-29c was significantly downregulated in remainder tissue at 24h following stroke and in infarct at 72h following stroke. The upreglation of miR-29b at 72h corresponded to a significant downregulation of miR-29 target genes MMP2, MMP9 and TGF-β1 in peri-infarct tissue at 72h following stroke. Modulation of miR-29b and miR-29c was achieved in a rat neuronal cell line but suppression of genes of interest was not observed following oxygen glucose deprivation. Several candidate miRNAs were then identified by microRNA Openarray analysis in stroke patient serum samples. Validation of these miRNAs was not demonstrated in the population studied, but assessment of these miRNAs in rat serum and isolated exosomes demonstrated that several of these miRNAs were significantly altered in SHRSP rats following stroke. Finally miR-21 was demonstrated to be significantly upregulated in SHRSP rat peri-infarct following stroke. This was associated with a change in miR-21 localization as determined by *in situ* hybridization. Modulation of miR-21 via the use of CAG-miR-21 mice demonstrated no difference in infarct size as measured by T_2-weighted MRI scan nor was any difference present in behavioural tests versus wild type. KO of miR-21 resulted in a reduction of survival rate compared with wild type.

This thesis demonstrates that miR-29 and miR-21 are modulated following stroke in animal models, and these are potential candidates for therapeutic intervention in the future. Analysis of clinical samples has illustrated difficulties in the identification of serum miRNA profiles and suggests that looking at the exosomal component of serum may provide better information regarding miRNA profiles after stroke.

Chapter 1 - Introduction

1.1 Epidemiology of Stroke

Stroke remains one of the major causes of mortality and is one of the leading causes of adult disability worldwide (Adamson et al. 2004). The burden that this places on public healthcare services, not to mention the severe negative effects of the affected individuals quality of life, cannot be overstated. Some of the most up to date important demographic information can be found in the 2014 update Executive Summary: Heart Disease and Stroke Statistics published by the American Heart Association. In this document it is stated that some of the biggest risk factors for the development of stroke are high blood pressure, smoking, lack of physical exercise and sedentary lifestyle (Go et al. 2014). These have long been known to be the major underlying causative factors of all cardiovascular disease. However, incidence of these risk factors continue to rise as the world increasingly adopts what has been referred to as the 'western lifestyle'. Although there has been a total decrease in mortality as a result of stroke by 22.8% when comparing death rates in 2010 to those of 2000, this does not reflect a decrease in stroke incidence, or morbidity. In effect more people are surviving stroke with worse clinical outcomes. This is a worst case scenario for stroke medicine and reflects the urgent need for improved therapeutics. Every year, around 15 million people will suffer from a stroke globally. It is estimated that every two seconds, somebody has a stroke, every six seconds someone dies of a stroke and every six seconds somebody will become permanently disabled due to stroke ("The Facts Behind '1 in 6' - World Stroke Campaign" 2015). As of 2012, stroke remains the second leading cause of mortality worldwide, second only to ischaemic heart disease (Figure 1.1)(Stroke Association 2015).

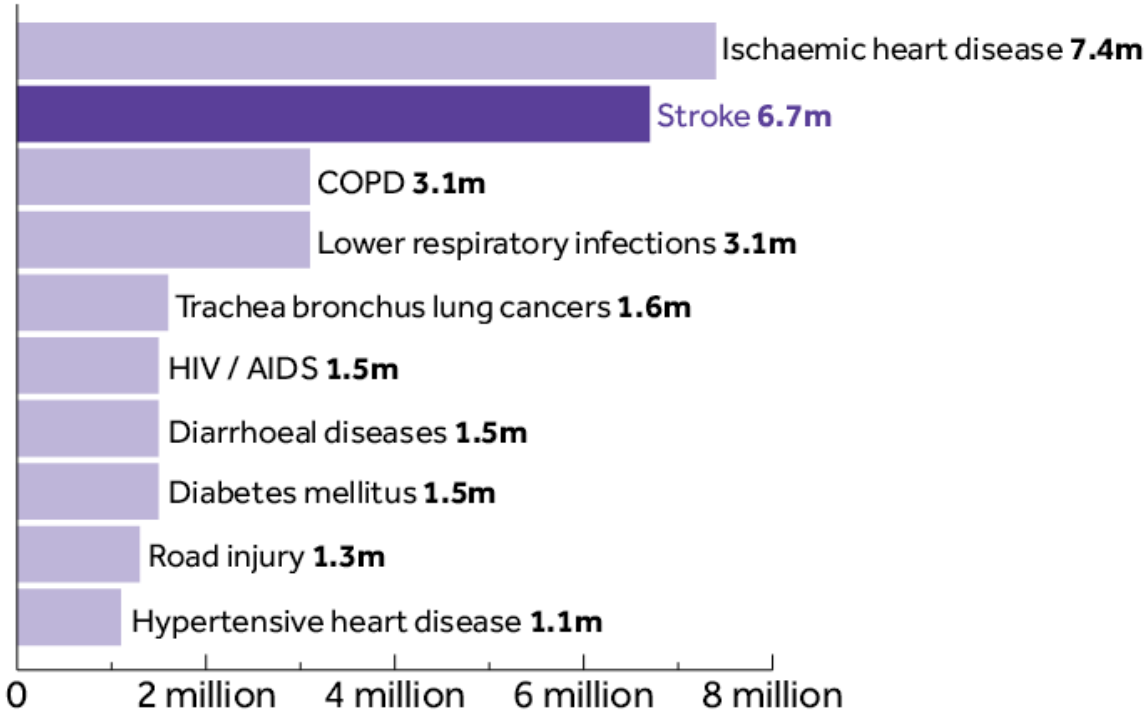

Figure 1.1– Top 10 leading causes of death worldwide (Stroke Association 2015)

Whilst stroke is a problem throughout the world, it is a major health concern in the UK due to the burden placed on the healthcare service. Within the UK stroke is most prevalent in the central belt/west coast of Scotland (Figure 1.2)(British Heart Foundation 2009).

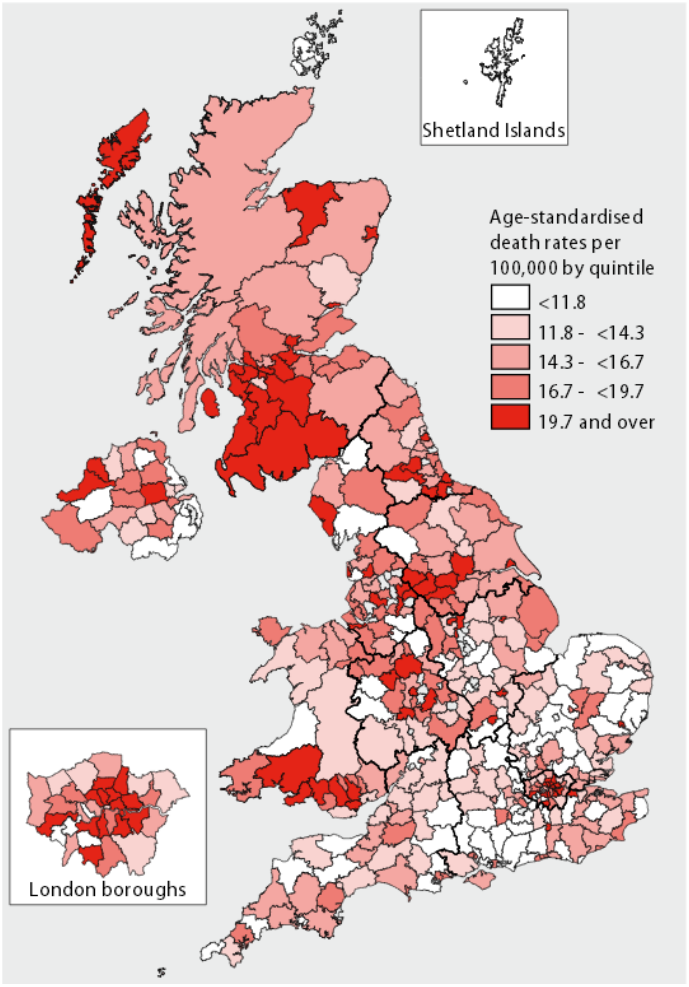

Figure 1.2 – Age-standardised death rates per 100,000 by quintile for every region in the UK (British Heart Foundation 2009).

1.2 History of Stroke

Although stroke currently poses a major burden on the landscape of modern public health knowledge of the disease is ancient. In one of the earliest medical documents, 'the Edwin Smith papyrus' which was written around 3000BC; Imhotep, the founder of Egyptian medicine described patients exhibiting symptoms of stroke (Greer 2008; Breasted 1930). In the pre-modern era stroke continued to affect many people, but understanding of the causes eluded doctors, whilst even the concept of a cure was inconceivable. It wasn't until the 1600s that Thomas Willis described, in detail, the arterial supply of the brain (Symonds 1955). In addition to his identification of the 'circle of Willis' he coined the term 'apoplexy' to refer to stroke. Understanding of the disease continued to improve and in the 1800s Matthew and Cruveilhier created several illustrations of apoplectic lesions. The esteemed Virchow introduced the concept of 'Virchow's triad' and in doing so became the first person to report that thromboembolism could be a cause of

vascular occlusion (Schiller 1970). The next major development in the understanding of stroke biology occurred in the 1900s when Charles Foix correlated lesion localization with clinical symptoms resulting in increased clinical interest in the disease (Caplan 1990). This foundation in anatomical localization of lesions laid the groundwork in constructing maps that can be used today by medical imaging to identify cerebrovascular lesions which are causative or potentially causative of a stroke.

The defining point of modern stroke history occurred in the 1960s. C. Miller Fisher produced detailed descriptions of stroke pathophysiology of many subtypes of the disease including lacunar strokes (Fisher 1969), carotid artery disease (Fisher 1969), transient ischaemic attacks (Fisher 1962) and intracerebral haemorrhage (Fisher et al. 1965). His student, Louis Caplan established one of the first stroke registry databases to collect and analyze important clinical data regarding the disease (Mohr et al. 1978). This rather prescient move was an early attempt in the true application of 'evidence based medicine' to stroke. The term 'Risk factors' was developed by Thomas Dawbee to indicate environmental conditions that could contribute to the development of cardiovascular disease in 1961 (Kannel et al. 1961). Following this, the Framingham heart study demonstrated a link between cardiovascular disease and stroke (Wolf et al. 1991). Risk factors of cardiovascular diseases and stroke continued to be refined as biological understanding of disease processes improved, whilst identification of novel risk factors also shed light on pathways involved in disease development. Mounting evidence supporting the use of preventative measures to reduce stroke risk accumulated and led to the concept of stroke prevention by the use of antihypertensive and antithrombotic agents (Hillen et al. 2000).

Rapid progress in the understanding of stroke has occurred over the last 60 years, including major improvements in the understanding of molecular mechanisms underlying stroke and improved medical imaging technologies for diagnostic purposes, but the single greatest coup achieved by medical professionals in the battle to beat stroke so far has been the successful introduction of intravascular recombinant tissue-Plasminogen Activator (TPA) (Werner Hacke et al. 1995) (Adeoyle et al. 2011). Provided the patient can be successfully diagnosed in the sub-acute period following stroke TPA can be administered for the purpose of thrombolysis (Hacke et al. 2008). Another recent development which has had a major impact on the goal of stroke medicine is the concept

of penumbra (Astrup et al. 1981). This is a region of the brain where obstruction of blood flow exists, but cell death has not yet occurred. It is this region of the brain which can potentially be rescued by thrombolytic therapy. Recent developments in stroke research have focused on neuroprotection (Majid 2014), improving upon existing thrombolytics (Hacke et al. 2005) or increasing implementation of the drug in patients who do not meet the approved therapeutic criteria (Simon et al. 2004), the development of specialised stroke units in hospitals for acute stroke care, mechanical thrombectomy (Fransen et al. 2014), and finally the use of gene and/or cell therapy to promote regeneration and recovery of damaged brain tissue (Fisher et al. 2007).

1.3 Classifications of stroke

Stroke has a variety of different subtypes, each of which manifests in the clinic slightly differently, and will benefit from different treatment strategies. As our understanding of stroke improves, so will the clinical ability to base treatment on physiological measurements of penumbra instead of relatively arbitrary criteria such as time since stroke. Basing clinical intervention strategies on this information will result in considerable improvements in the treatment of stroke.

The two main types of stroke are Haemorrhagic and Ischaemic stroke.

1.3.1 Haemorrhagic Stroke

Haemorrhagic stroke occurs following the breakdown of the vascular wall resulting in the flow of blood into the brain parenchyma or meninges, this accounts for approximately 13% of all stroke cases. Haemorrhagic stroke can be further subdivided based on location of the site of bleeding. Subarachnoid haemorrhage occurs when bleeding is located in the subarachnoid meningeal space between the arachnoid and the pial membranes Intracerebral haemorrhage refers to when the bleeding occurs in the brain parenchyma(“Hemorrhagic Strokes” 2014).

This type of stroke can also be classified based on the type of vascular pathologies which result in the onset of the injury. An aneurysm results when a region of the vasculature forms a balloon-like bulge. This region of vasculature displays weakened structural properties and if left untreated can rupture, resulting in haemorrhage. The other vasculopathy which can result in onset of haemorrhagic stroke is the presence of

arteriovenous malformation; this is when the cerebrovasculature develops abnormally. These anatomical variations can have compromised structural integrity, resulting in rupture and the onset of stroke (Parmet et al. 2004).

It is also possible for cerebrovascular haemorrhage to occur following traumatic brain injury, although most of the stroke literature focuses on non-traumatic haemorrhagic injury (Liu et al. 1999).

1.3.2 Ischaemic Stroke

Ischaemic stroke accounts for the vast majority of stroke cases (Adams et al. 1993). For this reason it is the most widely studied. Ischaemic stroke occurs when an obstruction becomes present in some region of the cerebrovasculature, this obstruction prevents blood flow to regions of the brain distal to the site of blockage resulting in hypoxic injury (Bornstein 2009). Ischaemic stroke can be said to be thrombotic or embolic (del Zoppo et al. 1992). Both of these types of stroke can result due to atherosclerosis (Chambless et al. 2000). Atherosclerosis is a vascular pathology where fatty deposits accumulate in the arteries forming plaques. If an atherosclerotic plaque destabilises, this may form a thrombus. If a sufficiently large thrombus is formed in the cerebrovasculature, blood flow may be occluded locally resulting in ischaemia. This is referred to as a thrombotic stroke. If a piece of thrombus or unstable atherosclerotic plaque breaks free and travels in the circulation, it can translocate into the cerebrovasculature causing occlusion and resulting in ischaemic injury. This type of stroke is classified as embolic. The most common presentation of embolic stroke is cardioembolic, caused by cardiac arrhythmia which results in the formation of a clot in the atria of the heart, which then moves towards the brain resulting in ischaemic injury (“Ischemic Strokes” 2014).

One of the major problems in the treatment of stroke is that the diagnostic criteria refer not to one disease, but a multitude of differential clinical phenotypes. In order to better understand the disease and generate more specific treatments much work has been done on improving descriptions of clinical phenotype of stroke. The TOAST (trial of ORG 10172 in acute stroke treatment) classification was one such organized attempt at a formal definition of stroke subtypes for the purpose of guiding stroke research (Adams et al. 1993), another scale of stroke assessment being the National Institutes of Health Stroke Scale (NIHSS) which characterises severity of stroke based on a score determined by

clinicians based on a number of different criteria assessing sensorimotor deficits and reduction in cognitive function (Brott et al. 1989).

As with haemorrhagic stroke, ischaemic stroke can also be subdivided based on site of ischaemia. This was not possible in the early days of stroke medicine, but with the advent of sophisticated medical imaging such as CT scans it became possible to pinpoint the location of the insult with some degree of accuracy. The most widely implemented system of stroke subtype identification is by TOAST (Trial of Org 1072 in Acute Stroke Treatment) classification. These criteria (Table 1.1) were determined in 1993 by the Trial of Org 10172 in Acute Stroke Treatment. These criteria were subsequently assessed in several clinical trials and provide an effective means of assessing stroke phenotype in patients (Adams et al. 1993).

Although the TOAST classification is informative for clinicians, it is dependent upon baseline CT scans which are often inconclusive. In an effort to overcome some of the caveats associated with use of the TOAST classification system, in 2005 the Causative Classification System (CCS) project was launched (Ay et al. 2005). As an alternative to TOAST classification the CCS utilized an array of different diagnostic systems (diffusion-weighted MRI, CT/MRI angiography, Echocardiography and Holter monitoring) in order to conclude the cause of stroke. Due to the increased requirement for clinical assessment in what is an extremely time-sensitive disease, implementation of this system has not been as widespread as that of the TOAST classification system (Ay et al. 2005). A recent comparison of the two classification systems found there to be “excellent agreement” between both classification systems, but failed to demonstrate in improvement in the reduction of patients assigned a diagnosis of unclassified (Lanfranconi and Markus, 2013). More recently two additional systems of classification have been developed: Atherosclerosis, Small-Vessel Disease, Cardiac Source, Other Cause (ASCO) system (Amarenco et al. 2009) and the Chinese Ischaemic Stroke Subclassification (CISS) system (Gao et al. 2011) . Each of these has potential advantages over the other more traditional methods; however their usefulness remains to be demonstrated by reliability and validity data.

Publication year	TOAST 1993	CCS 2007	A-S-C-O 2009	CISS 2011
Type of system	Causative	Causative and phenotypic	Phenotypic	Causative
Major subtypes	1. Large artery	1. Supra-aortic large artery atherosclerosis	1. Atherosclerosis	1. Large artery atherosclerosis
	2. Cardioembolism	2. Cardio-aortic embolism	2. Cardioembolism	2. Cardiogenic stroke
	3. Small vessel occlusion	3. Small artery occlusion	3. Small vessel disease	3. Penetrating artery disease
	4. Other determined etiology	4. Other causes	4. Other causes	4. Other etiologies
	5. Undetermined etiology	5. Undetermined causes		5. Undetermined etiology
Advantages	Worldwide useSimple, logic and easy to useValidation by independent groupsPredicting prognosis and risk of stroke recurrence	Rules and criteria based on published EvidenceUpdated criteria to stratify cardioembolism into high- and low-risk groupsWeb-based automated version availableReducing the ratio of "undetermined" category	Integration of diagnostic evaluation into the level of confidence for subtype assignmentsClarified diagnostic criteria to identify or rule out a stroke etiologyIntegration of noncausative factors into subtype assignments	Incorporation of the etiology and underlying mechanism into stroke subclassificationCreating a new subtype of PADLarge artery atherosclerosis further subclassified into four categories
Disadvantages	Only moderate inter-rater reliabilityOversized "undetermined" etiologyNot fit to recent advances in diagnostic technology	Depending on the availability of modern diagnostic technologyBased on evidence from diverse studiesAortic arch atherosclerosis belonging to cardio-aortic embolism	Further reliability and validity data neededInterpretation cautiously with the combination of causative and noncausative factorsDepending on the completeness of diagnostic toolsToo many phenotypic subtypes (n = 625) for research studiesToo restrictive definition to diagnose atherosclerosis and small vessel disease	Lacking reliability and validity dataDepends on the availability of brain and vascular imagingFuture imaging technology needed to verify the concept of PAD

ASCO, atherosclerosis, small vessel disease, cardiac causes, other uncommon causes; CCS, Causative Classification of Stroke System; CISS, Chinese Ischemic Stroke Subclassification; PAD, penetrating artery disease; TOAST, Trial of ORG 10172 in acute stroke treatment.

Table 1.1 - Characteristics of major etiologic classification systems for ischaemic stroke (P.-H. Chen et al. 2012)

1.4 Therapeutic Challenges of Stroke

It is undeniable that stroke is currently one of the biggest medical issues worldwide, yet we are presented with a relatively poorly funded area of disease research and a major unmet clinical need for interventions (British Heart Foundation 2009; Stroke Association 2015). Why is this? The reasons that stroke presents such a challenge to modern medicine are many-fold. Arguably the most important [definitely the most aggressively championed] is the factor of time in the treatment of the disease. If stroke cannot be successfully diagnosed within 3.5-4.5h of the onset of symptoms to the potential for

improve clinical outcome of the affected individual becomes limited. In this case the treatment of stroke becomes primarily an issue of care; ensuring the highest quality of life for the effected individual; ensuring that they have the highest degree of independence and dignity possible. Admission to specialised stroke hospital units upon stroke onset can significantly improve the chance of improved outcomes (Trialists'Collaboration 1997; Trialists'Collaboration 2001).

Another major hindrance in the treatment of stroke is successful application of thrombolytic therapeutics to stroke affected individuals. Only a minority of stroke patients qualify for this treatment, and of the few who qualify, even less receive it (Albers et al. 2011). Even if patients receive tPA, it is not always effective (Bhatia et al. 2010). It is for this reason that much of the clinical research of today is focussed on maximising the number of individuals who receive thrombolytic therapeutics, whilst minimizing the negative outcomes associated with haemorrhagic transformation (Fisher et al. 2007). The penumbra is of key importance during the acute phase following stroke and it is believed that by improving speed and quality of stroke diagnostics, the decision whether to move forward with thrombolytic treatment could be based on molecular measurements on which tissue is still alive rather than a relatively arbitrary time period. This would promote the advancement towards personalised medicine and maximize beneficial therapeutic outcomes.

In order to overcome some of the multitude of problems associated with clinical stroke research (predominantly problems regarding differences in stroke phenotypes in patients and the degree to which *in vitro* and *in vivo* models of disease directly translated to human disease) several initiatives have been imposed in order to establish standard practices of research, to identify priorities of stroke research and to overcome some of the technical issues posed by such studies. Perhaps the most widely cited instance of such a framework is the guidelines proposed through Stroke Treatment Academic Industry Roundtable (STAIR), this is an invitation only conference which assembles leading scientists, clinicians and government officials involved with stroke in order to discuss the best strategies and practices for stroke research and treatment. (Fisher et al. 2009)

1.5 Pathophysiology of Stroke

Stroke refers to a collection of heterogeneous diseases. Although at the macroscopic level there are considerable differences between each of these manifestations of the condition, at the molecular level there is considerable consistency with regards to the cell signaling pathways involved. Stoke pathophysiology tends to follow a set sequence of events which have varying impact at different times following onset of symptoms (Figure 1.3)(Dirnagl et al. 1999). Novel treatments have been developed based on observations made in molecular changes following stroke both in clinical settings as well as pre-clinical models (Mergenthaler et al. 2004). These novel treatments have been explored experimentally, but not yet clinically proven.

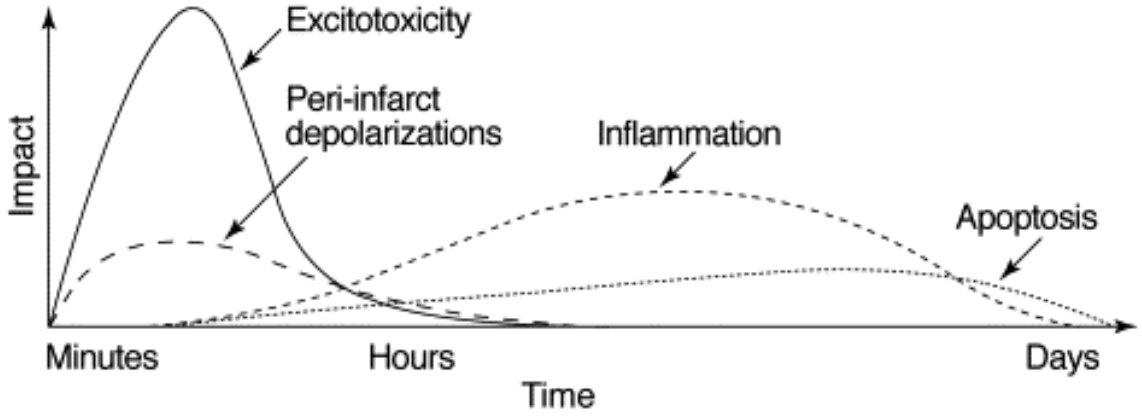

Figure 1.3 – Timeline of pathological events following ischaemic stroke. – The progression of stroke pathology follows a series of distinct events each with differing impact. The first stage of stroke pathology involves peri-infarct depolarizations and excitotoxicity which occurs acutely after injury. This is followed by a prolonged period of inflammation which occurs over a period of days(Dirnagl et al. 1999)

1.5.1 Penumbra

The concept of penumbra (Astrup et al. 1981) has become increasingly important recently in informing clinical interventions for stroke. Following occlusion of the vasculature there is necrosis of the adjacent brain tissue as a result of ischaemia. The penumbra is the region of tissue surrounding the evolving lesion supplied by the occluded vessel which is potentially salvageable if adequate treatment is received. There has been an attempt towards identifying the penumbra through use of perfusion-diffusion mismatch MRI imaging (Fisher and Bastan 2012) as it has been observed that there are cases in which patients may benefit from pharmacological interventions beyond the FDA approved time scale (Donnan et al. 2009; Davis et al. 2008).

1.5.2 Excitotoxicity

Excitotoxicity is a glutamate- mediated form of neuronal cell death which occurs following ischaemic injury (Lai et al. 2013). Interest in excitotoxicity began after the initial observation that monosodium glutamate was neurotoxic (Olney 1969). Subsequent studies identified the essential importance of excitotoxicity in stroke pathogenesis and several treatments have been developed with an aim to target specific aspects of this pathway (Asoh et al. 2002). Under normal physiological conditions glutamate is the major excitatory neurotransmitter and glutamatergic signaling is necessary for a diverse range of aspects of normal brain development and function including neuronal growth (Mattson et al. 1988), axonal guidance and synaptic plasticity (Abbott and Nelson 2000). Several glutamate receptor subtypes exist in the brain. The N-Methyl D-Aspartate receptor (NMDAR) is of particular interest in the context of stroke (Lees 1997). These receptors act as a sensor detecting levels of extracellular glutamate and processes these signals accordingly (Lai et al. 2013). Following ischaemic injury there is a profound increase in extracellular glutamate levels in the brain, this results in an influx of calcium ions through ionotropic receptors and release of calcium from intracellular stores. Increased intracellular calcium release results in the activation of a plethora of cell death signaling proteins which act to promote neuronal cell death.

1.5.3 Free Radicals

Following ischaemic injury there is an increase in free radical production in the brain resulting in oxidative stress (Radak et al. 2013). Generation of free radicals occurs predominantly as a result of reperfusion injury (Nour et al. 2013). A variety of different reactive oxygen species (ROS) are responsible for oxidative stress induced damage including superoxide, hydrogen peroxide and hydroxyl radicals. These reactive oxygen species are highly reactive and act to damage DNA in the nucleus of effected tissue, this in turn perturbs the normal functioning of the cellular machinery and triggers apoptotic signaling cascades. Oxidative stress is particularly harmful in the context of cerebrovascular injury as levels of endogenous antioxidant enzymes (such as superoxide dismutase) and antioxidant vitamins (such as α-tocopherol) are present at insufficient levels to ameliorate the increased oxidant production following stroke (Lo et al. 2003).

The brain is incredibly sensitive to oxidative damage as a result of reactive oxygen species due to a number of factors including the high basal aerobic respiration rate, high levels of peroxidisable lipids and abundant presence of iron which may act as a pro-oxidant during stress (Shirley et al. 2014). Primarily, the reactive oxygen species produced in the brain arise as a result of the mitochondrial respiratory chain, NAPDH oxidases and oxidase (Kahles and Brandes 2012; Sanderson et al. 2013; Vergeade et al. 2010). Under healthy conditions, superoxide is produced by mitochondrial metabolism as a byproduct of ATP generation. This superoxide is subsequently converted to hydrogen peroxide by superoxide dismutase before it is exported to cellular cytoplasm where it acts as an intracellular messenger (Rice 2011). Following stroke, oxygen levels are depleted prior to glucose driving a switch towards anaerobic respiration (Liu et al. 2004), this results in production of lactate acid and H^+ build up in mitochondria. Subsequently there is reversal of the H^+ uniporter on the mitochondrial membrane which results in H^+ accumulation in the cytosol (Ying et al. 1999). This acidic environment promotes oxidation as the positive hydrogen ions react with superoxide in order to produce hydrogen peroxide or hydroxyl ions. Superoxide may also react with nitric oxide producing the oxidant peroxynitrite and depleting cellular nitric oxide stocks. Glutamatergic signaling which occurs following stroke promotes increased intracellular production of nitric oxide, which contributes further to oxidant production. Peroxynitrite ions also promote apoptosis in the canonical fashion (Stanika et al. 2012). Following recanalization of the ischaemic brain after stroke the patient is subject to a well-documented reperfusion injury. Following recovery from ischaemia the mitochondrial respiratory chain is restored, this results in reversal of complex I of the chain and a subsequent overproduction of superoxide (Yamato et al. 2003; Peters et al. 1998; Chen et al. 2008). Additionally, as well as production of reactive oxygen species, nicotinamide adenine dinucleotide phosphate-oxidases (NOXs) make a considerable contribution to ROS production in the context of stroke. The role of these molecules is most prevalent in the reperfusion injury. Nox family contains seven members with NOX2, NOX3 and NOX4 displaying characteristic high levels of expression in the central nervous system. Under normal physiological conditions NOX proteins generate reactive oxygen species necessary for cellular function, but in ischaemic disease they produce an abundance of reactive oxygen species which contribute to pathological oxidative stress (Chen et al. 2009; Bokoch and Knaus 2003). Nox1 is of particular interest in the context of stroke. Nox1 is abundantly expressed in the cerebrovasculature

compared to other isoforms of NADPH oxidases suggesting potential functional relevance in the context of stroke (Ago et al. 2005). Knockout of Nox1 was found to reduce cerebral edema and infarct volume in mice subjected to 60 minutes of ischaemia with 23 hour of recovery. This was associated with improvements in the integrity of the Blood-Brain barrier (Kahles et al. 2010). It should be noted however, that other in other studies into transient and permanent cerebral ischaemia, ablation of Nox1 does not confer protection meaning that further studies are necessary to better characterise the role of this enzyme in ischaemia-reperfusion injury (Kahles et al. 2010; Vallet et al. 2005; Kleinschnitz et al. 2010). Suppression of Nox1 expression via the use of adeno-associated virus-mediated transduction of shRNA was recently demonstrated to have protective effects in the peri-infarct region of rats subjected to a transient 60 minutes of ischaemia (Choi et al. 2015).

Xanthine oxidase is an enzyme which promotes oxidation of hypoxanthine to convert it to xanthine, as well as oxidation of xanthine to uric acid. Under ischaemic conditions adenosine triphosphate is catabolized to hypoxanthine and xanthine oxidase is activated. When reperfusion occurs, the accumulated hypoxanthine and xanthine react with xanthine oxidase in order to produce reactive oxygen species superoxide and hydrogen peroxide (Granger et al. 1981; Parks and Granger 1986). Whilst xanthine oxidase catalyses the oxidation of hypoxanthine to xanthine producing superoxide (Ardan et al. 2004), xanthine dehydrogenase acts on the same substrates to produce NADH instead of $O2^{-}$. Due to the importance of ROS in stroke xanthine is of more interest in this context, but it is worth bearing in mind that xanthine dehydrogenase may be irreversibly converted to xanthine oxidase (Engerson et al. 1987).

Succinate is another player contributing to oxidative stress in the context of reperfusion injury. Under normal physiological conditions, succinate is an intermediary in the citric acid cycle of the mitochondria. However, following ischaemic injury there is an accumulation due to reversal of succinate dehydrogenase driven by excessive fumarate production from purine nucleotide breakdown. During reperfusion the accumulated succinate undergoes rapid reoxidization by succinate dehydrogenase. This results in the production of reactive oxygen species (Chouchani et al. 2014).

1.5.4 Inflammation

The glutamate mediated increase in intracellular calcium results in the promotion of several pathways relating to inflammation. Inflammatory transcription factors such as HIF (Mojsilovic-Petrovic et al. 2007), Stat3 (Suzuki et al. 2001) and NF-κB (Harari and Liao 2010) are activated following injury. Following this, several mediators of inflammation are upregulated and endothelial adhesion markers are expressed. These changes result in the recruitment of inflammatory cell types such as macrophages and monocytes which are recruited to the brain and this serves to contribute to the ischaemic damage worsening phenotype (Kochanek and Hallenbeck 1992).

In the early stages following obstruction of the cerebrovasculature by stroke, there is activation of proinflammatory signalling due to translocation of the adhesion molecule p-selectin in the membranes of endothelial cells and activated platelets which have been subjected to oxidative stress (Peerschke et al. 2010; Pinsky et al. 1996; Yilmaz and Granger 2010). Additionally, thrombin which is generated by activated platelets promotes the conversion of fibrinogen to fibrin. Accumulating fibrin acts to trap additional platelet cells and leukocytes resulting in clot formation which can in turn lead to further occlusion and exacerbation of ischaemic damage (del Zoppo et al. 1991).

Located within the brain parenchyma, microglia are responsible for the generation and release of the majority of inflammatory mediators in the brain. After onset of cerebral ischaemia there is an increase in extracellular levels of ATP and UTP in the parenchymal tissue of the brain occurring in response to excitotoxicity, oedema and membranous degradation of neuronal cells (Melani et al. 2005). The increased concentration of ATP in the extracellular space results in the activation of P2X7 receptors on the surface of the microglia, stimulating further release of additional pro-inflammatory factors such as NO, reactive oxygen species and cytokines (Korcok et al. 2004).

In the absence of pathological physiology, cell-cell communication within the brain acts to establish and maintain polarisation and quiescence of microglia. For instance, CD200 is a protein expressed on neuronal membranes which associates with the corresponding CD200R receptor on microglia. When this protein interacts with the equivalent receptor the effect is promotion of the resting phenotype (Lyons et al. 2007). Following ischaemic injury there is a deficiency in expressed levels of this protein which results in microglial

activation. Similarly, Fractalkine (CX3CL1) interacts with a microglial receptor CX3CR1 which allows for cellular quiescence under normal conditions, but during stroke diminished interactions occur due to matrix breakdown and microglia become activated promoting inflammation (Dénes et al. 2008).

Later on in the progression of ischaemic stroke, multiple molecular signals produced from the digestion of matrix proteins are released from dead cells. These proteins are called danger-associated molecular pattern molecules (DAMPs) (Chen and Nuñez 2010). DAMPs bind to toll-like and scavenger receptors on several cells in the neurovascular unit acting to stimulate release of additional pro-inflammatory signalling molecules like IL-6 and TNF via NFκB activation (Marsh et al 2009). DAMPs also result in the priming of dendritic cells for antigen presentation. This interaction illustrated the main cross over between innate and adaptive immunity after stroke. Despite the fact that the traditional view that the role of the immune system in response to ischaemic stroke emphasises the importance of infiltration of inflammatory signals from the circulation to the brain parenchyma, chemical signals from located in the parenchyma locally at the site of ischaemia are equally important, and that there are complex feedback loops based upon interaction between these two limbs of the immune response (Shirley et al. 2014).

1.5.5 Apoptosis

Following the initial bout of necrosis there is a second wave of cell death which occurs in the brain over 2-3 days following cerebrovascular injury. This phenomenon is referred to as delayed neuronal death (Kirino 2000; Nitatori et al. 1995). This postponed period of apoptotic cell death is of particular interest to stroke researchers as it provides an array of druggable targets for clinical interventions for stroke beyond the therapeutic window of traditional thrombolytic therapy . The main mechanism by which delayed neuronal death occurs is glutamate-calcium mediated excitotoxcitiy as described previously (Kirino 2000). Apoptosis pathways can be classed as intrinsic (Figure 1.4) and extrinsic pathways (Figure 1.5) (Broughton et al. 2009).

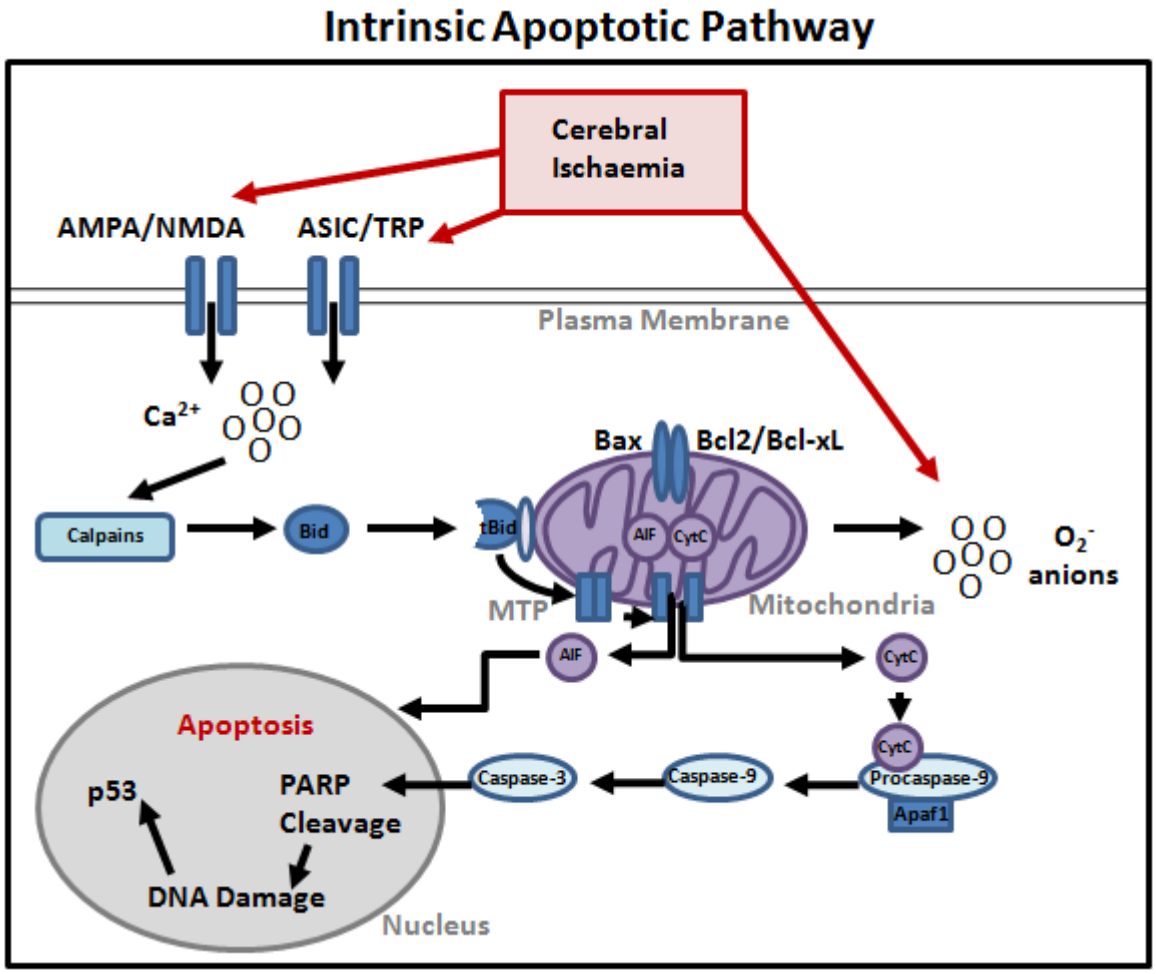

Figure 1.4 – Intrinsic apoptotic pathway – Cerebral ischaemia results in an influx of Ca^{2+} ions into neurons, this results in activation of calpains which in turn drive apoptosis pathways. There is also resultant generation of free radicals in the brain in the form of O_2^- ions which react with DNA causing damage and promoting apoptosis.(Broughton, Reutens, and Sobey 2009).

The most widely studied receptor which mediates post-ischaemic apoptosis is the NMDA receptor (Ikonomidou et al. 1999; Yu et al. 1999). Following stimulation by increased levels of glutamate there is an influx of calcium into neuronal cytoplasm. This results in an activation of calpain which mediates the cleavage of Bcl-2 interacting domain (BID) to an active form (tBID) (Raynaud and Marcilhac 2006). Activated BID translocates to the mitochondria and promotes cell death. Following the opening of mitochondrial pores apoptosis-inducing factor (AIF) and Cytochrome C (CytC) are released. CytC binds to Procaspase 9 promoting activation of the apoptosome. Procaspase-9 matures to form Caspase-9 which then cleaves Caspase-3, the cleaved caspase-3 is able to enter the nucleus where it promotes apoptosis. Another mechanism by which apoptosis occurs it via the caspase-independent translocation of apoptosis inducing factor (AIF) to the nucleus where it promotes apoptosis (Daugas et al. 2000).

Extrinsic mechanisms of apoptosis result as a result of stimulation of plasma membrane death receptors from the tumour necrosis factor receptor including Fas and TNFR-1 (Fulda and Debatin 2006; Beurel and Jope 2006). Once death receptors are activated, cleavage of procaspase-8 is initiated to form caspase-8. The activated caspase-8 is then released into the cytoplasm promoting cleavage of caspase-3 ultimately resulting in apoptosis (Broughton et al. 2009).

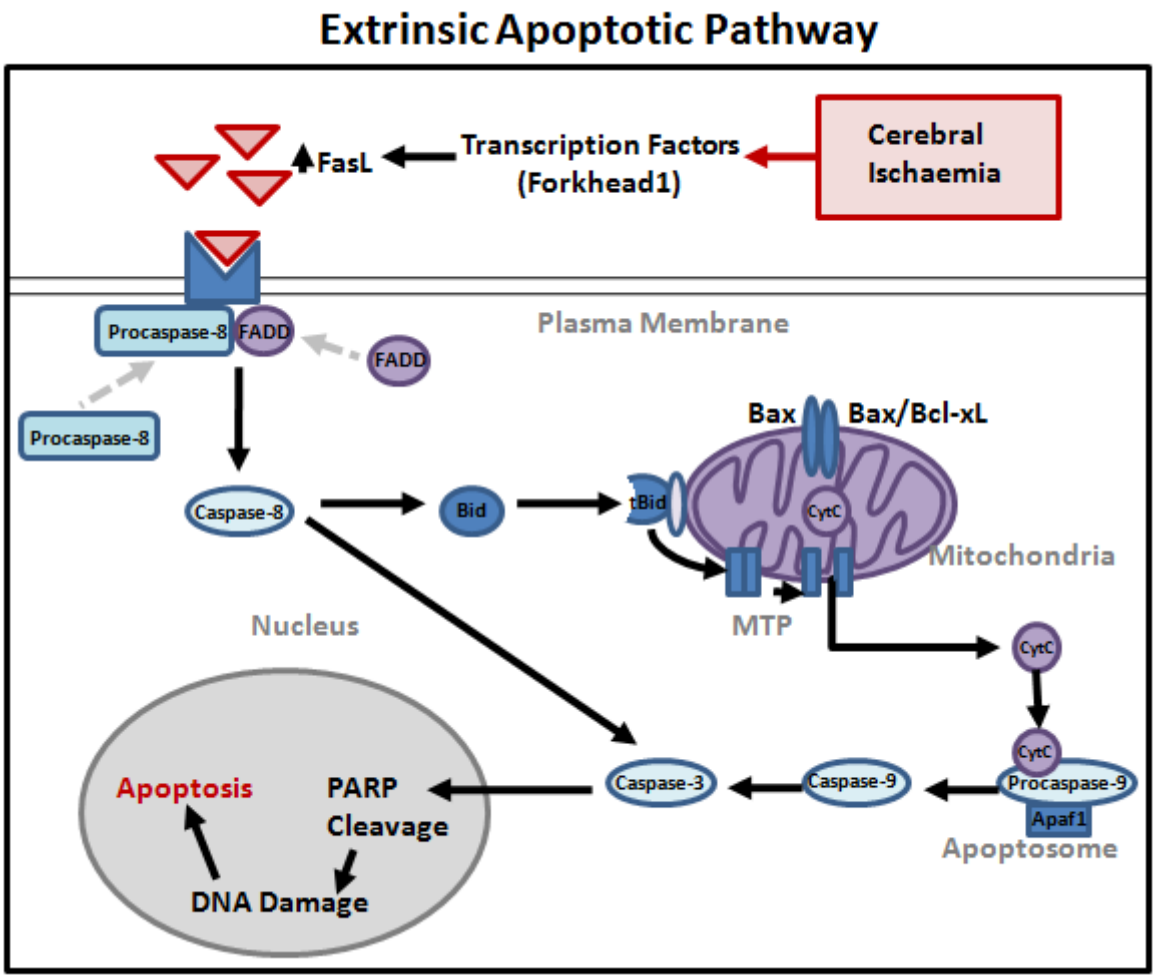

Figure 1.5– Extrinsic apoptotic pathway – Cerebral ischaemia results in the activation of transcription factors such as Forkhead1 which increase FasL expression. These ligands bind to Fas receptors and promote apoptosis via caspase activity (Adapted from Broughton, Reutens, and Sobey 2009).

1.6 Thrombolytic Therapy for Stroke

Currently the only clinically approved pharmacological treatment for ischaemic stroke is recombinant tissue plasminogen activator (rTPA) or Alteplase (Figure 1.6) (Hacke et al. 2008). Plasminogen activator is a molecule produced endogenously as part of the intrinsic thrombolytic pathways which regulate blood clot removal. The peptide was first

developed for thrombolytic treatment of Myocardial Infarction and was originally approved by the FDA for treatment of this disease (Rogers et al. 1987). Following the success of this treatment in context of another ischaemic disease, clinical researchers sought to determine whether this treatment could be of use in the treatment of ischaemic stroke.

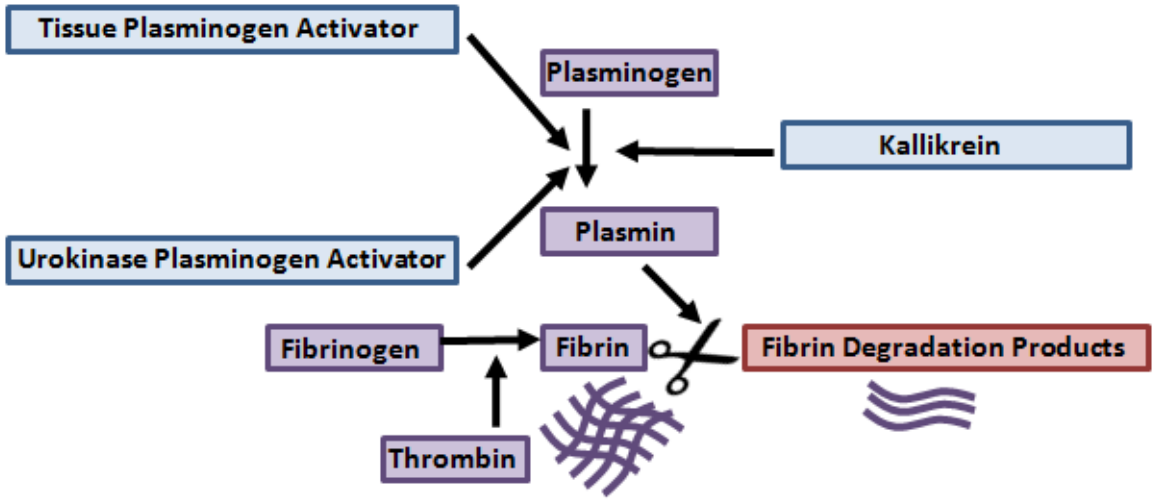

Figure 1.6 - Pathway regulating fibrin degradation – Tissue plasminogen activator acts to convert plasminogen into plasmin, this promotes the cleavage of fibrin to produce fibrin degradation products. In the context of stroke this results in the breakdown of clots with more specificity than traditional thrombolytics (Schäfer and Werner 2008)

Given that one of the complications associated with untreated stroke is haemorrhagic transformation and that haemorrhage is one of the potential side effects observed following Alteplase administration; great care was taken in order to ensure that the risks for patients were minimized (Adeoye et al. 2011).

To date there have been several trials assessing the safety and efficacy of Alteplase in the treatment of ischaemic stroke. Despite the fact that rTPA is widely used, the benefit of this treatment and the conditions under which it is beneficial remain under debate. Some criticisms have been made of some of these trials due to the involvement of Genetech, the company who produces the drug (Lindley et al. 2005).

The first rTPA for ischaemic stroke study was the NINDS trial which was published in 1995 ("Tissue Plasminogen Activator for Acute Ischemic Stroke" 1995). The observations made during this trial resulted in Alteplase therapy being granted FDA approval for the treatment of ischaemic stroke up to 3 hours following onset of symptoms and after

confirmation of ischaemic stroke by CT and/or MRI scan and consensus agreement of diagnosis by stroke clinicians.

The second successful Alteplase for ischaemic stroke study was the ECASS III study which demonstrated acceptable safety and efficacy when treatment was extended to 4.5h (Hacke et al. 2005, 2). Again, it was attempted to treat patients at greater time points following stroke in order to ensure that the greatest number of patients who may benefit from thrombolytic therapy may receive it. 821 patients were treated with alteplase (418) or placebo (403). At 90 days following treatment 52.4% of patients who received alteplase demonstrated favourable outcomes versus 45.2 in the placebo group. It was however noted that the alteplase treated patients exhibited a higher degree of haemorrhagic transformation following treatment (Hacke et al. 2008). Several subsequent trials have looked to extend the window of treatment to 6h, as well as administering treatment to older patients. The results of these studies are difficult to interpret. Whilst those who survive treatment seem to benefit from it, there is a significantly increase mortality period in late treated individuals in the first week following stroke (Simon et al. 2004; DeMers et al. 2012).

Some researchers are attempting to improve thrombolytic therapy for ischaemic stroke via the development of novel thrombolytic agents. Hoping for improved safety and or/efficacy. Desmoteplase is another, fibrin-specific, thrombolytic compound which has been extensively clinically investigated in relation to stroke, the initial desmoteplase in acute stroke (DIAS) trial was primarily a safety phase IIb trial which also sought to identify sensible doses for treatment of acute ischaemic stroke patients with perfusion-diffusion mismatch of MRI from 3 to 9 hours from onset of symptoms. Patients were randomised and received 25, 37.5 or 50mg of the drug compared to placebo. It was noted in this trial that there was a significant increase in the onset of intracranial haemorrhage in patients treated with desmoteplase. In order to address this, dosage was reduced and administered based on bodyweight. This trial demonstrated that 54.3% of patients who were treated within 3-6h achieved reperfusion and 40% of those treated from 6-9h achieved reperfusion associated with improved clinical outcomes (Werner Hacke et al. 2005). A follow up phase III trial (DIASII) further investigated the compound, looking at efficacy from 3-9 of symptom onset. Low NIHSS scores in the recruited patients meant that patients recruited had more severe stroke than the previous trial and as such the

evidence for efficacy at this time point was unconvincing (Werner Hacke et al. 2009). This thrombolytic is currently being investigated in two phase-3 clinical trials (DIAS-3 &DIAS-4)(Tsivgoulis et al. 2014)

1.7 Mechanical Clot Retrieval

Inspired by the success of endovascular catheterization based approaches to treating coronary artery disease, and also in an effort to circumvent the haemorrhagic side-effects of pharmacological thrombolytics, there has been an effort towards implementing mechanical clot retrieval in the treatment of ischaemic stroke (figure 1.7).

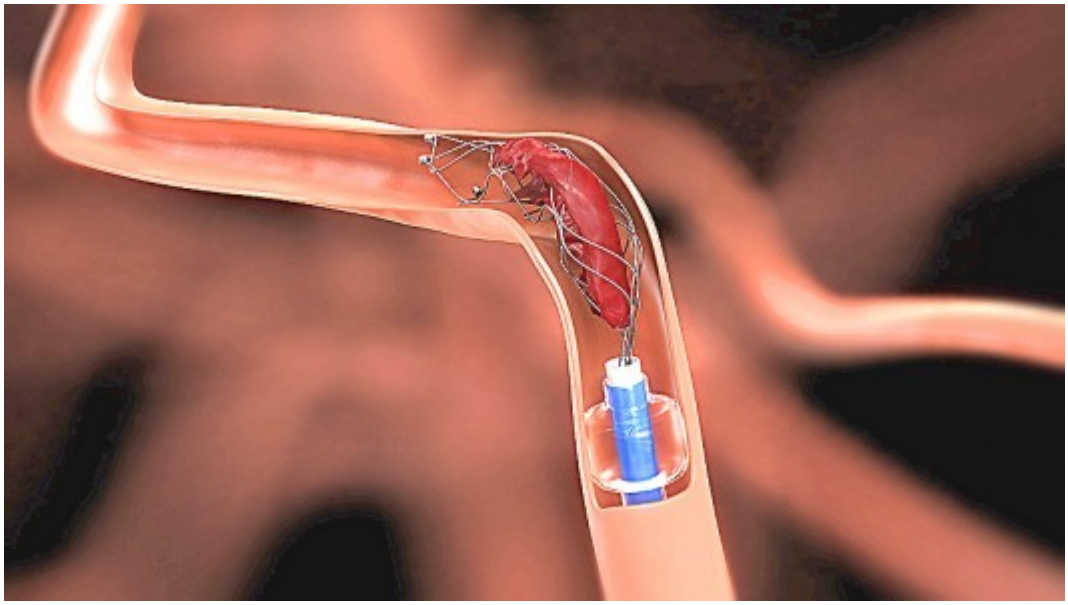

Figure 1.7 - Solitaire FR clot retrieval device – The solitaire clot retrieval device is one of the clinically approved clot retrieval devices on the market. A catheter is advanced to the site of occlusion and a balloon is inflated to prevent backflow of blood (which would potentially result in further distal occlusions) a mesh net is advanced around the clot and withdrawn to remove the clot. (Covidien 2015)

The principle of these devices is that once the site of the clot is identified, a catheter is inserted endovascularly and guided to the site of occlusion. A balloon on the catheter is inflated to provide structural support to the vessel and prevent flow disturbing the clot during the retrieval process. A wire net is extended from the tip of the catheter and used to surround the clot. Once the clot is secure, the catheter is removed and with it the source of ischaemia.

Though the use of these devices clinically for treatment of stroke has occurred only relatively recently, they do appear to offer promise as a therapeutic strategy. One group has demonstrated an impressive success rate with the use of the Solitaire FR clot retrieval device (Machi et al. 2012) demonstrating that recanalization was successful in 89% of the 56 patients treated. In this study, use of endovascular thrombectomy was used as a second line of treatment following tPA in patients who presented within 4.5h of onset of stroke; patients who presented 4.5-6h were treated by thrombectomy alone. Another more comprehensive multi-center study assessing efficacy of this device has reported similar results (Dávalos et al. 2012). Improvement of clinical outcomes in thrombectomy treated patients is greatest in those who were first treated with tPA. These early results suggest that there is a place for clot busting devices in the clinic, offered as an adjunct to unsuccessful thrombolytic therapy or for patients who do not meet the tPA criteria.

One of the problems associated with use of this device is that clots may become fragmented during retrieval and result in additional embolic ischaemia resulting from translocation of the dislocated fragments (Siu et al. 2014). Another issue associated with these devices is advancement of the catheter along tortuous regions of the cerebrovasculature. It is likely that as use of these devices is increased, refinement in design of these devices will result in improved safety and efficacy.

Another example of the successful implementation of clot retrieval in the clinic can be observed in the recent MR CLEAN trial. This randomised controlled multi-centre study used combinations of intra-arterial thrombolysis and mechanical clot retrieval with the MERCI device in order to attempt to treat 500 patients with ischaemic stroke (Fransen et al. 2014). 233 patients received intraarterial treatment, whilst 267 received usual care only, it was found that treated patients demonstrated a 13.5% improvement in the rate of functional independence versus controls suggesting that this treatment strategy is effective in treating proximal intracranial occlusion of the anterior circulation when administered within stroke onset (Berkhemer et al. 2015).

1.8 Neuroprotection

Whilst the majority of stroke intervention research has focused on thrombosis and platelet aggregation other alternative strategies have been considered. The most widely studied alternative treatment strategy for intervention in the context of stroke is arguably

that of neuroprotection. Virtually no publications relating neuroprotection to stroke exist prior to the early 1990s, but in the decade following hundreds of publications have entered the scientific literature (Ginsberg 2008). Stroke displays a complex sequence of events following onset of stroke. Recanalization in the acute phase following stroke aims to mitigate hypoxia-mediated necrosis following ischaemic injury (Rha and Saver 2007). Following the initial wave of cell death due to necrosis, there is a second wave of delayed neuronal cell death which is regulated by apoptosis and other cell-signalling pathways (Kirino 2000). The concept of neuroprotection is that through the use of pharmacological agents that target aspects of these cell signalling pathways in the sub-acute phase following stroke the extent of injury may be ameliorated and recovery promoted (Majid 2014).

Several drug classes have been assessed for potential usage in stroke treatment targeting several different pathways. Many of the pre-clinical studies demonstrated extremely promising results, though all failed in subsequent clinical trials. The most high profile failure has been that of NXY-059 (disodium 4-[*tert*-butyliminomethyl] benzene-1,3-disulfonate N-oxide)(Diener et al. 2008). This compound was derived from a generic nitrone spin-trap compound and had demonstrated convincing preclinical neuroprotection in models of stroke via reduction in the presence of free radicals. This compound was developed by Astra-Zeneca and entered into two phase I/II clinical trials. The first of which (SAINT-I)(Saver 2007) yielded positive results whilst the second (SAINT-2) was negative (Savitz and Schäbitz 2008). This was the closest that a neuroprotectant had come to entering clinical practice and the failure to demonstrate efficacy resulted in AstraZeneca halting development of the compound. Several reasons have been posited for the differences observed between the two trials. The researchers cite methodological differences whilst Peter H. Proctor (Proctor and Tamborello 2007) has suggested that the neuroprotection observed in the first phase I/II trial was the result of by-products of degradation of the notably unstable compound. Following these results, much of the industrial funding into stroke therapeutics was diverted elsewhere whilst many clinicians have expressed a lack of faith in preclinical research results in general. Following improvements which have been made in pre-clinical assessments of stroke therapeutics there have been new developments in the potential future use of neuroprotectants as new drug candidates are progressing through the clinical trial pipeline.

A recent candidate which is being investigated as a potential neuroprotectant for stroke is NA-1 (Hill et al. 2012). Increased glutamate release following stroke results in excitotoxicity contributing to delayed neuronal death (Choi 1992). This has long been well characterised, although several therapies which target this pathway have resulted in pathological effects due to the complexities in its mechanistic regulation. The excitotoxic effects of excessive glutamate signalling are mediated through its interaction with NMDA and AMPA receptors (Reynolds and Hastings 1995; Soundarapandian et al. 2005). Several neuroprotective compounds were assessed in pre-clinical models of stroke. Whilst initial evidence from animal models was promising, this failed to translate to clinical trials (Ikonomidou and Turski 2002). In addition to the pathogenic effects of these drugs, many of the NMDAR-antagonists had considerable psychological side effects which rendered this treatment unsuitable. In order to circumvent the negative effects of NMDAR targeted therapeutics it was determined that more specific drugs were necessary in order to dissect out the beneficial mechanisms from the negative ones. In order to achieve this goal, post-synaptic density-95 protein was targeted (Sun 2013). This scaffolding protein links NMDARs and effectors like NOS which are involved in the mediation of neurotoxic signalling. Fusion of the final 9 C-terminal residues of the NMDA NR2B subunit to the HIV Tat protein resulted in the production of a PSD-95 inhibitory molecule with considerable specificity and efficacy called NA-1 (Hill et al. 2012). In order to ensure a high degree of translation from lab to clinic, consideration of the STAIR guidelines was undertaken (Saver et al. 2009a). In addition to the commonly implemented models of stroke it was determined that use of higher-primate models was necessary in order to assess the efficacy of this group in models which effectively represented human pathology. Use of PSD-95 inhibitors in primate models of stroke where occlusion was achieved by use of titanium aneurysm clips demonstrated that treatment with NA-1 resulted in a 55% reduction in infarct size at 24h and 70% at 30 days determined by T_2-weighted MRI (Cook et al. 2012). The reduction in infarct size was associated with a functional improvement in behavioural measures including nonhuman primate stroke scale (NHPSS) and a sensorimotor battery of tasks including the hill and valley task, two-tube task and six-well task (Cook et al. 2012). This evidence supported the hypothesis that such a treatment would translate well to the clinic. The potential neuroprotective effects of NA-1 were addressed by the ENACT trial. In this double-blinded clinical trial patients with intracranial aneurysm were treated with the drug following surgical endovascular repair of their

aneurysm. Efficacy of the drug was assessed by MRI scan and a follow up clinical assessment 30 days following treatment. Of the 92 Patients treated with NA-1 at the end of their surgery a significant reduction in the presence of cerebral lesions was present (adjusted incidence rate ratio 0·53) when assessed by DWI and FLAIR scans compared to 93 controls who received placebo (Hill et al. 2012).

1.9 Stem Cells

An emerging and exciting future treatment for ischaemic stroke is stem cell based therapy. Currently, the focus of stem cell therapeutics is to use the administration of exogenously applied cells in order to promote repair and recovery of damaged tissue, although in the future stem cell therapy could potentially lead to the regeneration of damaged tissue. Historically, it was thought that neuronal cells displayed no regenerative capacity, in contrast to most other cell types in the human body. However, following the discovery of an endogenous adult stem cell population in the subventricular zone (SVZ) and olfactory bulb (Garcia et al. 1995; Kirschenbaum et al. 1999)(Ming and Song 2011) hopes were raised with regards to neuroregeneration. Subsequent studies have demonstrated that in animal models of stroke there is an increase in proliferation of this cell type. It has been suggested that proliferation of this cell population is regulated by a diverse range of cell signalling pathways including Notch (Hitoshi et al. 2002), Bone Morphogenetic Protein (Temple 2001), Tumour necrosis factor (Widera et al. 2006) and Sonic hedgehog (Lai et al. 2003). Administration of erythropoietin demonstrated an increase in SVZ stem cell proliferation in preclinical models of stroke (Shingo et al. 2001). However, a large clinical trial conducted on 522 patients with acute ischaemic stroke demonstrated that patients treated with EPO (Erythropoietin, a glycoprotein hormone) exhibited a higher mortality rate than placebo controls with no measurable improvement in phenotype, suggesting no efficacy for a potentially dangerous treatment (Ehrenreich et al. 2009). A subsequent in-depth analysis of these data has suggested that certain sub-groups in the study, namely those who had not received thrombolysis, may have benefitted from EPO as demonstrated by a reduction in the presence of circulating markers of damage post-stroke (Ehrenreich et al. 2011).

Exogenous neural stem cells may be generated from several sources including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and populations of adult stem cells isolated from various different sources (Okita et al. 2007). There are advantages and

disadvantages relating to each type of cell, and it is not yet clear which will be the best for use in the context of stroke in the future (Kalladka and Muir 2011). iPSC cells have the advantage of being allogeneic if taken from the patient's own tissue, however there are issues relating to terratoma formation (Oki et al. 2012). Embryonic stem cells have the advantage of pluripotency, but they are also oncogenic and difficult to obtain patient matched cell stocks. Adult stem cells can be isolated from bone marrow (haematopoietic stem cells) and adipose tissue (adipose tissue derived adult stem cells), these do not demonstrate the same degree of potency as ESCs or iPSCs, but it is possible to obtain patient specific cell populations, and there is little risk of oncogenesis (Haas et al. 2005).

The only clinical trial for use of stem cells in stroke to date is the PISCES trial, a safety trial where the ReNeuron ReN001 cell line was used to treat patients 6-24 months following the onset of stroke (Smith and Gavins 2012). The ReN001 cell line is a neuronal stem cell line immortalised by the fusion transgene c-mycERTAM to allow controlled expansion when cultured in the presence of 4-hydroxytamoxifen (Stroemer et al. 2007). This initial phase 1 clinical trial sought to determine the safety profile for this cell line and was successful in doing so. This trial has been approved for progress in order to determine whether efficacy of this treatment merits further research. Initial results have been promising and the stroke community awaits further results with anticipation. If functional improvement can be demonstrated in the clinic through the use of stem cell therapy, this would serve to revolutionise stroke medicine (Smith and Gavins 2012; Kalladka and Muir 2011).

1.10 Pre-clinical Models of Stroke

In order to determine novel potential therapeutic interventions for stroke it is essential to assess these treatments in pre-clinical models of stroke. There are several models available for such investigation.

1.10.1 *In vitro* models.

In vitro models provide an effective means of which to model mechanisms underlying therapeutic interventions in the context of stroke. In vitro models of stroke can utilise a variety of tissue sources in order to address specific aspects of the neurovascular unit.

Over the course of modern pre-clinical research a diverse landscape of *in vitro* techniques have been developed as researchers strive to identify the best models of stroke (Mehra et al. 2012). Currently there is no 'gold standard' for cell culture-based assessment of stroke interventions as every method is a trade-off between different strengths and weaknesses. The major advantages of these models are the low cost, and less ethical issues resulting from a reduction in the numbers of animals used. There is an increase in throughput due to the reduction in time it takes to perform a single experiment as well as the ability to perform multiple biological repeats in tandem. The biggest criticisms that may be levied at these models relate to the fact that they are not a true representation of what happens in the whole system of a stroke in situ.

Sources of tissue that are commonly implemented in vitro include primary cell cultures of neuronal cells (Goldberg and Choi 1993), organotypic cell cultures (Vornov et al. 1994) and entire slice sections of the brain (Dong et al. 1988). Ischaemic insult can be modelled by oxygen glucose deprivation followed by reoxygenation. Cells are placed in low-glucose serum free media and incubated in a hypoxic chamber for a pre-defined length of time before complete media is re-introduced and allowed to recover under normoxic conditions (Ord et al. 2013). This is widely used by stroke researchers as it is analogous to the ischaemia-reperfusion injury present in *in vivo* models of stroke. The neurovascular unit demonstrates considerable complexity with regards to the presence of highly heterotypic cell populations as well as a sophisticated cytoarchitecture. Neuronal, astrocytic, microglial and endothelial cells may all be assessed *in vitro* separately or in co-culture models. It is likely that moving forward in vivo models will be necessary to conduct experiments in combination with in vitro models of new therapies in order to ensure maximal translation of stroke research from bench to bedside.

1.10.2 *In vivo* models.

In order to assess stroke interventions, prior to the commencement of clinical trials it is necessary to obtain robust pre-clinical data in animal models of stroke (Albers et al. 2011). Earlier research made extensive use of cats, dogs (Corkill et al. 1978; Purdy et al. 1989) and non-human primates (Moseley et al. 1975; Laurent et al. 1975; Tranmer et al. 1992). However, due to animal costs and ethical issues associated with use of these mammals, most modern research is conducted in rodent models; primarily rats and mice. These

models can be subdivided into models of global ischaemia and models of focal ischaemia (Hunter et al. 1995). Global ischaemia is achieved by a temporary complete occlusion of cerebral blood flow followed by a period of reperfusion. This can be achieved by transient occlusion of both common carotid arteries with or without permanent occlusion of vertebral arteries. Global occlusion models of cerebral infarction results in the production of large reproducible infarcts due to selective and delayed neuronal cell death induced predominantly by apoptosis, however this model is criticized as being closer to the clinical manifestation of cardiac arrest than ischaemic stroke (Small and Buchan 2000). As a result of this, the focal ischaemia models are most commonly implemented today (Hossmann 2012). Focal ischaemia is achieved by occlusion of one of the terminal branches supplying the cerebrum. In the past the anterior cerebral artery was frequently occluded (Freemon 1971), however cerebral infarction following anterior cerebral artery occlusion is not commonly seen in the clinic. The most frequently occurring ischaemic stroke observed in the clinic is a result of occlusion of the left middle cerebral artery (Longa et al. 1989). For this reason middle cerebral artery occlusion models of stroke are the most commonly studied today (Macrae 2011). Both transient (tMCAO) and permanent (pMCAO) middle cerebral artery occlusions are studied by this model, transient occlusions allow for reperfusion into the brain and are representative of strokes where recanalization has been achieved. The monofilament model of tMCAO is the most frequently implemented. The advantages of this model include relative ease of use versus other models of stroke; however variation in infarct size with this model may be a problem. Whilst some groups preserve circulation in all arteries, others permanently occlude some branches by cauterization. Sealing blood vessels results in a worsened phenotype following stroke and also may influence variability in stroke size. For example, it has been demonstrated that permanent occlusion of the pterygopalatine artery alongside MCAO results in less variability than when this vessel is preserved (Chen et al. 2008).

Another model of stroke which is commonly implemented is the distal diathermy model of stroke which is reported to exhibit a higher degree of reproducibility (Tamura et al. 1981). Following anaesthesia, the surface of the cerebrum is exposed by cranioectomy. The middle cerebral artery is then occluded by cauterization by diathermy forceps. This model has been demonstrated to produce less variation in infarct size, but the surgical technique is more challenging to perform and reperfusion is not possible.

The embolic model of stroke is achieved by taking a blood sample from the subject animal and allowing it to clot, before injecting the clot back into the circulation to block the middle cerebral artery. This model is closer to that observed in the clinic due to the involvement of cell signalling events relating to embolus presence. However, this model is considerably more variable than other models (Hashimoto et al. 2010a).

Whilst the majority of research has been conducted in rodent models due to ethical issues and financial restraints, it is likely that to ensure maximal translation of stroke research pre-clinical trials in higher primates will be necessary. Each of the models and variations of these models has strengths and weaknesses; Employment of multiple models to assess potential therapeutic interventions will also be beneficial offering additional confidence for translation. The Multicentre Preclinical Animal Research Team (Multi-PART) is an initiative which is seeking to achieve this goal by international collaboration in order to establish and implement a platform for international multicentre stroke trials using randomized clinical trial design and a multi-centre, multi-model paradigm ("Welcome to Multi-PART" 2015).

There are many strengths and weaknesses of the animal stroke models versus *in vitro* techniques. The major strength of in vivo research over Petri dish based assays is that assessment of a complete physiological system of a living organism is a more accurate simulation of clinical stroke than a single cell. It also allows for assessment of more relevant physiological measures of stroke (i.e. lesion volume). Different animal models exist and each presents advantages and disadvantages. Non-human primates are arguably the closest organism to humans to study, but present the greatest ethical concerns regarding animal research. Rodents are the primarily used animals in early stage pre-clinical research. Whilst it is difficult to assess higher brain functions in rodent models, at the molecular level there is great similarity in the central nervous system across mammals. Different methods of stroke induction present different volumes of stroke, reproducibility and technical challenges. It is less technically challenging to modulate genes in mice than rats due to reduced amount of drug/viral vector/RNA mimic used, and the availability of transgenic mice, whilst the rat model benefits from less challenging surgery and greater reproducibility (Young et al. 2013; Gluck et al. 2002).

Using these models new and potentially revolutionary therapeutics can continue to be investigated by stroke researchers. Gene therapies in particular have hitherto untapped

potential, and within this class, non-coding RNAs remain one of the least understood therapeutics with arguably the most potential.

1.11 MicroRNAs

There was a time following the discovery of DNA, but preceding the complete sequencing of the genome that all DNA was responsible for coding a protein. Today this is still referred to as the 'central dogma' of molecular biology (Crick 1970). However, following the sequencing of many genomes (including that of the human) geneticists were faced with a very puzzling phenomena: only a tiny percentage of a genome was responsible for coding genes in humans (Venter et al. 2001). Initially this non-coding DNA which accounted for 98% of the human genome (versus the 2% of junk DNA in bacterial genomes) was deemed to be nothing more than an evolutionary artefact. However, several inquisitive minds started interrogating the mysterious genomic sequences and illuminated an entire microcosm of biology previously unknown to mankind (Doolittle 2013). The initial reasoning behind the human genome project was that 'if all diseases are genetic in nature' and 'we understand all of the genes' it would only be a matter of time before all diseases were understood and eventually cured. The truth was much less promising, though arguably much more interesting. There a whole host of types of non-coding RNAs, with novel functions being discovered every day, but my thesis is focussed on the class which arguably presents the greatest potential for medical interventions.

1.12 History

miRNAs are small non-coding molecules which act to post-transcriptionally inhibit expression of other genes. In that respect they are similar to the synthetic small interfering (siRNAs). The main difference (other than the fact that they are endogenously produced) is that whilst a single siRNA will act upon a single gene transcript, a single miRNA can potentially act upon thousands of different gene transcripts. Several other types of non coding RNAs exist which perform a variety of different functional roles in the regulation of gene expression including long non-coding RNAs, small nucleolar RNAs (snoRNAs), extracellular RNAs (exRNAs) and Piwi-interacting RNAs (piRNAs) which perform a diverse range of functions pertaining to the regulation of transcription and post-translational modification of gene products. Due to the relatively recent discovery of

these classes of RNA and the complexity of the operations they are involved in understanding in this area remains in its infancy (Morris 2012).

Whilst the first miRNA was described in *Caenorhabditis elegans* in 1993 (Lee et al. 1993), it was not until much later that the significance of this discovery was realised. The first miRNA discovered was initially named lin-4 and in 1993 it was described as interacting with a target gene lin-14 preventing translation to protein (Lee et al. 1993; Wightman et al. 1993). This was initially thought to be an unusual occurrence and was not considered much. This was the case until the discovery and description of let-7 (again in *C. elegans*)(Shabalina and Koonin 2008).It wasn't until this discovery that interest in post-transcriptional inhibition of gene expression by non-coding RNAs became more widely studied. A great deal of the recent progress in the understanding of epigenetic modes of control in the organism have been driven by the findings of the Human Genome project (Lander et al. 2001; Venter et al. 2001). Identification of large regions of highly conserved non-coding DNA sequences challenged the long-standing central dogma of molecular biology (Crick 1970). As it became apparent that there was considerably more complexity involved in the molecular mechanisms underlying life than was previously thought, interest in the epigenetic mechanisms of control became widely studied (Wolffe and Matzke 1999). Subsequently miRNAs were identified and described as being present in all eukaryotic organisms (Appasani 2008). The genetic age and relative simplicity of this class of genes raises some interesting philosophical questions about the evolution of life (Shabalina and Koonin 2008; Demongeot and Moreira 2007). But more interesting still are the questions miRNAs pose for modern medicine. As stated previously, a single miRNA can potentially act on thousands of transcripts, in many cases this single microRNA will inhibit several molecules in a single signalling pathway (Papagiannakopoulos et al. 2008). Therefore by modulation of a single miRNA species using pharmacological agents, the clinician could achieve potent modulation of entire pathways. As this form of regulation evolved, it could be argued that the effects of miRNA modulation would be synergistic and less prone to negative side-effects. It is for these reasons that proponents of miRNA-based medicines believe that RNA inhibition pathway (RNAi) based technologies are some of the most exciting novel therapeutic agents in development today (Nana-Sinkam and Croce 2012; Carmeliet and Jain 2011; Jamaluddin et al. 2011; Brown and Naldini 2009).

1.13 miRNA Biogenesis

The biogenesis of miRNAs involves synchronised activity of several aspects of cellular machinery and enzymatic reactions to take the miRNA from initial transcription through to ultimate inhibition of target transcripts (Figure 1.8). The initial step in the canonical process occurs when a pri-miRNA sequence is transcribed by RNA polymerase II (Cai et al. 2004). miRNA structure differs greatly between individual miRNAs across the genome. The miRNA can exist on its own, or with other miRNAs as part of a cluster. Following transcription of the pri-miRNA sequence, it becomes recognised and cleaved by the RNAse III-like enzyme Drosha (Lee et al. 2002) which is associated with the protein Pasha (Gregory et al. 2004). The double stranded pri-miRNA sequence which has been processed by the Drosha-Pasha complex is translocated from the nucleus into the cytoplasm via Exportin 5 (Bohnsack et al 2004). After it has been exported, the pre-miRNA sequence is cleaved by Dicer (another RNAse III-like enzyme) after which it forms a complex with RISC-loading complex subunit TARBP2. This results in the generation of a double stranded sequence ~22-23 bp in length. Traditionally the strands have been termed the active messenger strand (which is active and binds to target transcripts) and the passive guide strand (which is thought to degrade without any additional effects). This double stranded miRNA has a 3' 2 bp overhang and a 5' hydroxyl group (Grishok et al. 2001; Hutvágner et al. 2001). Following this cleavage the now mature miRNA sequence is loaded into an argonaute protein (generally Ago2). This protein-RNA complex then becomes associated with other aspects of the RNA-induced silencing complex (RISC), after which it associates with downstream target transcripts and can effect post-transcriptional inhibition of gene expression. Generally, only one strand of the miRNA duplex is able to associate with target transcripts (Matranga et al. 2005).Differences have been described in miRNA processing between vertebrate and invertebrate organisms relating to the function of Ago2 (Chendrimada et al. 2005; Jagannath and Wood 2009). It is unclear what differences in processing, if any, exist between mammalian species (Yoda et al. 2010). Suppression of target transcripts is mediated by interaction of a short 2-7 nucleotide sequence with a complementary sequence in the 3' Untranslated region of the target gene (Bartel 2009). In the case of a perfect match this can signal for degradation of the transcript, or else simply inhibition of translation (Huntzinger and Izaurralde 2011; Krol et al. 2010) (Figure 1.8).

Whilst the conventional wisdom of miRNA biology suggests that generally the 3' strand of the sequence is the messenger strand and the 5' sequence is the passenger strand, this is not always the case. There is also evidence which suggests that the active strand of a single miRNA can vary depending on cell type. For example, in one form of gastric cancer it was demonstrated that for some miRNAs the 3' strand was the active strand whilst the 5' strand was sometimes the active strand comparing healthy to cancerous cells (Li et al. 2012). A pertinent example of the passenger strand importance relating to cardiovascular biology is the discovery that miR-21* is the active form of the miRNA in exosomes derived from cardiac fibroblasts and that these miR-21 passenger strands mediate cardiac hypertrophy (Bang et al. 2014). This aspect of miRNA biology is little studied and in most cases remains poorly understood.

As more groups assess the complex role of miRNAs in health and disease, understanding of the cellular machinery which regulates these processes will also improve. Generally miRNAs are thought to be processed in the aforementioned 'canonical' process but emerging evidence is detailing instances where certain miRNAs under certain conditions can undergo 'non-canonical' processing. For example, if a miRNA exists in its own intron it can use the gene's intron splicing machinery to process itself (Okamura et al. 2007), whilst in some cases the Dicer slicing step can be performed directly by Ago2 (Cheloufi et al. 2010; Cifuentes et al. 2010). It is unclear in which context non-canonical processing of miRNA activity will be functionally significant.

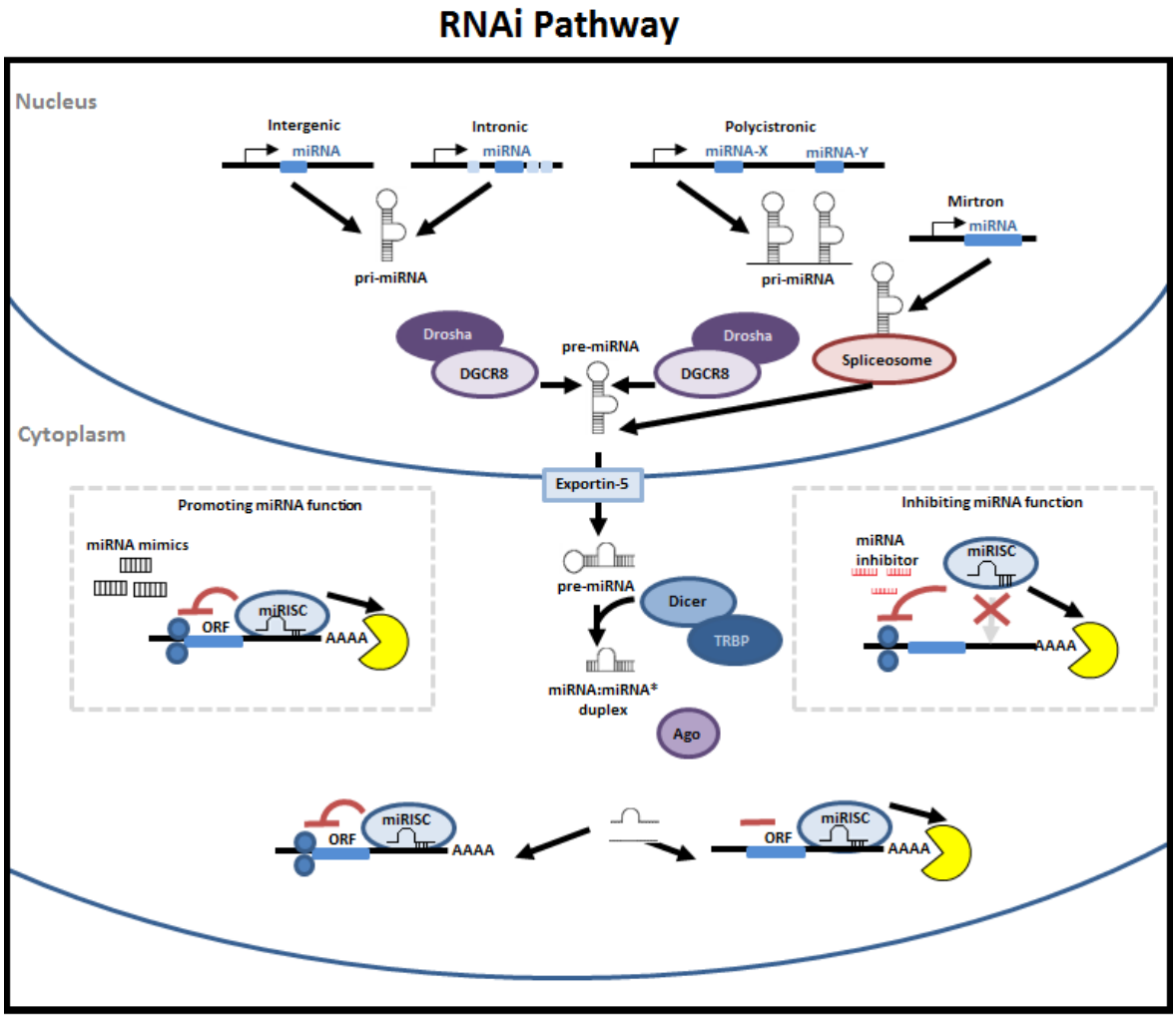

Figure 1.8 - miRNA biogenesis pathway (van Rooij and Kauppinen 2014) – miRNAs located in the genome in intergenic, intronic or polycistronic regions. Following transcription by the appropriate transcription factor the initial pri-miRNA sequence is transcribed. The pri-miRNA is then cleaved by Drosha to form the pre-miRNA sequence which is then exported from the nucleus to the cytoplasm via exportin-5. Following this, the pre-miRNA is cleaved by Dicer to form the mature miRNA sequence, Ago then promotes the dissociation of the two miRNA strands which then go on to interact with target transcripts via association with the RISC complex.

1.14 Therapeutic Applications

Following the identification of miRNAs, the description of the essential importance in many cellular pathways and their implication in several diseases, RNAi has become a widely studied avenue for potential novel gene therapies (Appasani 2008). The miRNAome offers thousands of miRNAs as potential agents of therapeutic modulation which potentially act on millions of genes (Jamaluddin et al. 2011). Traditionally gene therapy involves the modulation of a single gene to achieve increased or suppressed levels of a single protein. Whilst research in this field is ongoing, it remains to be widely implemented in clinical practice. One of the proposed difficulties of gene therapies is that

complex diseases are generally not regulated by a single gene. The added advantage of miRNA as a platform for gene therapy is that modulation of a single RNA sequence can effect a synchronized modulation in a variety of target genes. For example, miR-29 cluster members have been shown to interact with several aspects of the TGF-β signalling pathway including fibrogenic proteins such as collagen, fibrin and elastin (Zhou et al. 2012; Van Rooij et al. 2008). It has been demonstrated *in vivo* that miR-29 is a potent inhibitor of pro-fibrotic pathways and this may be of benefit in the treatment of fibrotic diseases (Kriegel et al. 2012; Cushing et al. 2011).

There are however, several caveats and potential pitfalls associated with RNAi based treatments (Castanotto and Rossi 2009). And it is for this reason that preclinical researchers need to ensure that we have a thorough, robust understanding of the mechanisms underlying the changes we observe before we try and take these treatments to clinic. Despite considerable scepticism from some contingents of the biomedical community, miRNA research continues to be translated towards the clinic with expediency. For example, Miravirsen (a miR-122 inhibitor) is currently undergoing Phase IIa clinical trials for the treatment of Hepatitis C Virus (Gebert et al. 2014; Janssen et al. 2013). Although this breakthrough may not at first seem to have direct implications in the use of miRNA therapeutics in the context of cardiovascular disease, the legal framework being develop and precedents set in getting this drug licensed for patient use will remove many of the obstacles in getting new drugs to market, whilst setting industry standards that subsequent studies can benefit from (Thum 2012).

1.15 Achieving miRNA Modulation.

There are a number of strategies which can be implemented in order to achieve therapeutic modulation of miRNA expression *in vivo* in preclinical models of disease with each of the associated technologies presenting different strengths and challenges. Though none of these strategies have yet made it into routine clinical practice, it is unlikely that one platform will be equally effective in all tissues and all disease states. For this reason it is likely that in the future such treatments will be tailored to the specific needs of the patient.

1.15.1 Viral-mediated miRNA Delivery

Perhaps the most efficient mode of miRNA transduction in vivo would be through the use of viral vectors (Yao et al 2011). Taking advantage of millions of years of genetic evolution viral vectors use the patient's own cellular machinery to transcribe and deliver a therapeutic payload. In the context of miRNA therapeutics this will either be the desired miRNA sequence, or a complementary miRNA inhibitory sequence. Viral vectors are based on several different commonly found viruses including adenovirus, lentivirus and others.

Adenoviral vectors are the most widely studied of the viral vectors and are based on the 'common cold' virus. In this class of viral vectors several different serotypes exist which account for differences in stimulation of the immune response as well as tissue specificity (Douglas et al. 1996).

57 different serotypes of adenovirus exist in humans meaning that there is a variety of vectors in this class that can be utilized in the treatment of disease (Buckwalter et al. 2012). If a patient has not been previously exposed to a given serotype, their immune system can be said to be 'naïve' to this serotype (Janeway et al. 2001). This means that there will be less of an immune response assisting the clinician in delivering the genetic payload. If the patient's immune system recognizes the serotype of the viral vector there will be a greater inflammatory response resulting in additional undesirable side effects and an increase in the required dose of viral vector required to treat the disease (Stolberg 1999).

An important concept in understanding viral gene therapy is trophicity. This refers to the tissue specificity of any given virus. Adenovirus is hepatotrophic meaning that it is highly active in the liver resulting in hepatotoxic effects (Mowa et al 2010). Ongoing research in the field of viral gene therapy is looking to identify serotypes with desirable trophicity, or to modify capsid proteins on the surface of the virus to modify trophicity (Everett et al. 2003; Shayakhmetov et al. 2005; Koizumi et al. 2007).

Lentiviral vectors are another widely utilized technology for gene modulation although they are unlikely to be used in clinical practice (Yi et al. 2005). Lentiviral vectors are based upon the retrovirus HIV-1 (Zufferey et al. 1997). The biggest advantage of the lentiviral vector is the high efficiency which exists, which results in a requirement for less virus to

be produced as well as less immune response from the recipient organism. Although modification of the viral genome has nullified the virus' ability to reproduce or mutate, the retroviral nature of the vector means that the therapeutic gene integrates with the host genome. Preservation of the transgenic sequence upon cellular division means that the modification of gene expression is potentially permanent. Another issue associated with the use of lentiviral vectors is that it is often not possible to predict the site of genomic integration. It is possible that integration of the transgene at a specific region may interfere with normal genetic regulation of the organism and promote oncogenesis or the development of some other undesirable pathology (Kay et al. 2001).

It is also possible to use viral vectors based on viruses from other species of animals. For instance canine adenovirus is a contender for use as a viral vector in the treatment of neurological diseases (Klonjkowski et al. 1997). As this virus predominantly infects dogs, many humans remain immunologically naïve to the virus. The other major advantage of this viral vector is that it displays marked neurological trophicity, using retrograde transport to enter the brain (Peltékian et al. 2002),successful delivery of pharmacological agents to the brain remains a major hurdle for researchers trying to treat disorders of the brain.

Another widely studied class of viral vectors is the class adeno-associated virus. This viral class results in no known diseases in humans, and the reduced immune response renders this vector highly desirable for use in the clinical arena as they are very successful for brain delivery (Murlidharan et al. 2014).

Due to the considerable cost of producing GMP compatible viral vectors as well as immunogenicity, despite the considerable advantage of the potent modulation viral vectors remain perhaps the least likely miRNA based treatment to enter routine clinical practice (Meyer and Finer 2001).

1.15.2 miR mimics

Another potential strategy for modulation of miRNA employs the use of miRNA mimics or miRNA inhibitors (van Rooij and Kauppinen 2014). These are short nucleotide sequences which imitate the miRNA *in situ*, but contain a modified nucleotide backbone which acts to inhibit degradation of these molecules. Though the transduction efficiency of using miRNA mimics would be greatly reduced in comparison to viral vectors, they may benefit

from circumvention of the immune system side effects and the regulatory aspects associated with implementation of viral vectors in humans (Castanotto and Rossi 2009). The goal of covalent modification of the synthetic miRNA mimics is to promote stability of the structure and to allow for cellular uptake of the construct (Lennox and Behlke 2011). In some cases the passenger strand is modified in order to prevent interaction with the RNA-induced silencing complex (RISC), whilst in other cases it is left unmodified in order to allow for rapid degradation (Chen et al. 2008). There are several different types of covalent modification available in the construction of synthetic miRNA mimics (Figure 1.9), although this is limited somewhat by the necessity for these sequences to interact normally with the RISC proteins.

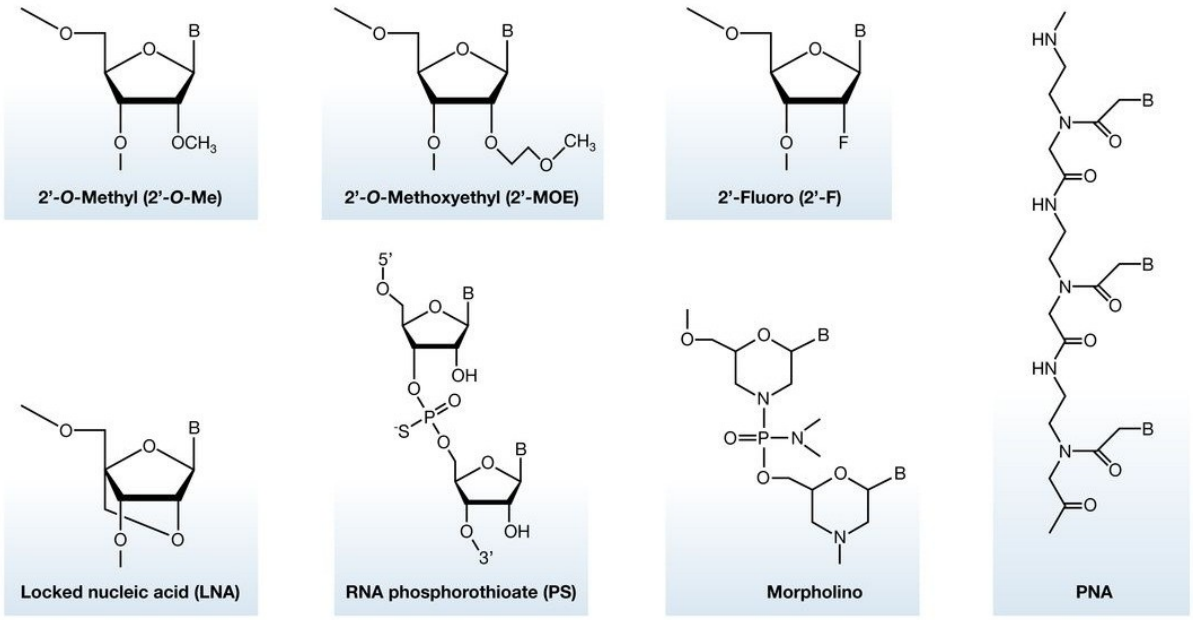

Figure 1.9 - Backbone modifications available for miRNA mimics –several different covalent modifications exist for the backbone of synthetic miRNA sequences. Each has its own characteristic effects on binding affinity and persistence in the system (van Rooij and Kauppinen 2014).

Another challenge associated with miRNA mimics is that of tissue specificity (Lagos-Quintana et al. 2002). Current technology means that it is not possible to direct expression to specific tissues with synthetic miRNA mimics as is possible with viral vectors. In clinical practice this would likely mean that administration of a mimic would result in considerable negative off target effects. Recent studies have suggested that in the future it may be possible to improve delivery of synthetic miRNA mimics through the use of microvesicles such as exosomes (Marcus and Leonard 2013; Lee et al 2012).

1.16 miRNAs in Cardiovascular Disease

Following the initial discovery and description of miRNA function there has been increased interest in the role that these molecules play in cardiovascular disease. The importance of miRNAs in the cardiovascular system relates to development and function of the heart, vasculature and other associated cell types (Urbich et al. 2008). Characterization studies conducted on cultured human endothelial cells have identified a variety of miRNAs which are specific to this cell type including let-7b, miR-16, miR-23a, miR-29, miR-100, miR-221, miR-222 and others (Suárez et al. 2007; Kuehbacher et al. 2007; Poliseno et al. 2006). Additionally, it has been determined that microRNAs play an essential role in the specification of cardiovascular cell lineage in the development of vertebrate organisms. The fundamental importance of miRNA biology in the development of the cardiovascular system was initially identified by experiments modulating genes responsible for miRNA processing. Ablation of Dicer activity in mice through the use of transgenic animals demonstrates that this mutation is not compatible for life. Dicer-knockout results in embryonic mortality during gestation between E12.5 and E14.5 due to defects on vasculogenesis (Yang et al. 2005). This observation was supported by similar findings in transgenic zebrafish deficient in Dicer (Giraldez et al. 2005).Abrogation of Drosha in HeLa cells demonstrated an accumulation of pri-miRNA transcripts and reduction of mature miRNA sequences in the cell resulting from a failure of miRNA maturation (Lee et al. 2003). Despite the perturbation which arises as a result of Drosha inhibition, the effects on endothelium were less pronounced than those seen following Dicer modulation (Kuehbacher et al. 2007). Although these experiments demonstrate the importance of miRNA activity in cardiovascular development, they offered little insight into the specifics regarding which miRNAs are responsible for the effect, and through modulation of which signalling pathways. Subsequent studies have sought to identify the specific mechanisms of individual miRNAs which mediate this. One example of this is the observation that miR-126 is abundantly expressed in $Flk1^{+}$ haematopoetic progenitor cells (Ivey et al. 2008). Futher evidence demonstrating the importance of miRNAs in cardiovascular function was demonstration that *In vitro* inhibition of let-7f and miR-27b promoted in vitro angiogenesis (Kuehbacher et al. 2007).

Although identification of abundant miRNAs in specific cell types is informative in identifying potentially important candidates in cellular function, it doesn't tell the whole

story. Relatively lower abundance miRNAs may be modulated in certain disease states resulting in differential effects following environmental insults. In disease states cardiovascular tissue is subjected to a range of different stress stimuli such as hypoxia, inflammation, shear-stress and other factors. miR-130a is an example of one such miRNA. Expressed at low levels in quiescent Human umbilical vein endothelial cells (HUVEC), there is a potent upregulation following exposure to foetal bovine serum which partially mediates angiogenic effects (Chen and Gorski 2008). Under these conditions miR-130a acts to post-transcriptionally inhibit expression of GAX and HoxA5 which ultimately results in pro-angiogenic effects through depression of proliferative, migration and tube formation pathways (Chen and Gorski 2008). Cancer studies have suggested that HIF-1α is a transcription factor responsible for promoting miRNA expression under hypoxic conditions and it is likely that this will be involved in changes observed in cardiovascular tissue in the context of ischemic cardiovascular diseases (Kulshreshtha et al. 2007).

In addition to the proangiogenic miRNAs, several miRNAs have been identified as inhibiting angiogenesis. Collectively the pro- and anti- angiogenic miRNAs have been referred to as 'angiomirs' (Wang and Olson 2009). Of the abundantly expressed miRNAs in HUVEC cells both miR-221 and miR-222 have been observed to play an important role in the inhibition of angiogenesis. For both of these miRNAs the anti-angiogenic effects are mediated by inhibition of c-kit. Some of the anti-angiogenic effects of miR-221/miR-222 are also partially mediated by the indirect inhibition of endothelial nitric oxide synthase. Nitric Oxide activity is important in the regulation of several aspects of endothelial cell growth, migration and remodelling. Additionally, miR-221/miR-222 have been found to modulate expression other miRNAs, several of which are predicted to interact with the 3'UTR of c-kit demonstrating that these miRNAs may share similar functions (Figure 1.10).

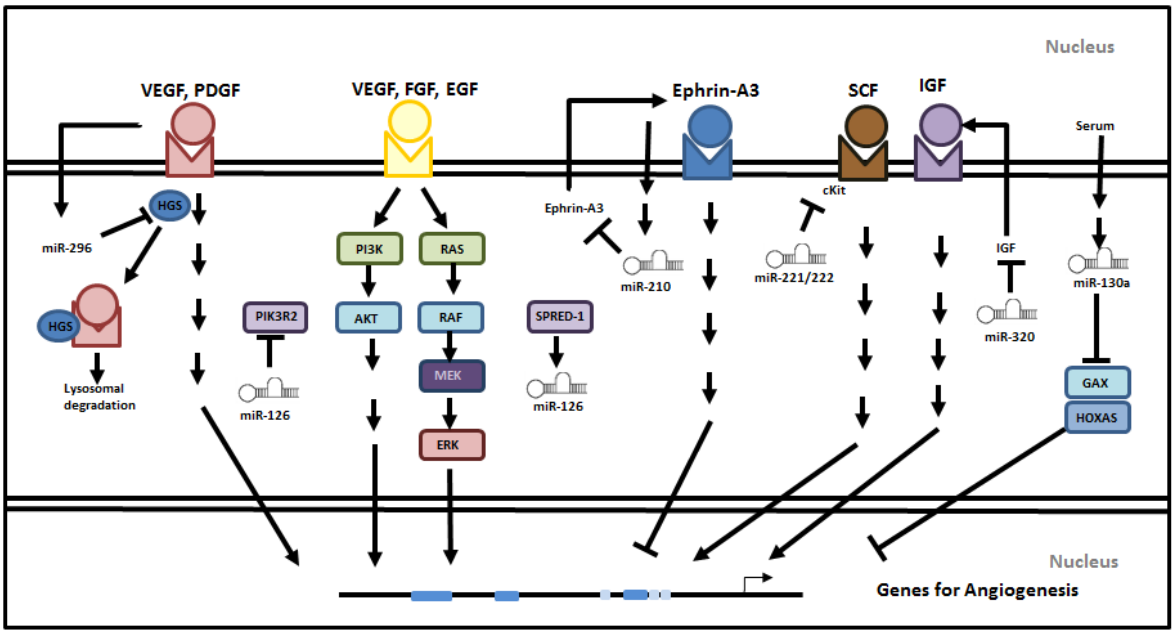

Figure 1.10 -Angiomirs - Diagram illustrating angiomirs and their mechanisms of action (S. Wang and Olson 2009)

1.17 miRNAs in ischaemic injury

The most common type of ischaemic disease is acute myocardial infarction and this is the leading cause of mortality and morbidity worldwide. This occurs when the coronary arteries supplying the heart become occluded either by embolus or thrombus formation. Due to the similarity in underlying causes many of the pathways underlying the pathogenesis of this disease can be assumed to display a great deal of overlap with stroke biology. Initial evidence regarding the importance of miRNAs in the development and progression of acute myocardial infarction (AMI) occurred following the identification of characteristic miRNA expression profiles in the border region of infarcted heart at 3 and 14 days following injury. Specific note was made of miR-29 which was downregulated following injury thus contributing to the post-injury development of cardiac fibrosis mediated through the TGF-β mediated pathway (Van Rooij et al. 2008). Others have assessed the role of miR-21 in acute myocardial infarction following observations that expression levels of this miRNA are perturbed following myocardial ischaemia. Promotion of miR-21 expression in the heart through the use of adenoviral vectors significantly reduced the size of myocardial infarct at 24h following experimentally induced acute myocardial infarction. Assessment of miR-21 target genes suggested partial mediation of the protective effect of miR-21 expression by modulation of PDCD4 and the downstream

molecule AP-1 (Cheng et al. 2010). More recently other miRNAs have been demonstrated to play an important role in the pathology of myocardial infarction such as miR-499. *in vitro* apoptosis mediated by hydrogen peroxide exposure in rat neonatal cardiomyocytes was abrogated by promotion of miR-499 expression. This protective effect was mediated by the modulation of several proapoptotic genes including Pdcd4, Pacs2 and Dyrk2 (Wang et al. 2014). Although the H_2O_2 stimulation of apoptosis is not a direct model of myocardial infarction, there is a profound production of free radicals following ischaemic injury which result in the generation of harmful metabolites such as hydrogen peroxide which means that this model is useful in characterizing mechanistic effects of some of the apoptotic pathways relevant to myocardial infarction. Another study demonstrated that *in vitro* and *in vivo* modulation of miR-99a in models of myocardial infarction demonstrated a functional improvement following injury, mechanistic analysis suggesting that this effect was partially mediated through modulation of mTOR. Emerging evidence has implicated a myriad of different miRNAs as being potential therapeutic targets in the treatment of myocardial infarction (Li et al. 2014). However, it remains unclear which of these (if any) will successfully make the translation to clinical practice. In addition to the potential therapeutic potential of miRNAs themselves, each of these studies is providing new information regarding the underlying molecular mechanisms with respect to the pathology of cardiac ischaemia and this will contribute to improved understanding of the disease in the future. miR-210 has been demonstrated to play an important role in ischaemic disease following the observation that it is upregulated in HUVEC cells following hypoxic challenge. *In vitro* modulation of miR-210 aided in the dissection of the molecular mechanisms at play here, demonstrating that upregulation of miR-210 promotes angiogenesis via tube formation and migration pathways partially mediated through the regulation of EphrinA3 (Fasanaro et al. 2008).

In addition to the functional assessment of miRNA modulation on the development of myocardial infarction, some groups have begun to study circulating levels of miRNAs as a biomarker for the disease. One study identified a significant increase in circulating levels of miR-133, miR-1291 and miR-663b in myocardial infarction patients versus healthy controls (Peng et al. 2014) whilst another identified an association with miR-328 and miR-134 levels which was associated with an increased risk of death and/or heart failure (He et al. 2014). It is unclear why observations in the modulated miRNAs differ between studies, but this may be an effect due to differences in the genetic background, or

demographics of the populations studied. The use of circulating miRNA biomarkers remains in its infancy. In order for this technology to enter the clinic it will be necessary to demonstrate that these biomarkers yield useful diagnostic and prognostic information to clinicians to a better degree of sophistication than traditional risk factors.

1.18 miRNAs in Stroke

There are many similarities in the underlying mechanisms governing ischaemic disease across the circulatory system as the fundamental signalling pathways regulating respiration are largely similar across tissue types. Ischaemic stroke presents considerable differences from the rest of the systemic circulation due to the specialised cell types in the central nervous system and complex interactions which exist between cells in the neurovascular unit.

Initial preclinical studies identifying the importance of miRNAs in stroke have demonstrated a myriad of different miRNAs to be altered in many different models of stroke. Adult male spontaneously hypertensive rats that were subjected to transient middle cerebral artery occlusion demonstrated alteration of several miRNAs at time points from 3h to 3 days of recovery. miR-140, miR-145 and miR-331 were observed to increase progressively with time following injury (Dharap and Bowen 2009, 2009). Follow up studies sought to investigate the role of miRNAs in ischaemic preconditioning as it has been shown that periods of mild exposure to ischaemia prior to a stroke event can have protective effects. Following a sub-lethal preconditioning period induced in mice, where the middle cerebral artery was occluded for either 15 or 120 minutes, expression levels of several miRNAs were assessed at 3 hours following recovery. After it was noted that miR-200 and miR-182 family members were significantly upregulated, functional assessment of manipulating these miRNAs was investigated by transfecting synthetic mimics into Neuro-2a cells prior to hypoxic challenge. miR-200b, miR-200c and miR-429 were shown to have neuroprotective effects via targeting of prolyl hydroxylase 2 (Lee et al. 2010). Further evidence for the usefulness of miRNAs as therapeutics for stroke can be found in experimental work where miR-181 levels were successfully modulated in astrocytic cell cultures. MiR-181 interacts with several aspects of the Bcl-2 protein family and directly inhibits the anti-apoptotic ligands Bcl-2 and Mcl-1 *in vitro* meaning that increased expression of miR-181 results in a more severe phenotype (Ouyan et al. 2012). Following

up this work, inhibition and overexpression of miR-181 was performed *in vivo* in a mouse transient middle cerebral artery occlusion model of stroke. It was shown that whilst overexpression of miR-181 *in vivo* exacerbated injury, inhibition resulted in a reduction of the damage caused. This was associated with increased levels of binding immunoglobulin protein (GRP-78) a HSP70 molecular chaperone which is targeted by miR-181. In C6 cells transfected with miR-181, the toxic effects were abrogated if GRP78 levels were maintained suggesting that this is a major mechanism whereby miR-181 effects stroke is by GRP78 inhibition (Ouyang et al. 2012).

After observing that miR-497 was significantly upregulated in the brain following experimental stroke, one group assessed its therapeutic potential. Ablation of miR-497 expression attenuates oxygen-glucose deprivation induced neuronal loss, whilst overexpression of miR-497 exacerbates this. miR-497 has been shown to bind directly to the 3'UTR region of bcl-2/-w genes. It was observed that there was a corresponding increase in bcl-2/-w protein levels in the ischaemic region of the brain following knockout of miR-497 and this was associated with a reduction in infarct size and improvements in neurological outcomes (Yin et al. 2010).

Further evidence of the importance of miRNAs comes from the observation that miR-15a is upregulated in an oxygen glucose deprivation model of stroke. miR-15a is upregulated by PPARδ which in turn acts on bcl-2. miR-15a is pathological in the context of stroke. It inhibits bcl-2 expression and results in cerebral vascular endothelial cell death which is a major event in the breakdown of the blood-brain barrier (Yin et al. 2010).

Investigation into the role of miRNAs in stroke remains in its infancy and as such the literature pertaining to this is comparatively limited compared to other disease areas. In recent years there has been growing interest in the role of miRNAs in stroke and the body of literature on this subject will continue to grow improving understanding of its nuances for the stroke research community.

Several miRNAs have been demonstrated to play an important role in stroke etiology, though to date none has definitively been shown to therapeutically improve outcomes when post-stroke treatment is administered despite considerable evidence demonstrating proof-of-concept. In addition to the miRNAs mentioned here, work has

been done on identifying the roles for miR-21 and miR-29 in stroke. These will be discussed in depth in subsequent chapters.

1.19 miRNAs in neurological disorders

The development of the central nervous system is a complex process requiring precise control of gene expression to specify cell fate decisions which result in the generation of the mature brain. It is likely that several miRNAs are integral to the healthy development of the central nervous system, but as research into this area is still in its infancy the specifics remain poorly understood. However, some progress has been made (Follert et al. 2014). miR-9 is a highly abundant brain miRNA which acts to modulate Nuclear receptor subfamily 2 group E member 1 (TLX) expression, this is important in the context of brain development as it inhibits the proliferation of neural stem cells and promotes differentiation to neural cell types (Zhao et al. 2009). Later during cortical development TLX expression is modulated by miR-137 via the transcriptional co-repressor LSD1 (Sun et al. 2011). One of the most abundant microRNAs expressed in brain tissue is miR-124. This plays an important role in the development of the brain through a zinc finger protein called RE1-silencing transcription factor which again leads to neuronal differentiation (Visvanathan et al. 2007). As it has been demonstrated that miRNA expression levels play an essential role in the normal development of the central nervous system, it can also be expected that perturbations in levels of specific miRNAs may be observed under certain pathological conditions and contribute to the development of disease. A recent comprehensive review summarises many of the findings relating to microRNA biomarkers for central nervous system diseases (Rao et al. 2013) Several sources of RNA have been assessed with regards to identification of biomarkers, but from a practical clinical perspective the most useful sources are bodily fluids as it is generally not advisable, or even possible to obtain biopsy tissue from the central nervous system. One of the landmark studies assessing miRNA biomarkers in the context of CNS diseases was a study analysing levels of miRNA expression levels of peripheral blood mononuclear cells between Alzheimer's disease (AD) patients versus healthy controls. This study demonstrated that there was a significant upregulation of both miR-34a and miR-181b in Alzheimer's Disease (Schipper et al. 2007). If these biomarkers can be validated as being specific to AD patients this would be a major coup for clinical researchers as currently diagnosis of AD is based on relatively subjective psychological testing which does not

effectively differentiate between this and other causes of dementia. Although currently no miRNA biomarker is routinely implemented in clinical practice, progress is being made with respect to a number of other challenging neurological diseases including schizophrenia (Lai et al. 2011) and Parkinson's disease (Martins et al. 2011) amongst others. Successful implementation of miRNA biomarkers indicative of central nervous system pathologies will facilitate clinical interventions by offering information to clinicians allowing them to identify disease at earlier stages and ultimately improve the quality of treatment received by the patients. Ultimately it will be possible to utilize miR modulation as a means of gene therapy in order to treat neurological diseases, as with cardiovascular medicine. However this is a long way off. Research in this area currently remains in the early stages of pre-clinical investigation. For example, several studies have identified miRNAs (including miR-7 and miR-153) as modulating expression of α-synuclein. This protein is of great interest in the context of Parkinson's disease as it forms fibrillary aggregates which accumulate to form Lewy bodies which contribute to neurodegeneration. It may be possible to use miRNA modulation to limit the rate of α-synuclein production and aggregation in order to slow or even halt progression of the disease (Ma et al. 2013). Another recent example of the ongoing preclinical research into central nervous system diseases can be observed with respect to Alzheimer's disease. miR-922 has been demonstrated to post-transcriptionally inhibit expression levels of UCHL1 which is associated with an increase in levels of phosphorylated tau proteins. Thus this study suggests that in patients with Alzheimer's disease, inhibition of miR-922 may act to prevent the progression of tauopathies and abrogate progression of the disease.

1.20 Hypothesis

miRNAs are modulated following stroke in response to ischaemic injury, this is functionally relevant in stroke pathophysiology and modulation of miR-21 and/or miR-29 will be effective targets for therapeutic injury in order to mitigate damage and promote recovery.

1.21 Aims

To characterise expression of specific miRNAs in experimental models of stroke.

To modulate specific miRNAs in cell culture models of hypoxic-reoxygenation injury.

To determine changes in target genes of specific miRNAs associated with miRNA modulation.

To characterise serum miRNA expression in a human patient population following stroke.

To modulate miR-21 *in vivo* through the use of transgenic animals. The role of miR-21 in stroke will then be assessed by subjecting these animals to the tMCAO model of stroke followed by investigation of physiological measures of stroke and behavioural testing.

Chapter 2 - Methods

2 Methods

2.1 Cell Culture

All tissue culture work was performed using a biological safety class II vertical laminar flow cabinet in sterile conditions. Cell lines were maintained in the appropriate media (Table 2.1) and incubated at 37^{o}C in a 5% CO_2 atmosphere unless otherwise stated.

2.1.1 Cell lines and Media conditions

Cell Line	Description	Cell Culture Medium
B50 (Sigma-Aldrich)	Immortalised neurons from rat brain	Dulbecco's Modified Eagle's medium (DMEM)/low glucose with no phenol red (Gibco, Paisley, UK) supplemented with 10% (v/v) foetal calf serum (FCS), 1% (v/v) penicillin, 100ug/ml streptomycin and 2mM L-glutamine
GPNT (Sigma-Aldrich)	Immortalised rat cerebral endothelial cell line	Hams-F10 nutrient mixture (Gibco, Paisley UK) supplemented with 10% FCS, 2ng/ml basic fibroblast growth factor, 80µg/ml Heparin, 2mM L-Glutamine, 100U/ml Penicillin and 100µg/ml Streptomycin
293T (Sigma-Aldrich)	Immortalised human embryonic kidney cell line	Dulbecco's Modified Eagle's medium (DMEM) (Gibco, Paisley, UK) supplemented with 10% (v/v) foetal calf serum (FCS), 1% (v/v) penicillin, 100ug/ml streptomycin and 2mM L-glutamine

Table 2.1 – Cell lines used and media used.

2.1.2 Maintenance of Established Cell Lines

Cells were grown in monolayers on appropriate plastic ware/glassware and media was refreshed every 2-4 days as necessary. Cells were observed twice daily in order to ensure that they demonstrated healthy phenotype and absence of microbial contamination. Cells were routinely passaged at approximately 80% confluency in order to maintain cells in exponential phase of growth as this is optimal for cell culture conditions. In order to passage cells, they were initially twice washed with sterile PBS to remove improperly adherent cells and any debris that may be present in flask. Cells were then incubated in the presence of trypsin-EDTA (Gibco, Paisley, UK) for 5 min at 37^{o}C or until cells appear to dissociate. Once cells have been sufficiently trypsinised one volume of complete media was added to neutralize the action of trypsin-EDTA. Cells were pelleted by centrifugation at 1500rpm for 5 min before resuspension in complete media for passaging or plating. Before plating, cells were counted using a haemocytometer to ensure the required seeding density was observed.

2.1.3 Cryo-preservation and Recovery of Cultured Cell Lines

Recovery - Cells were placed in liquid nitrogen for long term storage. Cryovials containing desired cells were removed from cold storage and allowed to defrost at room temperature. Once cells are thawed, they were resuspended in a volume of media in order to neutralize DMSO (which is toxic). Cells were spun down at 1500rpm for 5 minutes and resuspended in pre-warmed media before being seeded in a T150cm^{3} flask.

Cryopreservation – In order to ensure that adequate numbers of low passage cells were available for experimentation it was necessary to freeze down excess cells regularly. In order to do this, cells were trypsinized as described previously and resuspended in 5mls of complete media supplemented with 10% dimethyl sulphoxide (DMSO) per T150cm^{3} flask. Following this,1ml of cell suspension was aliquoted into cryo-preservation vials and cooled at a constant -1^{o}C/min to -80^{o}C using isopropanol. Frozen vials were stored indefinitely in liquid nitrogen. In order to ensure that future researchers faced ease of location of cryopreserved cells up to date detailed records of the contents of the liquid nitrogen storage were maintained.

2.2 General Molecular Biology Techniques

2.2.1 Preparation of Plasmid DNA

An ampicillin (100μg/ml) containing Luria base (LB) agar plate (0.5g/l, sodium chloride; 10g/l, tryptone; 5g/l Yeast extract; and 15 g/l agar) was streaked with bacteria containing the plasmid DNA to be amplified from a glycerol stock and incubated inverted overnight at 37^{o}C. A single colony was then picked from the plate and used to inoculate a starter culture of 10 ml Luria broth (LB) containing ampicillin (100ug/ml). The starter culture was incubated in an orbital shaker for 8h at 37^{o}C at 180rpm. The cloudy starter culture was either taken for mini-prep extraction or then added to 500ml of LB with ampicillin (100ug/ml) in a 2l flask and incubated in an orbital shaker overnight at 37^{o}C at 180rpm for a maxi-prep. The bacterial cells were harvested by centrifugation at 6000*g* for 15min at 4^{o}C. Glycerol stocks of positive colonies were produced by mixing 150ul sterile glycerol with 850ul of culture and vortexing before stroking at -80^{o}C

2.2.2 Small-scale Isolation of Plasmid DNA (Miniprep)

Plasmid DNA was isolated from *e. coli* using the QIAGEN Miniprep Kit. Bacterial cells from starter cultures were harvested by centrifugation at 6000g for 15min at 4^{o}C, and the pellet resuspended in 300ul lysis buffer P1. 250ul buffer P2 was added and solution mixed thoroughly by inversion. 250ul buffer N3 was added, mixed immediately by inversion (4-6 times), and mixture was centrifuged at 13000rpm for 10min. Supernatant was applied to the QIAprep spin column then washed by addition of 750ul buffer PE followed by centrifugation at 13000g for 60 seconds. The flow through was discarded and DNA was eluted in 30ul dH_2O.

2.2.3 Large-scale Isolation of Plasmid DNA (MAXIPREP)

The plasmid DNA was extracted from the bacteria using the Plasmid Maxi Kit (QIAGEN, Crawley, UK) as per manufacturer's instructions. Briefly, the bacterial pellet was resuspended in 10ml of the lysis Buffer P1. Buffer P1 contains Tris and EDTA. EDTA chelates divalent metals (primarily magnesium and calcium). Removal of these cations destabilises the cell membrane, producing lysis of the bacterial cells, and also inhibits

DNases. In addition, P1 also contained RNAse A (a ribonuclease to degrade RNA). 10ml of Buffer P2 was added, the solution mixed thoroughly by inverting 4-6 times incubated at room temperature for 5 min. Buffer P2 contains sodium hydroxide and SDS. SDS is a detergent that disrupts the phospholipids of the cell membrane and sodium hydroxide disrupts the cell walls. This results in release of plasmid DNA and sheared cellular DNA from the cells. Sodium hydroxide also denatures the DNA, producing linearization of cellular DNA. 10ml of Buffer P3 was added (chilled to $4^{O}C$), the solution mixed thoroughly but inverting 4-6 times and incubated on ice for 20 min. Buffer P3 is a neutralisation buffer containing potassium acetate and SDS). The solution was then centrifuged at 20000g for 30min at $4^{O}C$ and the supernatant containing the plasmid DNA was removed. A QIAGEN-tip 500 was equilibrated by addition of 10ml buffer QBT and the column allowed to empty by gravity flow. The supernatant was applied to the anion-exchange QIAGEN-tip and allowed to enter the resin by gravity flow, where the plasmid DNA selectively binds under low-salt and pH conditions. The QIAGEN-tip was washed twice with 30ml Buffer QC, a medium-salt wash to remove RNA, proteins, metabolites and other low-molecular weight impurities. The plasmid DNA was then eluted from the QIAGEN-tip by addition of 15ml Buffer QF, a high-salt buffer. As DNA is negatively charged, the addition of salt masks the charges and allows DNA to precipitate. The plasmid DNA was then concentrated and desalted but isopropanol precipitation. 10.5ml of isopropanol was added to the plasmid DNA and subsequently removed, and the pellet washed by additional 70% (v/v) ethanol and air-dried for 5-10 min. The dried plasmid DNA was re-dissolved in 100ul dH_2O before quantification of yield by NanoDrop^{TM}

2.2.4 Cloning

pMA-T constructs containing either miR-29b2 (Figure 2.1) or miR-29c (Figure 2.2) were purchased from Geneart.

MiR-29b2 Stem loop sequence

Forward

5’ AAGCTT GGATCC ACCATGG

CTTCTGGAAGCTGGTTTCACATGGTGGCTTAGATTTTTCCATCTTTGTATCTAGCACCATTTGAAAT
CAGTGTTTTAGGAG

TAG TTTTTT CTCGAG GATATC 3’

MiR-29c Stem loop sequence.

Forward

5’ AAGCTT GGATCC ACCATGG

ATCTCTTACACAGGCTGACCGATTTCTCCTGGTGTTCAGAGTCTGTTTTTGTCTAGCACCATTTGAA
ATCGGTTATGATGTAGGGGGA

TAG TTTTTT CTCGAG GATATC 3’

10µg of each miR construct was subjected to restriction digest by EcoRV and HindIII at 37^{o}C overnight for ligation into linearized pcDNA3.1(+) backbone (Figure 2.3). pcDNA3.1(+) backbone was linearized by EcoRV, HindIII double digest. Digested pcDNA3.1(+) was dephosphorylated using the NEB Antarctic Phosphatase enzyme (NEB) in order to reduce autoligation.

pSFFV (pHR'SIN-cPPT-SFFV-MCS-WPRE) (Figure 2.4) backbone was linearized by Xho1, BamHI double digest at 37^{o}C overnight. Digested pSFFV was dephosphorylated using Antarctic Phosphatase (NEB) in order to reduce autoligation. The dephosphorlyation reaction was incubated at 37^{o}C for 15mins, enzyme was inactivated by incubation at 65^{o}C for 15mins.

Digests were run on a 2% agarose gel at 100V for 35 minutes. Bands were visualised on an ultraviolet lamp and excised by scalpel. Gel extraction was performed by Qiaquick gel extraction kit (QIAGEN).

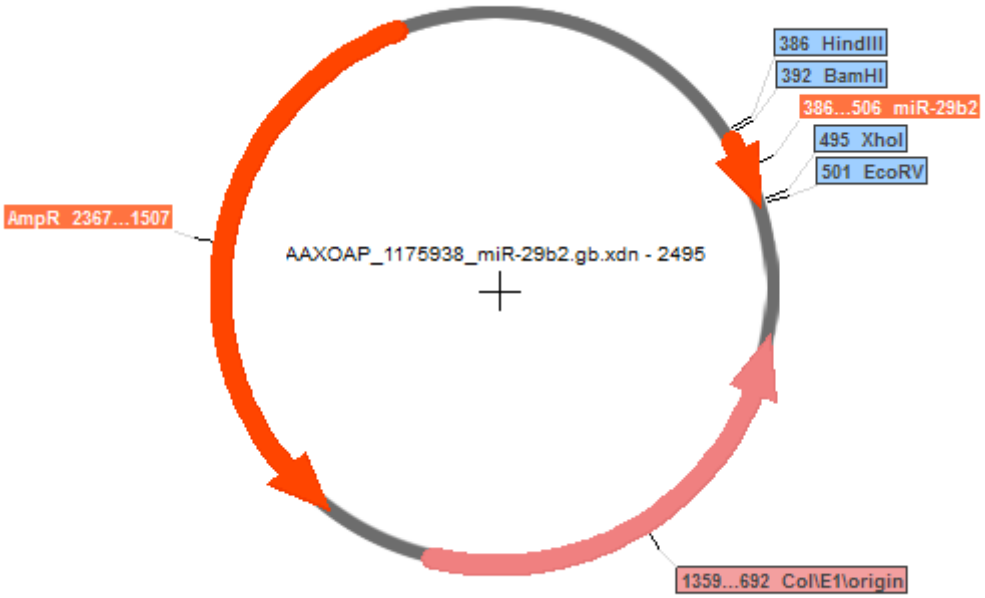

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Figure 2.1 - Geneart miR-29b2 plasmid vector map – Illustration of the pv-miR-29b construct used in cloning with restriction sites of interest labelled.

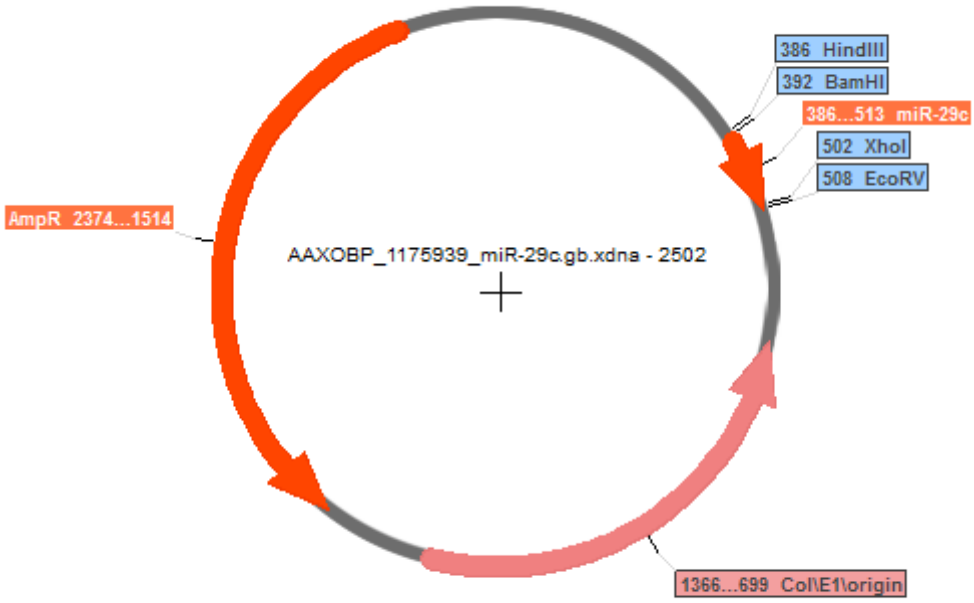

Figure 2.2 - Geneart miR-29c plasmid vector map - Illustration of the pv-miR-29c construct used in cloning with restriction sites of interest labelled.

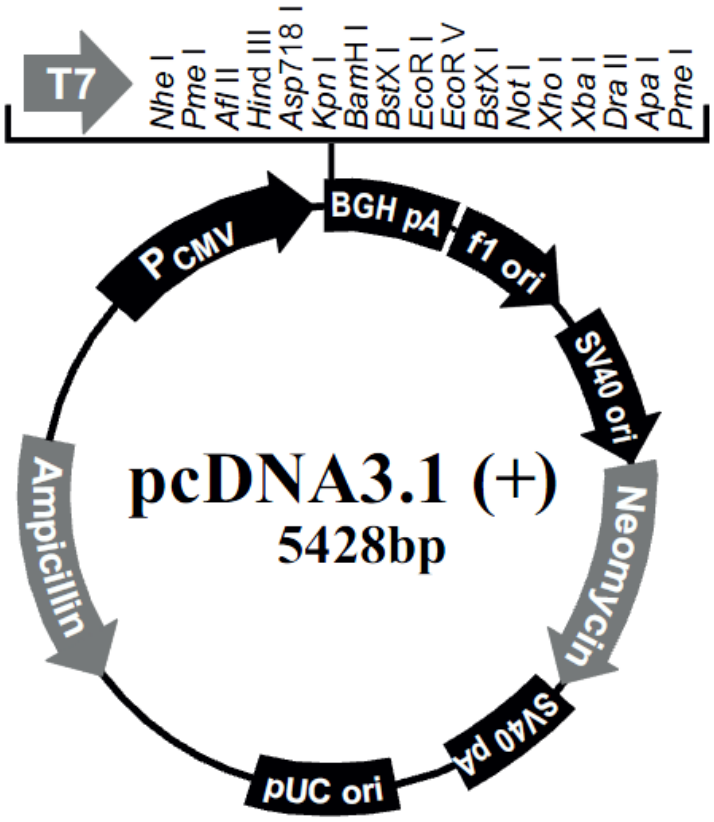

Figure 2.3 - pcDNA3.1(+) vector map - Illustration of the pcDNA plasmid used as a backbone in cloning with restriction sites of interest labelled.

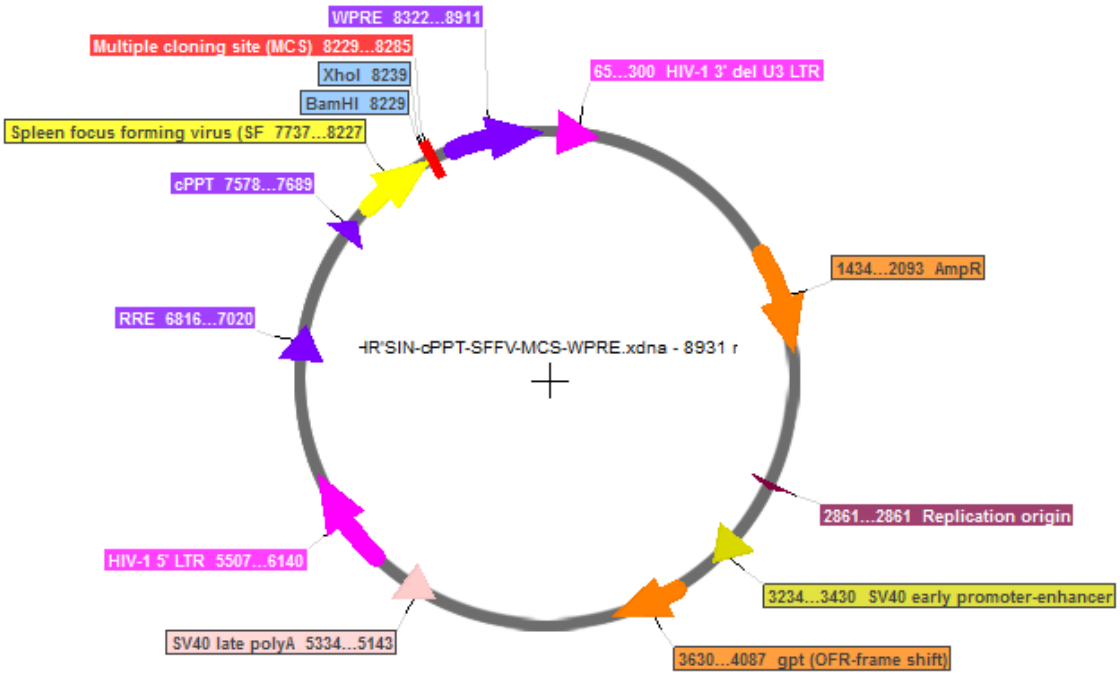

Figure 2.4 - pSFFV vector map - Illustration of the pSFFV plasmid used as a backbone in cloning with restriction sites of interest labelled.

2.2.5 Enzymatic Digestion of Cloning Vectors

Digestion of pSFFV and pcDNA3.1 backbones was achieved by restriction digest enzyme digestion. In each case a double digest was performed BamHI-HF and Xho1 for pSFFV, whilst EcoRV and HindIII-HF was used for pcDNA3.1. Reactions were incubated at 37^{o}C overnight. Successful digestion was confirmed by running products of reaction on a 1% agarose gel containing ethidium bromide and visualised by ultra-violet (UV) light.

2.2.6 Dephosphorlyation of Digests

In order to minimize the risk of autoligation, linearized backbones were dephosphorylated by use of Antarctic phosphate enzyme. A reaction was prepared using optimized concentrations of digest, enzyme and buffer. This reaction mix was incubated at 37^{o}C for 15minutes, followed by a 65^{o}C incubation for 15 minutes to terminate the reaction.

2.2.7 Extraction of DNA Digest Products from Agarose Gel

DNA fragment of interest was excised under UV light with a clean sharp scalpel. Gel slice was weighed in a colourless tube and 3 volumes of buffer QG was added per 1 volume of gel (100mg gel ~100ul). Gel was incubated in buffer QG at 50^{o}C for 10 minutes until gel slice had completely dissolved. Following dissolution the mixture had turned a yellow colour indicative of pH necessary to proceed. 1 volume of isopropanol was added and the sample was thoroughly mixed by vortex. DNA was allowed to bind to QIAquick column membrane by centrifugation at top speed for 1 min. Eluent was discarded. DNA was then subjected to a wash with buffer PE followed by elution in 30ul of buffer EB. Purified DNA was then ready for downstream applications.

2.2.8 Ligation

Ligation reactions were performed at a range of molar ratios using T4 DNA Ligase (NEB) as per manufacturer's instructions. Digested insert and dephosphorylated linearized

backbone were combined and reactions were incubated at 16^{O}C overnight. Ligations containing backbone alone or insert alone were used as controls to identify background autoligation. Ligation products were transformed into top 10 competent *E. coli* by heat shock.

2.2.9 E. coli Transformation

Top 10 competent *E. coli* was defrosted on ice, and incubated for 30 minutes with ligation products and then heat shocked in a water bath at 42^{O}C for 30 seconds. This was cooled on ice for 2 minutes, and then incubated at 37^{O}C with SOC medium for 1h. *E. coli* was plated on agar plates containing 100µg/ml ampicillin and incubated at 37^{O}C overnight.

2.2.10 Sequencing of plasmid

Plasmid identity was confirmed by sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit as per manufacturer's protocol. Primers were used to sequence across the pcDNA3.1or pSFFV as appropriate. Monoclonal colonies were selected from agar plates and DNA was extracted using the QIAGEN Miniprep kit as per manufacturer's instructions. The desired clones were then amplified using the QIAGEN Maxiprep kit.

2.2.11 Production of miRNA containing constructs by cloning

In order to produce vectors containing miR-29b2 and miR-29c the pcDNA3.1(+) backbone was used (Figure 2.5). Both pcDNA3.1(+) and miR-29x inserts were linearised by double digest using EcoRV and HindIII (Figure 2.7, 2.8). Following digestion, pcDNA3.1 backbone was dephosphorylated to reduce the risk of auto-ligation during the subsequent ligation reaction. For production of the lentiviral construct, the pSFFV backbone (Figure 2.6) was linearised by XhoI, BamHI double digest (Figure 2.7). Backbone was again linearised by dephosphorylation reaction to prevent autoligation. Following ligation reactions competent e. coli were transformed and DNA extracted using minipreps for several clones of each construct. Cloned plasmids were validated by sequencing across region of interest.

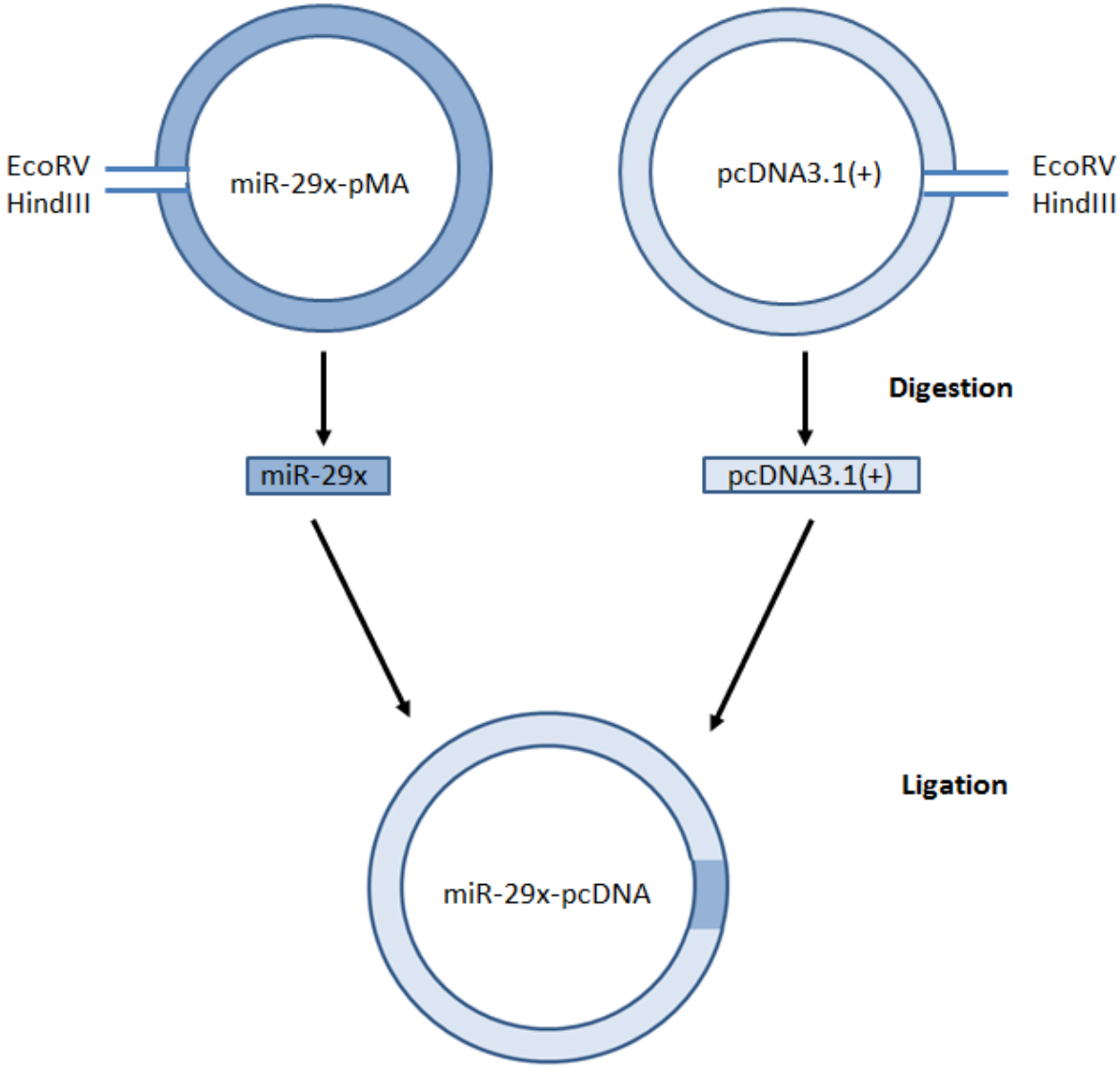

Figure 2.5 – Illustration of cloning strategy for production of miRNA-29x containing pcDNA plasmid. - miR-29x (miR-29b2 or miR-29c) was ligated out of the pMA vector by double digest with EcoRV and HindIII. pcDNA3.1(+) was linearised by double digest with EcoRV and HindIII. Resultant products of double digestion were recombined using DNA ligase to form desired construct.

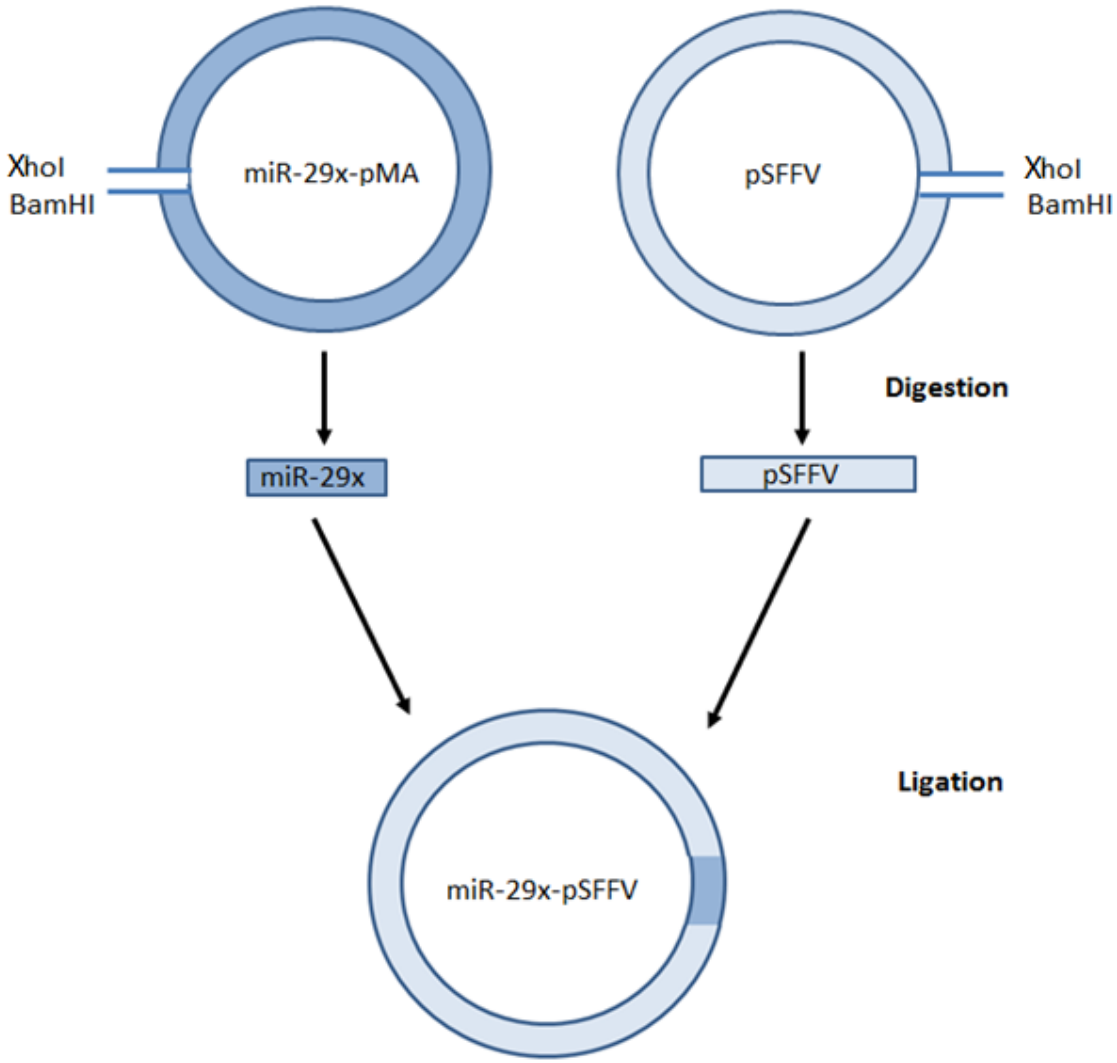

Figure 2.6 - Illustration of cloning strategy for production of miRNA-29x containing pSFFV plasmid. - miR-29x (either miR-29b2 or miR-29c) was ligated out of the pMA vector by double digest with xhoI and BamHI. pSFFV was linearised by double digest with xhoI and BamHI. Resultant products of double digestion were recombined using DNA ligase to form desired construct.

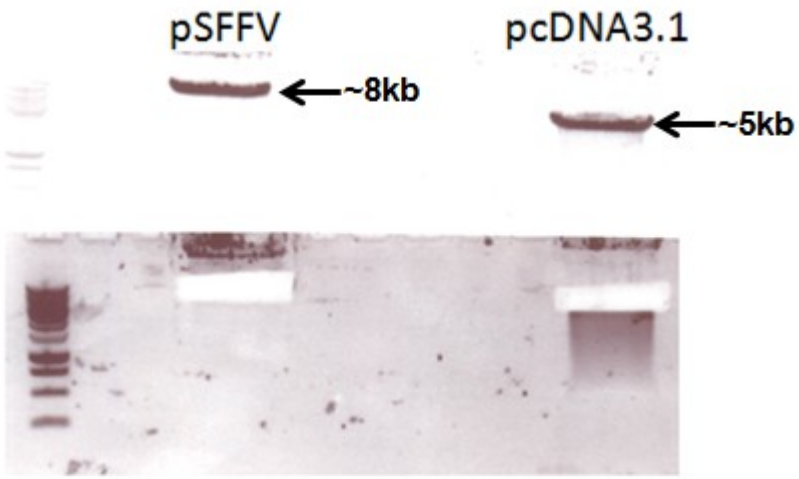

Figure 2.7 – Linearization of plasmid - pSFFV and pcDNA3.1 were linearised by double digestion with Xho1/BamHI or EcoRV/HindIII, respectively. The products of these double digests were run on agarose gel. Bands were visualised under ultraviolet light and bands of appropriate size were excised and DNA isolated by gel band extraction.1kBP ladder on left-hand side for reference.

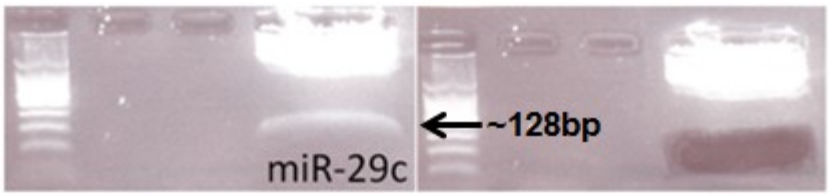

Figure 2.8 – Digestion of miRNA insert - 10µg of each miRNA construct were digested by appropriate restriction enzymes (Xho1/BamHI for construction of the pSFFV plasmid and EcoRV/HindIII for construction of the pcDNA3/1 plasmid) to isolate the miRNA sequence from the pMA vector. The resultant digests were run on a 2% (w/v) agarose gel containing ethidium bromide and visualized under ultraviolet light before excision for purification.100bp ladder on left-hand side for reference.

2.2.12 Extraction of RNA

RNA was extracted from cells, tissue and serum using appropriate manufacturer's protocols using the QIAGEN miRNEasy miRNA extraction kit (QIAGEN, Crawley, UK). Cells and/or tissue were homogenized by incubation in presence of QIAzol followed by mechanical disruption by pipetting up and down or placement in a Qiagen tissue homogenizer as appropriate. Following homogenization, 140µl chloroform was added, mixture mixed thoroughly by vortex and samples spun at >10000rpm at 4^{o}C for 15 minutes. The aqueous layer was removed and placed in a fresh RNAse free tube. RNA was precipitated by addition of ethanol and application to spin column. If necessary, samples were treated with DNase (See section DNase Treatment of RNA). Columns then underwent a series of washes to remove impurities before being eluted in 30ul of H_2O. RNA was stored at -80^{o}C. RNA obtained from cells and tissue were DNase treated to remove genomic DNA, but this was not necessary in serum samples. RNA extractions were performed in fume hood in order to prevent exposure to toxic chemicals. The quantity of RNA in each sample was quantified by NanoDrop^{TM} (ND-100 spectrophotometer [Labtech International, Ringmer, UK]).

2.2.13 DNase Treatment of RNA

DNase digestion was necessary before analysis of RNA by qPCR in order to remove genomic DNA contaminants which would be detected during qPCR and potentially confound results. DNase treatment was performed by incubation of samples in on-

column DNase digestion set (Sigma-Aldrich) according to manufacturer's protocol. For each sample 10µl of DNase I stock solution was mixed with 70µl Buffer RDD, applied to column and allowed to incubate at room temperature for 15 minutes. This step was performed during the first column wash of the miRNEasy protocol. Following DNAse treatment the miRNeasy wash protocol was continued.

2.2.14 cDNA Synthesis

500ng of RNA was used to synthesise cDNA using the Applied Biosystems TaqMan Reverse Transcription kit. For each reaction a mastermix was prepared containing 2µl of 10x buffer, 4.4µl of 25mM $MgSO_4$, 4µl of dTNPs, 1µl of Random hexamers, 0.4µl RNAse inhibitor and 0.5µl of Multiscribe Reverse Transcriptase. 500ng of RNA was loaded per reaction. Reverse transcription was achieved by incubating reactions at 16^{o}C for 30minutes, followed by 42^{o}C for 30 minutes and 85^{o}C for 5 minutes before storing samples at 4^{o}C or -20^{o}C for long term storage.

2.2.15 cDNA Synthesis for miRNA

For miRNA analysis: RNA was diluted to a concentration of 2ng/µl. Reverse transcription was performed using the TaqMan miRNA Reverse Transcription Assay as per manufacturers protocol. 5ng of RNA was used per reaction. For each reaction a mastermix was prepared containing 0.15µL of 100mM dNTPs (with dTTP), 1µl of 50U/µl Multiscribe Reverse Transcriptase at 0.19µl 20U/µl RNAse inhibitor and 4.16µl of Nuclease-free Water. Reverse transcription was performed for appropriate miRNA transcripts and rat U87 or human RNU48 depending on which reference gene was relevant for the cell types used. Exogenous reference genes used were determined based on previous experimental data in the lab which has demonstrated that these genes remain expressed at a constant level consistently. Serum samples were spiked by a known concentration of *c.elegans*miRNA-39 for use as reference gene during qPCR analysis.

Following reverse transcription, qPCR was performed using the appropriate TaqMan probes and TaqMan Universal II Mastermix as per manufacturer's protocol.

2.2.16 qRT-PCR

TaqMan™ qRT-PCR (Applied Biosystems, ABI Prism 7900HT Sequence Detection System) was used to quantify the relative concentration of mRNA present in both cells and tissue. This quantitative measurement is based on the detection of a fluorescent signal produced proportionally during amplification of a PCR product. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle and can be measured directly. Acquisition of data occurred when PCR amplification was in the exponential phase. The TaqMan detection system (Applied Biosystems, Warrington, UK). The reaction was initiated by heating the reaction mixtures at 95^{o}C for 10 minutes followed by 40 cycles of 95^{o}C for 15 seconds, followed by 60^{o}C for 1 minute. Relative expression was calculated following normalisation to an appropriate housekeeper using the ΔΔCt method. Analysis of qPCR was predominantly performed by Microsoft Excel and statistical analysis was performed in Graphpad Prism where necessary.

2.2.17 Extraction of Genomic DNA for Genotyping

Extraction of genomic DNA for genotyping was achieved by using the Invitrogen Pureline Genomic DNA mini extraction kit. Mouse ear clips or tail tips were homogenized by incubation at 55^{o}C overnight in Homogenization buffer with Proteinase K. Homogenized tissue was clarified by spinning at >10000rpm for 3 minutes. Lysate was transferred to a new tube and precipitated in a mixture of genomic lysis/binding buffer and 100% ethanol. Sample was treated with RNAse to remove transcript copies from sample. Solution was applied to a spin column and spun at >10000rpm to allow sample to bind membrane. Following a series of washes to remove impurities, sample was eluted for subsequent analysis. All handling of DNA was performed in a laminar flow fume hood in order to minimize risk of contamination of samples with atmospheric GFP.

2.3 In vitro assays

2.3.1 Hypoxic Challenge

Medium on transfected cells was replaced by serum free media; cells were then placed in a hypoxic incubator (1% O_2, 5% CO_2 and balance N_2) and incubated for 9h. Cells were then placed under normoxic conditions in full serum media to recover for 24h before harvesting.

2.3.2 Transfection with siPort

24-hours prior to transfection cells were seeded in 12-well plate at a density of $4x10^4$ cells/well and allowed to recover in normal media conditions.

siPORT™ NeoFX™ Transfection Agent was diluted in Opti-MEM medium (3µL siPORT up to 50µl media per well) and incubated at room temperature for 10 minutes. Ambion® miRNA Mimic or Cy3-labelled scrambled control (Life Technologies) was diluted to desired concentration in *Opti*-MEM medium (up to 50µL media per well). siPORT and RNA were mixed in 1:1 ratio and allowed to complex at room temperature for 30mins. Cells were then washed with PBS and 100µl of RNA:transfection reagent complex was added to each well.

Cells were incubated at 37^oC for 48h to achieve transfection before being subjected to further experiments (i.e. Hypoxic challenge).

2.4 Viral Vectors

2.4.1 Production of Lentiviral Vectors

Lentivirus was produced by triple transfection of 293T cell with a packaging plasmid (pCMVΔ8.74), a plasmid which produces vesicular stomatitis virus envelope (VSVg)(pMDG) (Plasmid Factory, Bielefeld, Germany), and an expression plasmid (pHR'SIN-cPPT-SFFV-eGFP-WPRE) containing the gene of interest (Obtained from Prof. Adrian Thrasher, Institute of Child Health, University College London, London, UK). Polyethylenimine (PEI, Sigma-Aldrich) was utilised as a transfection reagent. 293T Cells were seeded in T150cm^3

flasks 24h prior to transfection at a confluence of 70%. Per flask 17.5µg envelope plasmid, 32.5g packaging plasmid and 50µg of expression plasmid were added to 5ml OptiMEM I reduced serum media with GlutaMax I. This mixture was filtered through a sterile 0.22µM filter and combined with 5ml filtered OptiMEM containing PEI. Solutions were combined and allowed to incubate at room temperature for 20 minutes to allow formation of polyplexes. Media was aspirated from cells and cells were subsequently washed with PBS before incubation with transfection reagents. Supernatant was harvested at 48 and 72h. Lentivirus was concentrated by ultracentrifugation or 45mins at 23000rpm, 4^{o}C, before being resuspended in OptiMEM and aliquoted into eppendorphs before being stored at -80^{o}C.

2.4.2 Lentiviral vector titration

293T cells were plated in a 12-well plate at a density of 5x10^{4} cells/well 24 hours prior to transduction. Cells were then transduced with serial dilutions of the unknown concentration lentivirus ranging from 10^{-2} to 10^{-6} and incubated for 72h. DNA was harvested and qPCR performed. Original titre of lentiviral infectious units was calculated by counting the copy number of transgenic inserts present in the transduced cells.

To determine the molecular weight of the expression plasmid in Daltons the length of the construct in base pairs (bp) was multiplied by 330x2nt/bp. Daltons gives the molecular weight of the construct in g/mol. The mass of one copy of plasmid was calculated by dividing the mass of plasmid in g/mol by Avagadro's constant to obtain g/molecule. The copy number of plasmid/ml was calculated by dividing the concentration of plasmid in g/ml by the mass in g/molecule. A top standard was prepared to desired concentration and serial dilutions were used to prepare subsequent standards.

2.4.3 Viral Transduction Assay

Cells were seeded at in a 12-well plate at a density of 5x10^{4} cells/well 24h prior to transduction. Following this, the media was replaced and the desired Multiplicity of infection (MOI, virus particles per cell) of GFP virus was added. For lentiviral titration MOIs of 10, 25, 50 and 100 vp/cell were used. For Adenovirus MOIs of 5000, 10000 and 20000vp/cell were used.

2.4.4 Transfection of 293T cells

In order to assess transduction of pcDNA constructs 293T cells were transfected using Lipofectamine reagent as per manufacturer's protocol. Cells were seeded at 70% confluence in a 12-well plate and allowed to recover for 24h. Subsequently, 1µg of plasmid DNA was allowed to complex with 5µl of Lipofectamine reagent per well in optiMEM. Cells were then washed with PBS and incubated with the complexed DNA for 24h before replenishing cells with complete media. Cells were harvested with QIAzol at 72h hours for RNA extraction and qPCR analysis.

2.5 *In vivo*

All *in vivo* experiments were carried out with ethical approval and under authentication granted under the Animals (Scientific Procedures) Act 1986.

Transgenic animals were bred under licence PPL 60/4286 or PPL 60/4429.

Animals were housed under controlled environmental conditions. Temperature was maintained at ambient (15-25^{o}C) temperature with 12h light/ dark cycles. Animals were fed standard chow (rat and mouse No.1 maintenance diet, Special diet services) and water was provided *ad libitinum*.

Aseptic surgical conditions were used at all times whilst animals were on procedure.

2.5.1 Rat & Mouse Strains

2.5.2 Rat

2.5.3 SHRSP

Stroke- Prone spontaneously hypertensive rats are an inbred strain of animals based on a Wistar-Kyoto background. These animals demonstrate many physiological markers of cardiovascular disease and were used to introduce comorbidity to the study of stroke. Animals were maintained in house by selective brother-sister mating and routine lab microsatellite screening was used to confirm homozygosity of all loci within a random group from each strain. 16-week old male rats were used throughout weighing ~290-310g

2.5.4 Mouse

2.5.5 ICR (CD-1®)

Male ICR outbred mice were bought from Harlan at the desired weight (25g) and allowed 1 week locally to acclimatize before being placed on procedure.

2.5.6 CAG-miR-21 (or littermate WT)

Mice overexpressing miR-21from a mixed genetic background of B6/C3/F1 were obtained via collaboration with Mark Hatley at St. Jude USA. A local colony which had been set up previously meant that the animals used were bred in house and males were placed on procedure when they weighed 25-30g. Heterozygosity for transgene was determined by PCR of GFP.

2.5.7 miR-21 KO (or littermate WT)

Mice deficient for miR-21 from a mixed genetic background of S129/C57 were obtained through collaboration with Cheryl Nolen/Rhonda Bassel-Duby Department of Molecular Biology, UT Southwestern Medical Center at Dallas TX. A local colony which had previously been set up meant that animals used were bred in house and males were placed on procedure when they weighed 25-30g. Genotype was determined by PCR of miR-21 flanking region.

2.5.8 Genotyping of CAG-miR-21 mice (Memphis colony)

Ear clips and/or tail tips were obtained from Memphis colony mouse pups. Genomic DNA was extracted from tissue using the Purelink Genomic DNA mini extraction kit. Ear clips were suspended in a lysis mix containing 1ml of Lysis Buffer and 10µl of Proteinase K and incubated at 55^{o}C overnight until tissue was fully lysed. 5µl of RNase A was added to the lysate and the samples were incubated at room temperature for 5 minutes. DNA was precipitated by addition of 200µl of 100% ethanol and bound to the spin column by centrifugation at 10000g for 1 minute at room temperature. Eluent was discarded and spin column placed in a new collection tube. The spin column was washed with 500µl of Wash Buffer 1 by centrifugation at 10000g for 1 minute at room temperature before a second wash with Wash Buffer 2 by centrifugation at 10000g for 3 minutes at room temperature. Spin columns were then transferred to nuclease free eppendorph tubes and

DNA was eluted by centrifugation at 10000g for 1 minute. PCR was then performed on extracted DNA using GoTaq PCR Mastermix and primers specific for GFP tag (EGFP Primers Fwd 5'- TCT TCT TCA AGG ACG ACG GCA ACT -3', Rev 5' – TGT GGC GGA TCT TGA AGT TCA CCT -3'). For each reaction a mastermix was prepared containing 5µl 5X Flexi Buffer, 1µl 10mM dNTPs, 2µl $MgCl_2$, 1µl 10µM primer mix. 0.25µl GoTaq Flexi Polymerase and 14.75µl dH_2O. Amplification of transcript of interest was achieved by heating samples at 95^oC for 2 minutes, followed by 32 cycles of 95^oC for 30 seconds, 60^oC for 30 seconds and 72^oC for 60 seconds. The reaction was then terminated by heating at 72^oC for 5 minutes and reaction products were then stored at 4^oC or -20^oC as required. PCR products were run on a 2% (w/v)agarose gel containing ethidium bromide at 90V for 20 minutes before being visualised on the Bio-Radgel viewer. Genotype was determined by absence or presence of specific 216bp GFP band on gel (Figure 2.7).

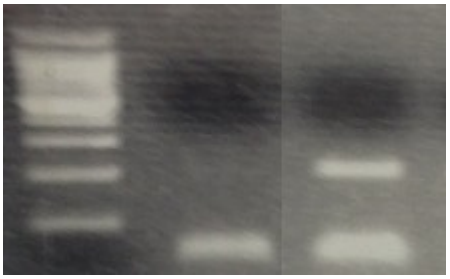

Figure 2.9 - Example of miR-21 Memphis Genotyping Products run on agarose gel- The band on the left is a 100bp ladder, the middle band is indicative of the primer-dimer formation present in the wild-type animals whilst the band on the right is a doublet with characteristic band at 216bp indicating presence of transgene.

2.5.9 Genotyping of miR-21 KO mice

Ear clips and/or tail tips were obtained from miR-21 KO colony mouse pups. Genomic DNA was extracted from tissue using the Invitrogen Pureline Genomic DNA mini extraction kit. PCR was then performed using GoTaq PCR Mastermix and primers specific for miR-21 region of genome (miR21PrimersFwd 5'- GGG CGT CGA CCC GGC TTT AAC AGG TG-3', Rev 5' –GGG CGT CGA CGA TAC TGC TGC TGT TAC CAA G-3'). For each sample a mastermix was prepared containing 5µl Gotaq Flexi Buffer, 3µl 25mM $MgCl_2$, 0.5µl of 10mM dNTPs, 1ul of 10µM miR-21 Fwd Primer, 1µl of 10µM miR-21 Rev Primer, 0.125 GoTaq Flexi Polymerase and 12.875µl dH_2O. Amplification of the transcript of interest was achieved by heating the reaction mixture at 95^oC for 2 minutes, followed by 35 cycles of 95^oC for 15 seconds, 62.3^oC for 35 seconds and 72^oC for 30 seconds. The reaction was

then terminated by heating at 72^{o}C for 5 minutes and reaction products were then stored at 4^{o}C or -20^{o}C as required. PCR products were run on a 2% agarose(w/v) gel containing ethidium bromide at 140V for 30 minutes before being visualised on the Bio-Rad gel viewer. Genotype was determined by size of specific band on gel. KO allele 330bp, Wild Type allele 469bp (Figure 2.8).

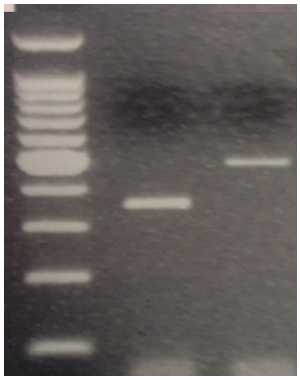

Figure 2.10 - Example of miR-21 KO Genotyping Products run on agarose gel. Left band is 100bp ladder, Middle band is the 330bp KO band whilst the band on the right is the larger 416bp band.

2.5.10 Temperature Monitoring

Throughout surgical procedures, body temperature was monitored using a rectal thermometer probe and kept within physiological limits (37.5±1 ^{o}C) using a heat lamp. Swabs were implemented as a form of insulation to prevent injury from direct heat exposure.

2.5.11 Blood Pressure measurement by tail-cuff plethysmography

Animals were placed in box and body temperature was raised by use of heat lamp. Once animals were sufficiently warmed they were removed from box, wrapped in cloth restraint and blood pressure was measured via tail cuff plethysmography. Whilst undergoing plethysmography mice were kept on heat mat to ensure that vasodilatation was maintained in order to facilitate recordings of accurate blood pressures. Several readings were taken from each animal in order to ensure accurate readings of blood pressure were obtained. Following acquisition of five consistent readings an average systolic blood pressure was calculated. Average heart rate (bpm) was also measured through the use of this device.

2.5.12 Study Design

Studies were randomised and blinded where possible, to prevent bias in animal selection and data analysis. All data analysis was performed under blinded conditions in accordance with the most recent pre-clinical STAIR guidelines (S.T.A.I.R 2009). For the mouse study n=12 was indicated by power calculation to observe a 20% change in infarct size using variability recorded by pilot study.

2.5.13 Anaesthesia

Anaesthesia was induced in 16-week old SHRSP rats with 5% isoflurane in 1.5l oxygen before intubation. To achieve intubation rats were rendered unconscious in induction box then transferred to surgical platform, held in place by a suture around their teeth. A lamp was shone upon the throat to illuminate the oesophagal-tracheal junction. The rats mouth was held open with a custom made speculum and once the tracheal opening was visible, care was taken to advance the ventilation tube along the trachea. Upon intubation isoflurane was reduced to 2.5% (in 300ml oxygen). Ventilation was maintained at a rate of 67 breaths per minute. After ensuring animal was sufficiently anaesthetized a rectal thermometer was inserted to observe body temperature which was maintained at 37±1^{o}C using a heat lamp.

Anaesthesia was induced in 25-30g mice with 5% isoflurane in 1.5l oxygen before being transferred to a face mask. Upon induction isoflurane was reduced to 2.5% (in 300ml oxygen). Anaesthesia was maintained in mice on face mask. After ensuring animal was sufficiently anaesthetized a rectal thermometer was inserted to observe body temperature which was maintained at 37±0.5^{o}C using a heat lamp.

2.5.14 Pre-stroke Stereotactic Surgery

Prior to tMCAO in rats, hemicraniectomy was performed as the associated durotomy greatly reduces mortality rate following ischemic injury (Ord et al. 2012). Rat was prepared for surgery as described above and placed in a prone position on a stereotactic scaffold. The rat's head was secured in place using the stereotactic scaffold's ear bars.

Once securely in place, an incision was made along the midline of the skull, periosteum was removed from the surgical area with cotton buds. Retractors were used to keep surgical area open as required. A burr hole was made posterolateral to the bregma using a bone micro-drill. Bone was pierced using forceps ensuring that the dura mater was pierced. The burr hole was subsequently sealed using dental cement (Prontolute, Wright Cottrell) and allowed to set. The surgical area was then sutured and the rat was allowed to recover.

2.5.15 Transient Middle Cerebral Artery (tMCAO) model of stroke

2.5.16 Rats

4 days after burr hole surgery the rat was prepared for surgery as described above and transient middle cerebral artery occlusion was performed (tMCAO). This method of tMCAO is a modified version of Longa and colleagues (Longa et al. 1980). Under an operating microscope (M651, Leica Microsystems, UK). An incision was made on the left side of the rat lateral to the midline, superficial to the carotid artery bifurcation. Branches of the carotid artery were surgically isolated. The occipital artery was cauterized. All other carotid branches except the middle cerebral artery were temporarily ligated using 5/0 silk suture. Once ligations were in place and adequate tension was placed on the vessels an incision was made in the common carotid artery. A silicon coated mono-filament 0.37mm in diameter (Doccol Corporation, USA) was then advanced 22mm along the internal carotid artery to block the origin of the middle cerebral artery and left in place for 45 minutes, animals remained anaesthetised for the duration of the procedure (Figure 2.11).

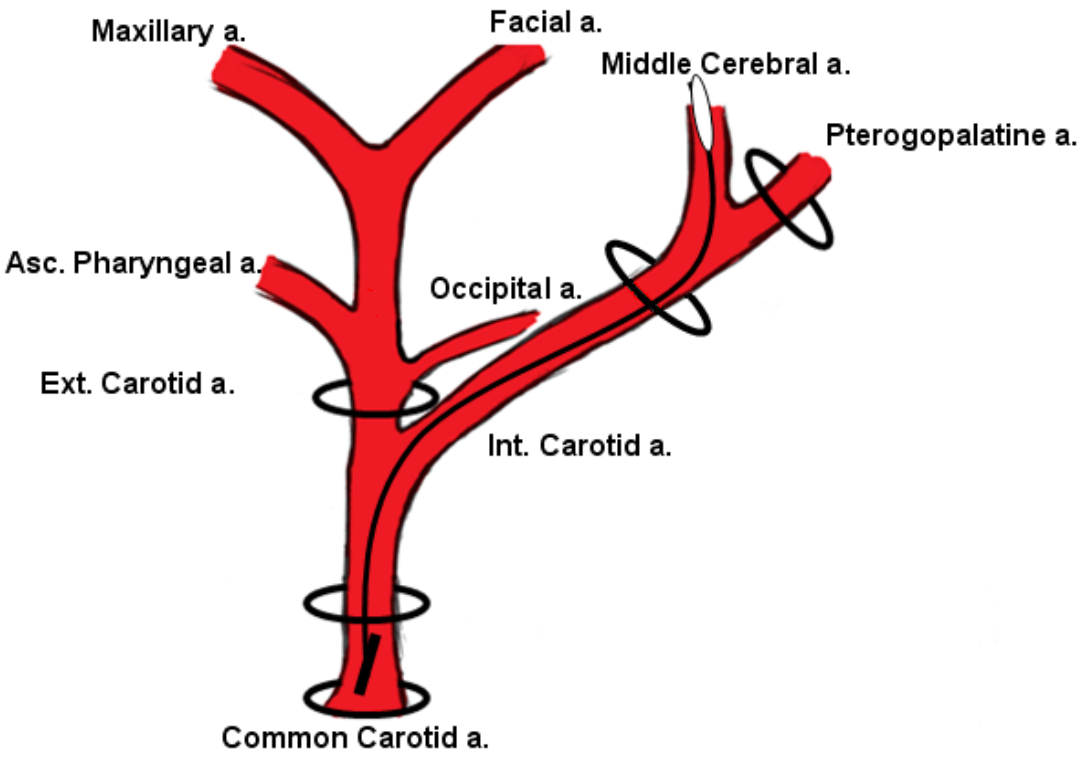

Figure 2.11 - Illustration of vascular ligations for transient middle cerebral artery occlusion – A silicone coated monofilament was advanced 22mm along the common carotid artery to temporarily block the origin of the middle cerebral artery.

Following occlusion the filament was removed, the incision was cauterized, and temporary ligations removed. After ensuring the structural integrity of the vessel the surgical area was cleaned with saline and sutured. 2mls of subcutaneous saline was administered and the rat was allowed to recover for 3h, 24h, 48h or 72h.

2.5.17 Mice

Mice were not subjected to hemicraniectomy as rats were, but tMCAO surgery in this animal is identical in principle, albeit on a smaller scale. Under an operating microscope and incision is made along the midline of the neck. All branches of the carotid were surgically isolated and temporarily ligated with 6/0 silk suture. A Doccol monofilament of diameter 0.23mm was advanced 10mm from the bifurcation of the common carotid. Occlusion was maintained for 45mins and mice were allowed to recover for 72h.

2.5.18 MRI quantification of infarct size

Infarct size was quantified in mice through T2-weighted MRI imaging. Light anaesthesia was induced and animals were placed in core of Bruker Pharmascan. Temperature and respiration rate were monitored for duration of procedure. Body temperature was maintained in scanner through use of a heat jacket. Anaesthesia was achieved as described in section 2.6.16 and the procedure typically lasted no longer than an hour.

Images obtained by MRI were analysed by Spin and ImageJ as required. Scale was set on 2dseq files. In order to calculate infarct size, the area of each hemisphere was measured followed by the area of the site of lesion. 15 coronal sections were used to determine the infarct volume. Volume of lesion was determined as being the area of the lesion across the length of the brain. Lesion size was calculated as the infarct as a percentage of total ipsilateral hemisphere normalized to the contralateral hemisphere in order to account for swelling/oedema (Figure 2.12).

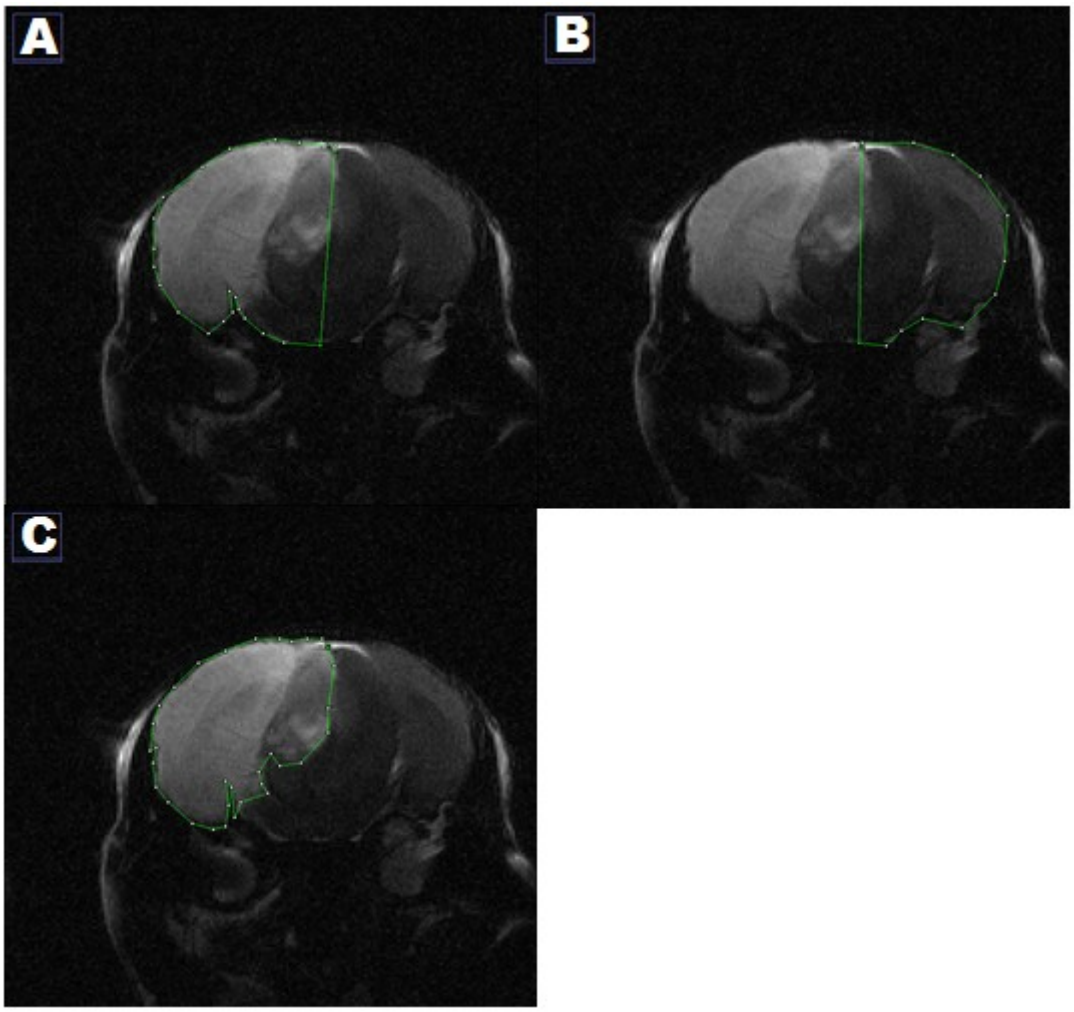

Figure 2.12 - Representative image demonstrating method of quantification for one slice of brain. (A) Ipsilateral hemisphere area is measured, followed by (B) Contralateral hemisphere and finally (C) infarcted region.

2.5.19 Transcardiac perfusion

After the assigned recovery period each animal was anaesthetized before perfusion fixation under physiological pressure. Animals were induced as described previously and terminated by exsanguination by perfusion via the heart of heparinized saline, once exsanguination was complete animals were fixed using paraformaldehyde (PFA). Chemical fixation is a the common method used to preserve tissue and results in less distortion of the tissue than methods such as fresh, freezing or heat based techniques. Fixation with 4% PFA maintains cellular definition of the tissue and, if carried out carefully, will produce few artefacts. Furthermore it is possible to perform in situ hybridization for miRNAs on tissue which has been paraffin embedded following PFA fixation, which is very attractive for miRNA research.

Animals were anaesthetised as described previously. Animals were placed on a facemask in the supine position, limbs were held in place by tape. Once sufficient level of anaesthesia was achieved an incision was made in the thorax at the level of the xiphhoid process. Thorax was then dissected superiorly along the ribcage in order to isolate the heart. Once the heart was isolated and pericardium removed a blunt cannula was inserted into the apex of the heart and advanced into the aorta. Saline was used to perfuse the systemic circulation following incision in the left atria allowing for adequate circulation. Once fluid leaving the atria is transparent, this means that the animal is sufficiently perfused. Following this perfusion by 4% PFA in saline was performed until rigor mortis sets in.

2.5.20 India ink staining of cerebrovasculature

Animals were euthanized by perfusion with PBS as described previously. Animals were then perfused with a mixture of 2.5% (v/v) India ink in 10% (w/v) porcine gelatine. Stained cerebrovasculature was then photographed with a digital camera and the gross anatomy described accordingly.

2.5.21 Behavioural Testing

All behavioural testing was carried out in a dedicated mouse behavioural testing room in the Veterinary Research Facility (VRF). Each animal was trained on the neurological assessments prior to middle cerebral artery occlusion (MCAO) to ensure that animals had familiarity with each test from the outset in order to ensure greater reproducibility. Following training, animals were assessed on a single occasion 3-4 days prior to experimental stroke in order to ascertain a baseline score. Animals on procedure were then subsequently assessed at 24h, 48h and 72h before sacrifice.

In order to determine sensorimotor deficit the Garcia 18-point neuroscore was implemented as described elsewhere (Table 2.2)(Garcia et al. 1995).

2.5.22 - 18-point Garcia Neuroscore

Test	0	1	2	3	Score
Spontaneous activity (in cage for 5 min) Note: normal movement is for rat to approach all sides therefore a score of 3 should indicate this behaviour.	**No Movement**	**Barely Moves**	**Moves but does not approach at least 3 sides of cage**	**Moves and approaches all sides of cage**	
Symmetry of movements (four limbs)	**Right side: no movement**	**Right side: slight movement and splays to side a lot resulting in sideways movement.**	**Right side: moves slower & splays to side. General movement still forward**	**Both sides: Move symmetrically**	
Symmetry of forelimbs (outstretching to bench-top while held by tail)	**Right side: no movement, no outreaching**	**Right side: slight movement to outreach: Attempts to place right forelimb but often left hanging.**	**Right side: moves & outreaches less than left side: Contact with both forelimbs but less able with right resulting in missing bench-top**	**Symmetrical outreach: no preference given to left or right forelimb in making contact with bench-top**	
Reaction to touch on either side of trunk Note: Start with gentle touch on right side then left. If equal, score =3. If no response right, increase force of stimulus on right to determine if weak or no response.		**No response on right side**	**Weak response on right side**	**Symmetrical response**	
Response to vibrissae touch Note: as with trunk start with gentle touch on extremities of whiskers (right then left) and move inwards if no response on right side.		**No response on right side**	**Weak response on right side**	**Symmetrical response**	
Climbing wall of wire Cage Note: If right side is weak they tend to attempt climb and then turn to left side and not move.		**Fails to climb**	**Right side is weak**	**Normal Climbing**	

Table 2.2 – Neuroscore Criteria – This is the scoring system which was implemented in order to assess sensorimotor ability of mice on procedure(Garcia et al. 1995)

2.5.23 Ladder Rung Walking Test

In order to quantify sensorimotor deficits following stroke, mice were analysed using ladder rung walking test (Metz and Whishaw 2009a) (Figure 2.13). Animals were required to walk along a horizontal ladder between two sheets of Perspex. The spacing of the rungs was altered regularly in order to ensure that animals were not successfully placing feet as a result of memory. Mouse were allowed three trials each of which were filmed. Data was collected from all animals at baseline prior to experimental stroke then again at 24h, 48h and 72h after they were placed on procedure. Video footage was then analysed in VLC media player in order to count errant footfalls as a percentage of steps taken.

Figure 2.13- Ladder Rung Walking Test was used to measure sensorimotor deficit pre and post-stroke. Mouse was placed into ladder rung apparatus at one end (right side of image) and allowed to traverse the ladder until it exited the ladder on the other end (left side of image). This was taken to be one run of the ladder.

2.6 Patient Study

2.6.1 Patient Recruitment

55 stroke patients and 20 stroke mimics were recruited as study participants from February 2012 to March 2013 from the Western Infirmary Acute Stroke Unit in Glasgow. All participants gave written informed consent and the study was approved by the Scotland A Research Ethics Committee. After consent, patients had a baseline 48hours, Day 7, 1 month, and 3 months assessment. At each of these, a clinical assessment was performed and peripheral blood samples were taken. The day 7 blood samples were analysed in the original population. Upon the addition of further subjects, day 7 and 1month blood samples were analysed. At the baseline visit, a cardiovascular history was taken (including past medical); a record of current medications kept, and investigations

planned and under-went were noted. At each visit stroke severity was measured using the National Institutes of Health Stroke Scale (NIHSS) and at 1 and 3 months, outcomes were measured using the modified Rankin scale Score (mRS: a global disability measure), the Barthel Index (B.I. – measures ability to complete activities of daily living), and the Stroke Impact Scale (SIS: a measure of the strokes effect on the patients quality of life). All participants were placed into 3 diagnostic categories, viz. Definite Ischaemic Stroke, Possible Ischaemic Stroke, and Non-stroke. To place patients in these categories senior stroke physicians carried out extensive assessments including complete medical histories, systemic examinations and received assistance from investigations: carotid doppler, ECG, CT/MRI, angiography, echocardiograms, and cardiac monitors. If there was any ambiguity with regards to which group each patient belonged to, other expert opinions were sought. Definite stroke patients were further categorised according to the internationally recognised TOAST criteria: large-artery, cardioembolism, small-vessel, indeterminate, and undetermined aetiology (Adams et al. 1993b)[Adams 1993]. Stroke mimic patients were used as a control group. These were patients who presented at clinic with symptoms of stroke, but upon medical imaging and consensus by stroke clinicians were determined to have a differential diagnosis (i.e. TIA, migraine etc.).

2.6.2 Sample Collection

Blood from patients was withdrawn before being centrifuged at 3000xg for 15 minutes to isolate serum. This isolated serum was then extracted and aliquots were stored in eppendorf tubes -20^{o}C prior to RNA extraction.

2.6.3 Applied Biosystems miRNA OpenArray

In order to identify candidate miRNAs for validation from the human population the miRNA OpenArray platform was used. Due to low RNA yield from serum RNA extractions the low sample input optimized protocol was used (https://tools.lifetechnologies.com/content/sfs/brochures/cms_097637.pdf). RNA was quantified by Nanodrop and of each sample was used to prepare the reverse transcription reaction. Samples and reagents were kept on ice at all times to preserve integrity of RNA and enzymes.

A Mastermix was prepared for all samples prepared with 10% excess to account for any potential pipetting error. For each reaction 0.75µl of 10X Megaplex RT Primers, 0.15µl of 100mM dNTPS with dTTP, 1.50µl of 50U/µl Multiscribe Reverse Transcriptase, 0.75µl of 10X RT Buffer, 0.9µl of 25mM $MgCl_2$, 0.09µl of 20U/µl RNAse inhibitor and 0.35µl of Nuclease-free water were prepared. Two RT reactions were prepared per sample, one for use with Pool A primers, and the other with Pool B primers. Samples were added to a 96-well plate and subjected to reverse transcription reaction by 40 cycles of heating at 16^{o}C for 2 minutes, 42^{o}C for 1 minute and 50^{o}C for 1 second, followed by deactivation of enzymes by heating at 85^{o}C for 5 minutes before finally storing the reaction products at 4^{o}C. After reverse transcription of samples, sensitivity for lower abundance miRNAs was increased by performing a pre-amplification reaction for all miRNAs in cDNA. A Mastermix was prepared for each sample containing 20µl of 2X TaqMan Preamp Mastermix, 4 µl 10X Megaplex PreAmp Primers and 8.5 µl Nuclease-free water. 32.5µl of this mastermix was added to 7.5µl of RT reaction to give a final volume of 40µl. Reactions were prepared for all samples and added to a 96-well plate for preamplification. The preamplification reaction was achieved by heating at 95^{o}C for 10 minutes, followed by 55^{o}C for 2 minutes and 72^{o}C for 2 minutes. The reaction then underwent 16 cycles of 95^{o}C for 15 seconds followed by 60^{o}C for 4 minutes. The reaction was terminated by heating at 99.9^{o}C for 10 minutes and products were then stored at 4^{o}C. Prior to commencement of qPCR reactions each amplified sample was diluted 1:20 by taking 3µl of the preamp mix and adding 57µl of 0.1xTE buffer. In order to prepare qPCR reactions PCR Master Mix was added to each well. For each primer pool set 5µl of reaction mixture was added to an OpenArray 384-well sample plate from which it was loaded to an OpenArray card by use of the OpenArray Accufill system. After reactions were run on the OpenArray cards data was then analysed by DataAssist software or Microsoft Excel as appropriate (Figure 2.14).

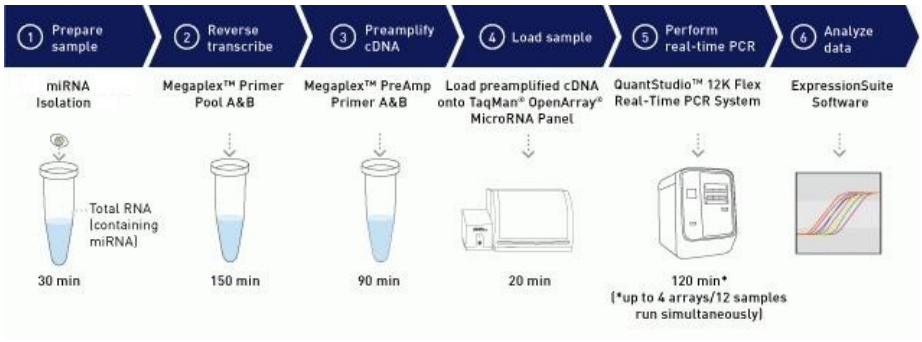

Figure 2.14 - Workflow of Openarray system – RNA is isolated from samples before reverse transcription, preamplification and finally qPCR.

2.6.4 Exosome isolation and quantification

Exosomes were isolated from serum using the Invitrogen total exosome isolation kit and counted by nanosight. Serum samples were thawed and kept on ice whilst in use. Samples were centrifuged at 2000xg for 5 minutes in order to ensure no particulates were present in sample. Following this, 100µl of the sample was transferred to a fresh tube and 20µl of exosome isolation reagent added. This mixture was vortexed to ensure that the solution was homogenous. Samples were incubated at 4^{o}C for 30 minutes to precipitate out exosomes. Following this samples were placed in centrifuge and spun at 10000xg for 10 minutes at room temperature. Supernatant was aspirated and discarded and subsequently exosomal pellet was resuspended in PBS for downstream analysis. miRNAs were extracted from the isolated exosomes and analysed by qPCR. Some of the candidate microRNAs identified by OpenArray were analysed in exosomal RNA in comparison with total RNA.

2.7 Histology

2.7.1 Processing and Embedding

Brains were cut into approximately 7 (~ 2 mm) coronal slices using a matrix (Harvard Apparatus, UK) placed in individual cassettes in a tissue processor (Shandon Excelsior, Thermo Scientific), and dehydrated through a serial alcohol gradient and xylene before embedding in paraffin wax ready for subsequent histological analysis. Conditions for embedding were as follows:

1. 70 % (v/v) EtOH 2 h

2. 80 % (v/v) EtOH 3 h

3. 95 % (v/v) EtOH 4 h

4. 100 % (v/v) EtOH (1) 4 h

5. 100 % (v/v) EtOH (2) 5 h

6. 100 % EtOH (3) 5 h

7. 100 % EtOH (4) 6 h

8. 50 % EtOH / 50 % Xylene (v/v) 4 h

9. Xylene (1) 5 h

10. Xylene (2) 5 h

11. Wax (1) 5 h

12. Wax (2) 5 h

13. Wax (3) 6 h

2.7.2 Sectioning of Tissue

Serial 6 μm sections of paraffin embedded brains were cut on a manual rotary microtome (RM2235, Leica) at 7 pre-determined coronal levels, throughout the middle cerebral artery territory, and collected onto poly-L-lysine coated slides (Starfrost® Adhesive Microscope Slides). Three slides were obtained from each level with three or four sections on each slide. Sections were baked on to slides at 60 °C for 4 h followed by 40 °C overnight. Once slides were prepared, they were stored at room temperature until required for histological analysis.

2.7.3 In situ hybridisation for miRNA expression

Paraffin embedded rat brain sections were dewaxed in Histoclear and rehydrated by immersion along a decreasing alcohol gradient. Sections were unmasked by boiling in 10mM citric saline buffer, pH 6. Slides were subsequently immersed in 0.2M hydrochloric acid for 20 minutes before incubation in 0.3% triton-X at room temperature for 15 minutes. Following this sections were incubated at 37^{o}C with 0.5U of proteinase K for 15 minutes. Sections were refixed in 4% paraformaldehyde before incubation in hybridization buffer. After being allowed to equilibrate with hybridization buffer sections were hybridized using double-labelled LNA *in situ* probes and incubated at appropriate temperature overnight (58^{o}C for Scrambled control, 52^{o}C for miR-29b, miR-29c and 51^{o}C for miR-21). Sections were subjected to a series of stringency washes using decreasing concentrations of Saline sodium citrate (SSC) buffer in order to reduce non-specific binding of probe before finally being incubated at 4^{o}C overnight using an anti-digoxigenin

AP fab antibody. The following day non-specific binding of antibody was removed by multiple washes with PBS and Alkaline Phospate (AP) buffer. Finally MiR expression was visualised by staining with Anti-Digoxigenin-AP. Precipitation was allowed to occur over a 48h period. Sections were mounted using Histoclear taking care to ensure that no water was present under coverslip (Figure 2.15). For the duration of the in situ protocol it was of upmost importance to use sterile technique as even the slightest presence of RNAse will result in failure of the protocol. When possible positive controls were employed to validate that the protocol had worked even if staining in experimental samples was not observed. Scrambled-probe negatives were used for every experimental sample to ensure that staining observed was miRNA specific.

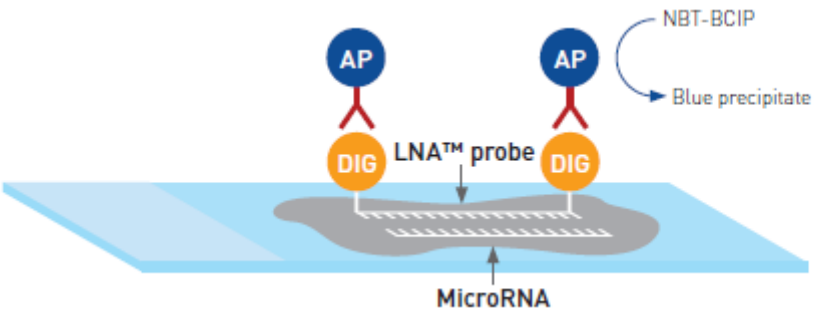

Figure 2.15 - Illustration of Anti-Digoxigenin-AP antibody binding to DIG conjugated oligonucleotide probe (Exiqon 2011).

2.7.4 Immunohistochemistry

6µm paraffin embedded brain sections were dewaxed in Histoclear and rehydrated by immersion along a decreasingly concentrated alcohol gradient. Once rehydrated antigen retrieval was performed by boiling samples in citrate buffer (10mM, pH 6) for 15 minutes. Slides were then cooled in water and washed 3x5 minutes in TBS. Sections were then blocked by TBS+0.05% (v/v) Tween-20 (TBS-T) + 15% (v/v)goat serum. After blocking, sections were incubated with primary antibody or appropriate IgG control. Hexaribonucleotide Binding Protein-3 (NeuN) at a concentration of 5ug/ml and Glial Fibrillary Acidic Protein (GFAP) at a concentration of 7.25ug/ml. Slides were incubated with primary antibody at 4^{o}C overnight, corresponding samples were incubated with the same concentration of IgG overnight to confirm specificity of antibody binding. The following day, sections were washed 3x5 minutes with TBST-T before being incubated in a 1:500 concentration of the appropriate secondary antibody in TBS-T+15% (v/v) goat serum 2h at room temperature. Following incubation with secondary, slides were washed

3x5minutes with TBS and mounted with DAPI prolong gold. Slides were visualised by confocal microscopy.

2.7.5 Haematoxylin & Eosin staining

6µM paraffin-embedded sections from perfusion fixed brain were rehydrated by moving down an alcohol gradient. Paraffin was removed from sections by to sequential washes in Histoclear for 7 minutes. This was followed by washing in 100% ethanol for 7 minutes, 95% ethanol for 7 minutes, 70% ethanol for 7 minutes and finally water for 7 minutes to rehydrate the sections. Sections were then stained by incubation in Haematoxylin at room temperature for 2 minutes, followed by a 5 minute wash under running tap water. Slides were dipped into 70% ethanol three times prior to staining with Eosin for 5 minutes followed by a 3 minute wash under running tap water. Sections were dehydrated again by running them up a concentration gradient of ethanol: Two 7 minute washes of 95% ethanol, followed by 7 minutes in 100% alcohol. Slides then undergo two washes in Histoclear before being mounted using Histomount. Slides were then viewed using bright-field microscopy.

2.8 Solutions

0.1% DEPC water (1L) was prepared by adding 1ml of DEPC per 1l of distilled water. The prepared solution was incubated at room temperature prior to autoclaving. 5l of DEPC water was prepared before starting an *in situ* run.

10x PBS (DEPC-treated) (1l) was prepared by dissolving 80g NaCl, 2g KCl, 14.4g Na_2PO_4(Na_2HPO_4) and 2.4g KH_4PO_4 and made up to around 800mls with DEPC-treated water. NaOH was added to the solution until a of pH 7.4 was obtained. The solution was then autoclaved and diluted to 1x prior to use as required.

1M sodium citrate buffer was prepared by dissolving 14.705g of sodium citrate (molecular weight = 294.10g) into around 40mls of DEPC-treated water in a falcon tube. NaOH was added to obtain a pH of 6 and the solution was made up to 50mls. Sodium citrate buffer was diluted to 10mM with DEPC-treated water prior to use as required.

0.2M HCl was prepared by adding 8.58ml of 11.64M HCl(density – 1.18 or 36%) to a 500ml bottle and made up to 500ml with DEPC-treated water.

A 1M stock of **Tris buffer** was prepared by dissolving 24.228g of Tris-HCl in 200ml of DEPC-treated water. NaOH was added to the solution until a pH of 9.5 was obtained. The solution was then autoclaved and diluted to 0.1M with DEPC-treated water as required.

Hybridization buffer was prepared by adding 25ml formamide, 10ml of 20x SSC, 2.5ml of 50x Denhart's solution, 5ml of 2.5mg/ml salmon sperm DNA, 3ml of 10mg/ml yeast transfer RNA, 500ul of 2.5% SDS and 500ul of 10% DIG blocking reagent to 3.5ml DEPC-treated H_2O. Hybridization buffer was aliquoted into 1.5ml Eppendorph tubes and stored at -20^{o}C.

2M Tetramisole Hydrochloride (mwt= 240.75g) was prepared by dissolving 0.481g in 1ml of 1xPBS before being aliquoted and stored at -20^{o}C. This solution was diluted to 2mM when needed.

10x Blocking reagent was prepared by dissolving 10g blocking reagent in 100ml maleic acid before being autoclaved and transferred into 5ml aliquots before being stored at -20^{o}C. Blocking reagent was diluted to 1x with DEPC-PBS when needed and fresh FCS added.

PFA was prepared in a fume hood by weighing out 4g of paraformaldehyde into a glass jar. 100ml of DEPC-PBS was added to the powder and the mixture was heated at 50^{o}C in a water bath until fully dissolved. PFA was then aliquoted and stored at -20^{o}C

AP Buffer (500ml) was prepared of 0.1M Tris HCl (6.57g), 0.15M NaCl (4.38g), 5mM $MgCl_2$ (2.5ml of 1M $MgCl_2$) and 0.05%-1% TWEEN-20 (250ul) to a bottle before adding NaOH until a pH of 9.5 was obtained.

2.9 Statistical Analysis

2.9.1 In vitro

All in vitro results are expressed as mean ±standard error of the mean (±SEM). In vitro experiments were performed in triplicate on at least three independent occasions and analysis was by unpaired Student's t-test. In the case of multiple comparisons repeated measures ANOVA was used with Bonferroni's post test. A p-value of less than 0.05 was considered to be significant.

2.9.2 In vivo

In vivo experiments were performed with at least 12 mice in each the transgenic study. Comparison between the groups was performed by repeated measures analysis of variance (ANOVA), as described previously (Davidson *et al*., 1995) with Bonferroni's post-test. A p-value of less than 0.05 was considered to be significant

2.9.3 Human Study

Clinical data is presented as raw Ct values when normalization to reference gene was not possible or mean RQ±RQMax/Min. Statistical analysis was performed by unpaired Student's t-test taking a p-value of less than 0.05 to be statistically significant. Number of patients required for the study was determined by discussion with clinicians and statisticians who performed a power calculation in order to ensure that this study was appropriately powered.

Chapter 3 – the Role of miR-29 in Ischaemic Stroke

3.1 Introduction

3.1.1 The miR-29 cluster

The miR-29 cluster is comprised of 4 species located across two chromosomes (Figure 3.1). The first discovered member of the miR-29 family was miR-29a which was discovered in mice following screening experiments to search for short non-coding RNAs in *Drosophila melanogaster* (Lagos-Quintana et al. 2001). As miRNAs are named based on the order in which they were discovered, miR-29 is amongst the first to be studied and has been widely investigated. Following the discovery of miR-29a, two distinct isoforms were subsequently identified: miR-29b (Dostie et al. 2003) and miR-29c (Lagos-Quintana et al. 2002). Both miR-29b1 and miR-29a are produced from a single primary transcript and processed from the intronic sequence from a locus located on chromosome 7q32 which is located adjacent to the common fragile site FRA7H (Mishmar et al. 1998; Schneider et al. 2007) and separated by 652 bases (Wang et al. 2013), whilst miR-29b2 and miR-29c are transcribed from chromosome 1, locus site 1q32 where they are separated by 507 bases (Garzon et al. 2009).miR-29b1 and miR-29b2 are isoforms of each other in that they have an identical sequence to each other. Although the other members of the cluster differ in terms of sequence, they all share an identical seed sequence. As the seed sequence is the region of the mature miRNA that binds target transcripts, we can expect that the targets of all 4 types of miR-29 will have identical targets. Differences occur with regards to transcription, cleavage and processing of each of the miRNA (Noetel et al. 2012). Across the literature we can see that fold changes vary for miR-29 subtypes in response to different stimulus (Chung et al. 2013; Maegdefessel et al. 2014; He et al. 2013; Wang et al. 2013). It has also been observed that these miRNAs degrade at different rates. A relatively rapid rate of decay is observed for miR-29b compared to the other two family members. This has been proposed to be a result of the uracil rich region towards the end of the sequence (Kriegel et al. 2012). The miR-29 cluster is highly conserved across species suggesting fundamental importance in all organisms. It is possible that the complex, multiple mechanisms which regulate expression of this miRNA protect the organism against negative effects which may arise as a result of some single aberrant mutation.

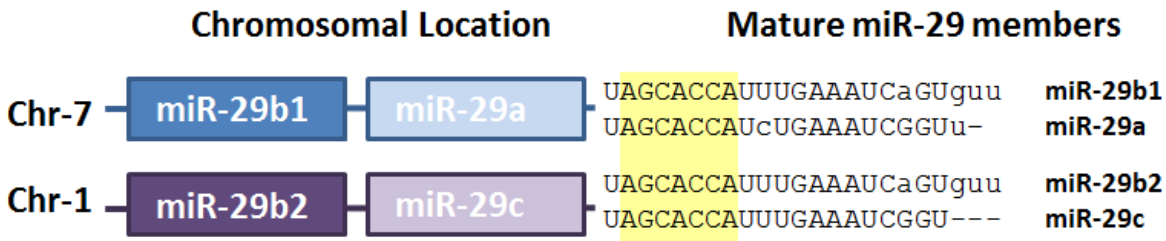

Figure 3.1 - miR-29 family members - chromosomal location and mature sequence with seed sequence highlighted (Noetel et al. 2012).

3.1.2 miR-29 in disease

Several groups have identified miR-29 cluster members as being dysregulated in a variety of diseases and disease models (Kriegel et al. 2012, Wang et al. 2013). Following this, there has been a great effort in order to identify underlying mechanisms with a view to developing novel therapeutics.

As is often the case with disease studies for novel genes, initial medical research regarding miR-29 was conducted in the context of cancer. Many studies in several forms of the disease have identified differential regulation of miR-29 family members (Castilla et al. 2011; Li et al. 2011; Zhao et al. 2010; Calin et al. 2005; Iorio et al. 2005) although these studies indicated that it was highly likely that miR-29 is functionally involved in the pathogenesis and progression of cancers, no evidence was presented to delineate the mechanisms by which this may occur.

It has been demonstrated that following myocardial infarction, there is a downregulation in miR-29 expression in the infarcted region. Following *in vivo* inhibition of miR-29 an increase in several members of the collagen protein family were observed (Van Rooij et al. 2008). Post-infarct fibrosis is associated with negative clinical outcomes in patients as scarring of the myocardium results in a decline in healthy cardiac function. This group went on to demonstrate through the use of 3'UTR mutants that COL1A1, COL2A2, COL3A1, FBN and ELN1 all directly interact with the seed sequence of miR-29 cluster members (Van Rooij et al. 2008).

Others have also focused on the potent fibrotic effects of miR-29 cluster members in renal disease. For example, it has been demonstrated that miR-29 is a potent modulator of TGF-β mediated fibrosis in a manner similar to what was observed in the context of

myocardial infarction (Ramdas et al. 2013). Following ischaemic stroke, the brain undergoes a period of glial scarring, which is largely mediated through the TGF-β signalling pathway (Silver and Miller 2004). It does remain to be demonstrated what role miR-29 plays in glial scarring (if any), though it may be hypothesized that one might expect this strong pro-fibrotic modulator to play a role in this process under some conditions.

3.1.3 The role of miR-29 in Stroke

The miRNA-29 cluster has recently been implicated as playing a potential role in the development and progression of cardiovascular disease, although to date there is still considerable work to be done in improving understanding of the role it plays in stroke. Initial evidence of an important role for the miR-29 cluster in stroke comes from an experiment performed *in vivo*. Transient middle cerebral artery occlusion (tMCAO) was induced in Sprague-Dawley rats and changes in the miRNAome were assessed by microarray. At time points 24h and 48h post-tMCAO there was an approximate 40 fold decrease in expression of miR-29b and miR-29c (Jeyaseelan et al. 2008a). A similar phenomenon was observed in spontaneously hypertensive rats following stroke, attenuation was observed at all time points in this study, but the reduction in miR-29 expression was most pronounced at 12h and 24h post-tMCAO (Dharap and Bowen 2009).

A peripheral blood miRNA screen conducted on patients between the ages of 18 and 49 years demonstrated that miRNA expression profile can be used to identify stroke subtypes. Several miRNA species were found to be differentially regulated when compared to age matched 'healthy' controls. The miR-29 cluster was found to be expressed highest in patients with large artery occlusion stroke. This provided early clinically based evidence for the prospective role of the miR-29 cluster in ischaemic stroke (Tan et al. 2009).

It has been suggested that upregulation of Hepatocyte growth factor (HGF) promotes recovery from stroke by long term neuroprotection and neuroregeneration (Hayashi et al. 2001). Others have demonstrated that the beneficial effects of HGF activity occur via miR-29 mediated reduction in collagen expression in hepatic stellate cells (Kwiecinski et al.

2011). Although this has not been addressed directly in the neurovascular unit, the aforementioned observations provide indirect evidence in support of the potential therapeutic use for miR-29 modulation following stroke. The potent fibrotic effects of the miR-29 cluster can also be expected to play an important role in the neurovascular unit following stroke. miR-29b has also been demonstrated to promote apoptosis in neuronal cells following hypoxic insult *in vitro* due to its interaction with the Bcl2 pathway demonstrating the potential functional significance in stroke (Shi et al. 2012). Whilst several cancer publications implicate these miRNAs as playing an important role in cell cycle and apoptosis; the relationship between the miR-29 cluster and these pathways remains unstudied in stroke but one would expect there to be importance, especially when considering aspects of the disease such as delayed neuronal death and neurodegeneration.

It was hypothesized based on the reported experimental observations that miR-29 dysregulation occurs following ischaemic stroke and that this modulation impacts upon subsequent pathophysiology.

3.1.4 Hypothesis

miRNA-29 is altered in the context of stroke and this is functionally important.

3.1.5 Aims

Characterization of miR-29b and miR-29c expression in SHRSP brain tissue following stroke by qPCR and *in situ* hybridization.

Assessment of changes in miR-29 targets in SHRSP brain tissue following stroke by qPCR.

Modulation of miR-29b and miR-29c *in vitro* and assessment of target genes in a hypoxic challenge experiment.

3.2 Results

3.2.2 Overexpression of miRNA using pcDNA plasmid

In order to validate that cloned vectors could achieve overexpression of desired miRNAs, 293T cells were transfected with the miRNA containing plasmids. Transfection reagent only (lipofectamine) and transfection with pcDNA3.1(+)-GFP were utilized as negative controls. No change in miR-29 expression levels was observed between non-treated controls, lipofectamine only control and pcDNA3.1(+)-GFP control. However, a significant upregulation in miR-29b was present in cells transfected with pcDNA-miR-29b and a significant upregulation of miR-29c was observed in cells transfected with pcDNA-miR-29c. Transfection efficiency was determined to be approximately 70% by looking at GFP transfected cells under a fluorescent microscope (Figure 3.2).

3.2.3 Viral Titration in B50 and GPNT cells

In order to determine what the optimal transduction efficiency in cell types of interest; a viral titration was performed with several viral vectors. Lenti-GFP was effective at transducing cells at all MOI attempted (Figure 3.3). Maximal transduction of B50 cells appeared to occur when MOI = 50. GPNT cells were also treated with lenti-GFP, although no transduction was observed at any concentration of virus (data not shown).

Ad5-GFP was also assessed as a potential vector for transduction of miRNA. This vector does not appear to be effective at transducing B50 cells, whereas there is considerable transduction at all MOI attempted in GPNT cells. Maximal transduction appears to occur at an MOI of 10000. (Figure 3.4)

Due to unexpected problems regarding the efficacy of available viral vectors in cell types of interest (B50 and GPNT cells), as well as the considerable time and resources taken to produce these vectors, subsequent modulations were achieved by transfection of cells with commercially available miRNA mimics. This will be discussed in depth later.

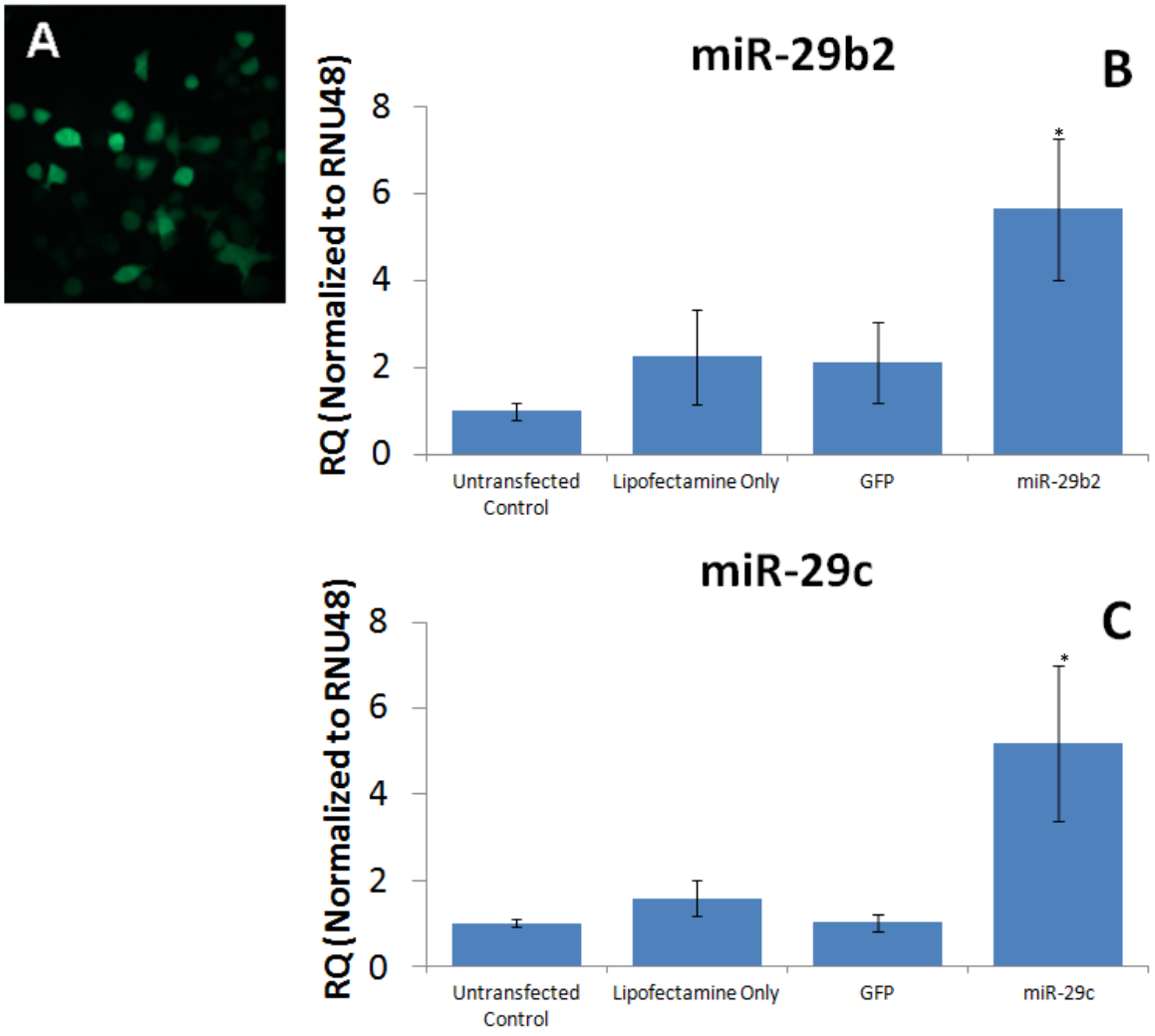

Figure 3.2 – pcDNA3.1(+) Transfection –293T cells were transfected with 2.5µg of DNA in 3.75µl lipofectamine with a transfection efficiency of approximately 70% (A**).** Levels of (B) miR29b2 and (C) miR29c were determined using TaqMan RT-PCR using specific miR probes and normalized to RNU48. RQ is presented relative to untreated control. n=3, data presented as mean RQ ±RQmax/min, *=p<0.05 Student's two tailed t-test.

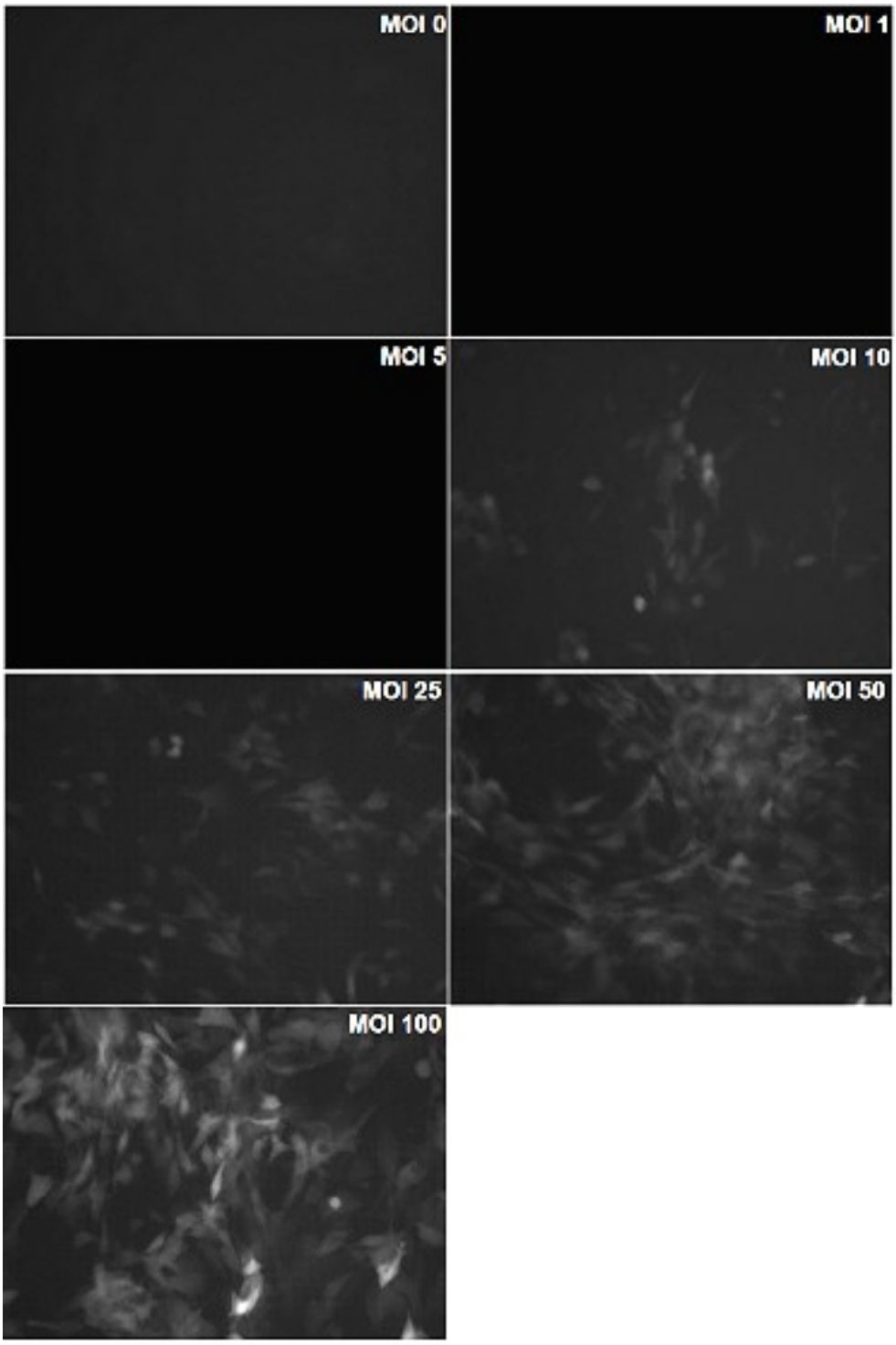

Figure 3.3 – Lentiviral Transduction Assay – Representative black &white images ofB50 cells transduced by lentiviral particles expressing GFP at increasing concentrations of lentiviral vector. n=3

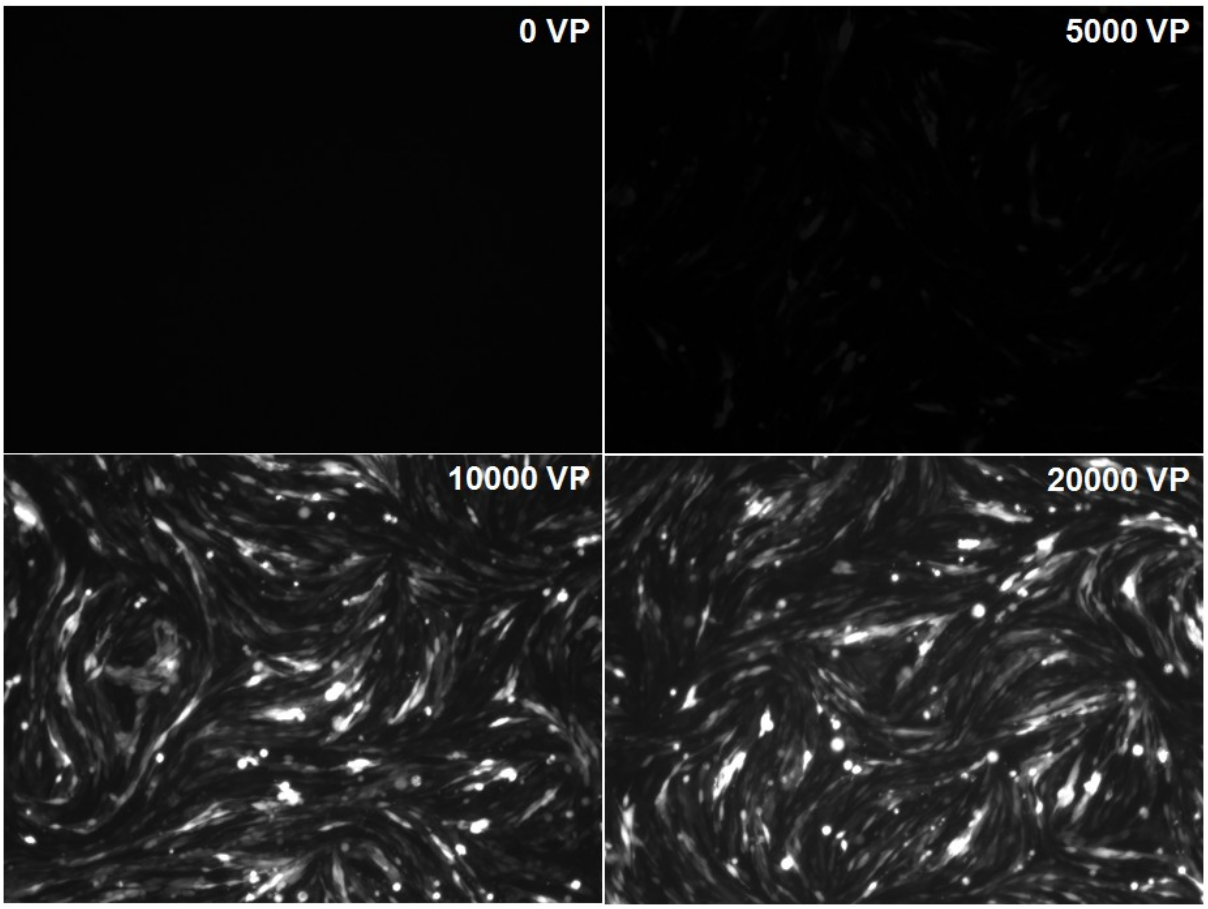

Figure 3.4 – Adenoviral Transduction Assay – Representative images of GPNT cells transduced by adenoviral vectors expressing GFP at increasing concentrations of Ad5 viral vector. n=3

3.2.4 miR-29b/c expression in infarcted rat brain as determined by qPCR analysis

In order to determine whether or not there was an endogenous change in the level of miR-29 expression following ischaemic stroke, qPCR analysis of infarcted rat brain was conducted at 24h and 72h following tMCAO (45mins) in SHRSP. SHRSP rats were used as opposed to the more widely used strains as this inbred population displays the kind of comorbidity present in human stroke and therefore this model can be expected to be more translatable to human stroke. The infarct was identified as a region of hyperintensity on T_2-diffusion weighted MRI and the brain was dissected into regions of infarct, peri-infarct and border (Fig 3.5). For miR-29b (Figure 3.6) no difference in expression was observed between stroke and sham controls in the un-infarcted border tissue. However, a significant upregulation in miR-29b was evident in peri-infarct tissue at 72h following stroke. Mir-29b was significantly downregulated in the infarct region at 24h and 72h following stroke. All qPCR was normalized to U87.

MiR-29c was determined to be significantly downregulated in border tissue at 24h following stroke (Figure 3.7). No difference was observed in miR-29c expression in peri-infarct tissue at either time point following stroke. Finally, a significant decrease was observed in miR-29c in the infarct region at 72h following stroke.

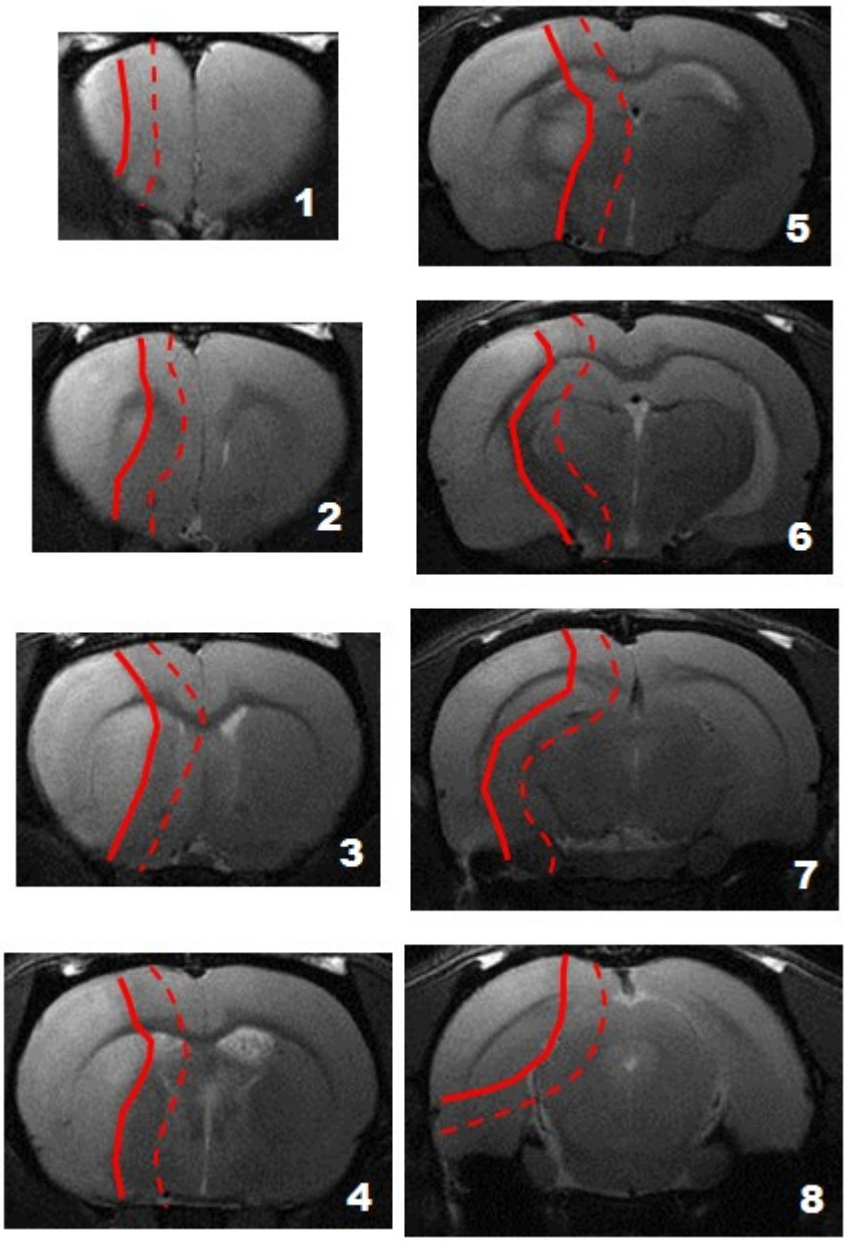

Figure 3.5 MRI Demarcation of Infarct, Peri-Infarct and Remainder tissue - T2-weighted MRI images across 8 coronal levels within MCA territory demonstrating the region of infarct [within bold red line], peri-infarct [between bold and dotted red lines] and border [between dotted red line and midline of brain at 72h after tMCAO (45 mins) in SHRSP. **(Image courtesy of Dr. Emily Ord)**

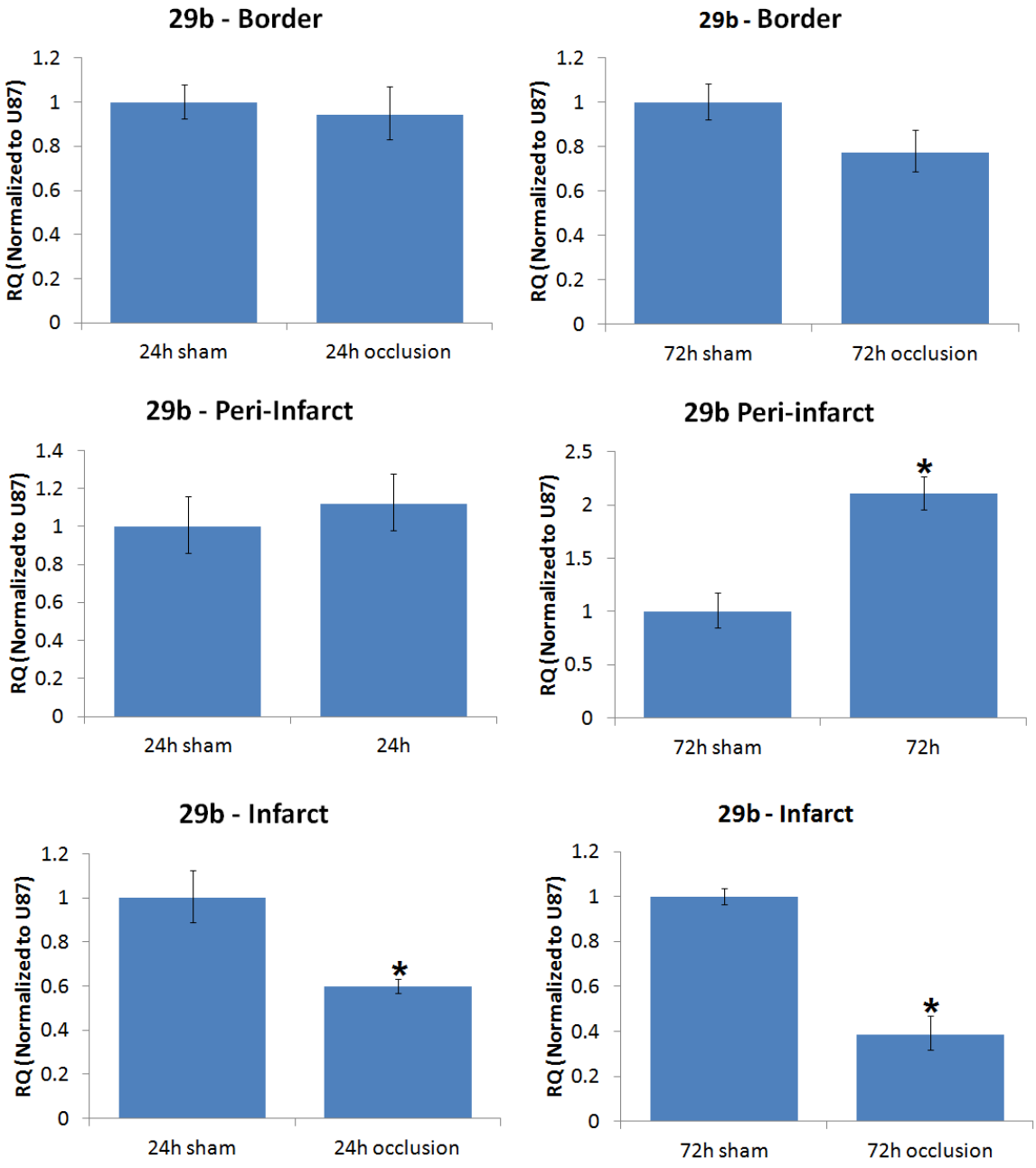

Figure 3.6 - qPCR analysis of rat brain tissue for expression of miR-29b at 24h and 72h following stroke. Infarct, Peri-infarct and Border regions were defined by MRI. Expression is significantly (*, $p<0.05$) decreased in the infarct region at both 24h and 72h following stroke. Expression is significantly increased at 72h in the peri-infarct tissue. Data are presented as RQ (±RQMax/Min) normalized to U87, n=4. Statistical analysis was conducted by two-tailed unpaired Student's t-test.

3.2.5 Localization of miR-29b and miR-29c in peri-infarct region of stroke brain.

A characteristic pattern of mRNA-29 expression can be observed on *in situ* hybridization stained rat brain sections in the absence of stroke. The purple staining is indicative of miRNA presence. Localization of miR-29b appears similar to that of miR-29c. In the images presented (Figure 3.8) it appears that there may be a higher expression level of 29b to 29c. However, as this was not reflected in the Ct values of the qPCR data and as ISH is not a quantitative measure of expression this cannot be assumed.

Anatomically both miRNAs appeared are most abundant in cell bodies of pyramidal neurons of the hippocampus and cortical layers II/III. It is unclear whether or not this is functionally significant or merely reflective of the fact that there are more nuclei present in this region when compared with the axonal projections present in surrounding non-stained tissue. Identification of the location of miR-29 expression will be important to fully understand the role that this miRNA plays in specific cell types in specific regions of the brain. This will guide researchers when considering whether to target therapeutic interventions to specific cell types. Another aspect that will need to be considered is that different pathways will need to be targeted for therapy in different cell types. Whilst overexpression of miR-29c may be protective in neurons, it may have iatrogenic effects in astrocytes for example. This aspect of miRNA activity in the neurovascular unit remains almost unexplored.

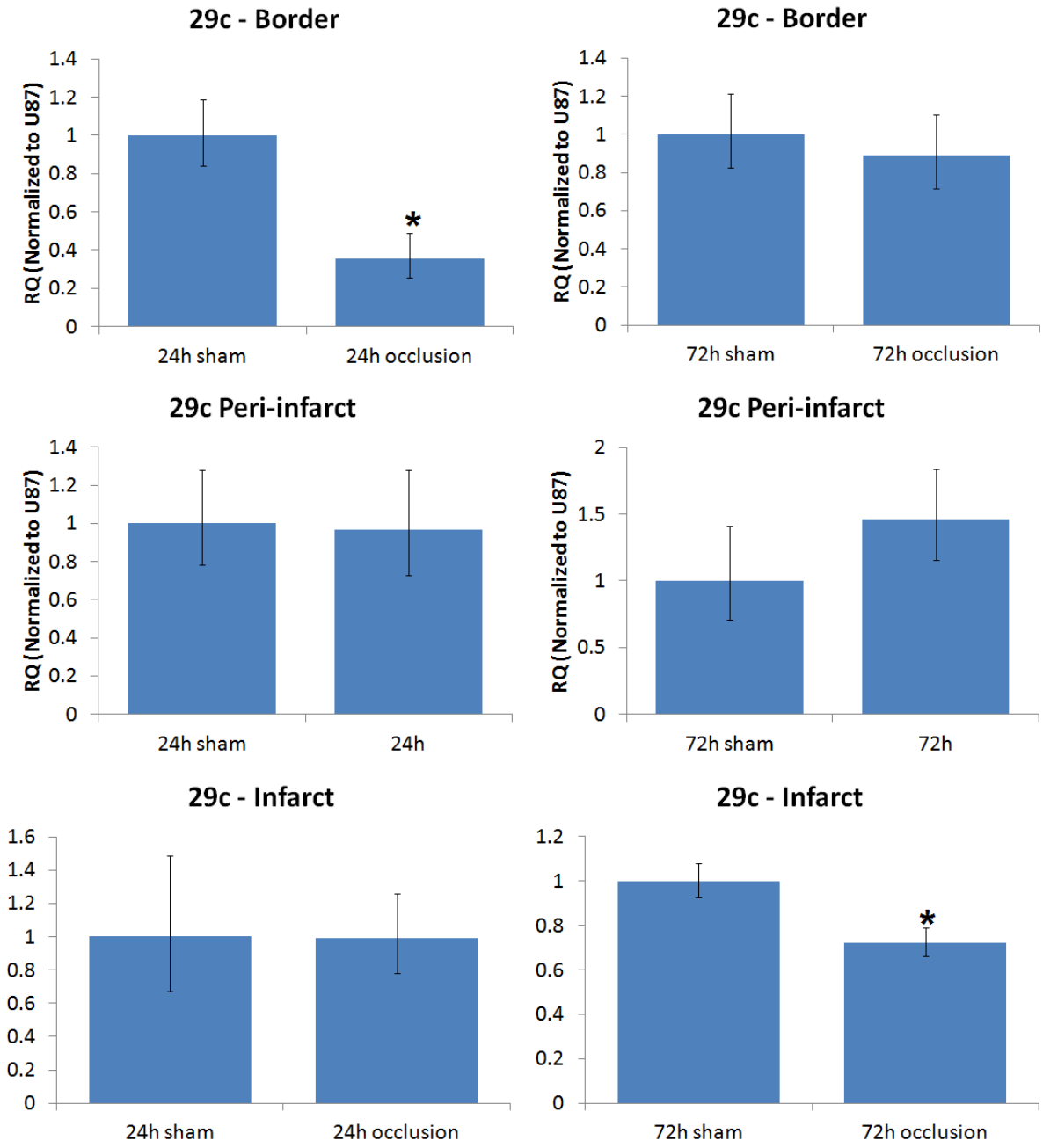

Figure 3.7 qPCR analysis of rat brain tissue for expression of miR-29c at 24h and 72h following stroke. Infarct, Peri-infarct and Border regions were defined by T2 MRI. Expression of miR-29c was measured in Border region, Peri-infarct and Infarcted region at 24h and 72h following stroke. Data are presented as RQ (±RQMax/Min) normalized to RNU48, n=4. Statistical analysis was conducted by two-tailed unpaired Student's t-test, * $p < 0.05$.

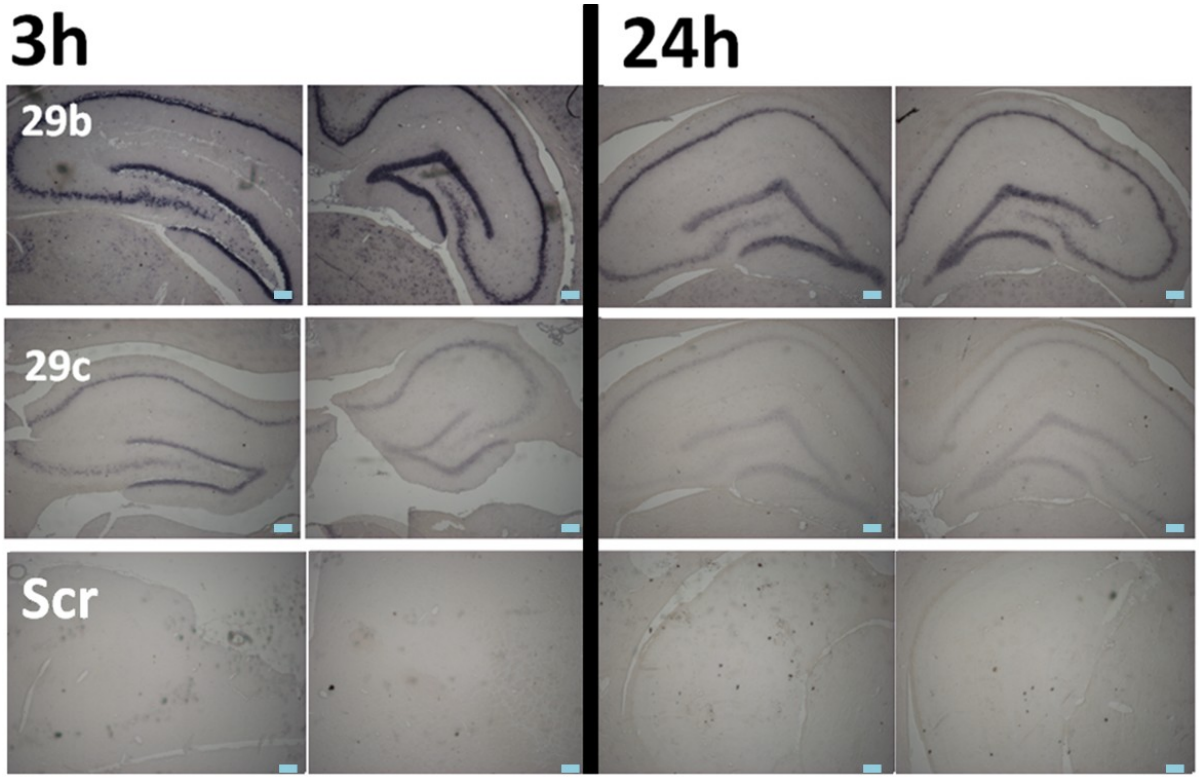

Figure 3.8 – Localization of miR-29 in brain tissue - miR-29b, miR-29c and Scrambled in situ hybridization for miRNA expression in paraffin embedded rat brain sections at the level of the dorsal hippocampus at 3 & 24h following tMCAO. Scale bar 100µm.

3.2.6 Analysis of validated miR-29 cluster targets in rat peri-infarct tissue by qPCR

In order to ascertain whether there was any complementary difference in miR-29 targets qPCR was performed in the same tissue used to analyze endogenous changes in expression levels (Figure 3.9). MMP2, MMP9 and TGFβ1 are all significantly downregulated in the peri-infarct region at 72h following stroke indicating that these genes may be under the regulation of miR-29b under these conditions. Col3A1, Col11A1, HDAC2, HDAC4 and Bcl2 were also assessed, but no significant difference was observed.

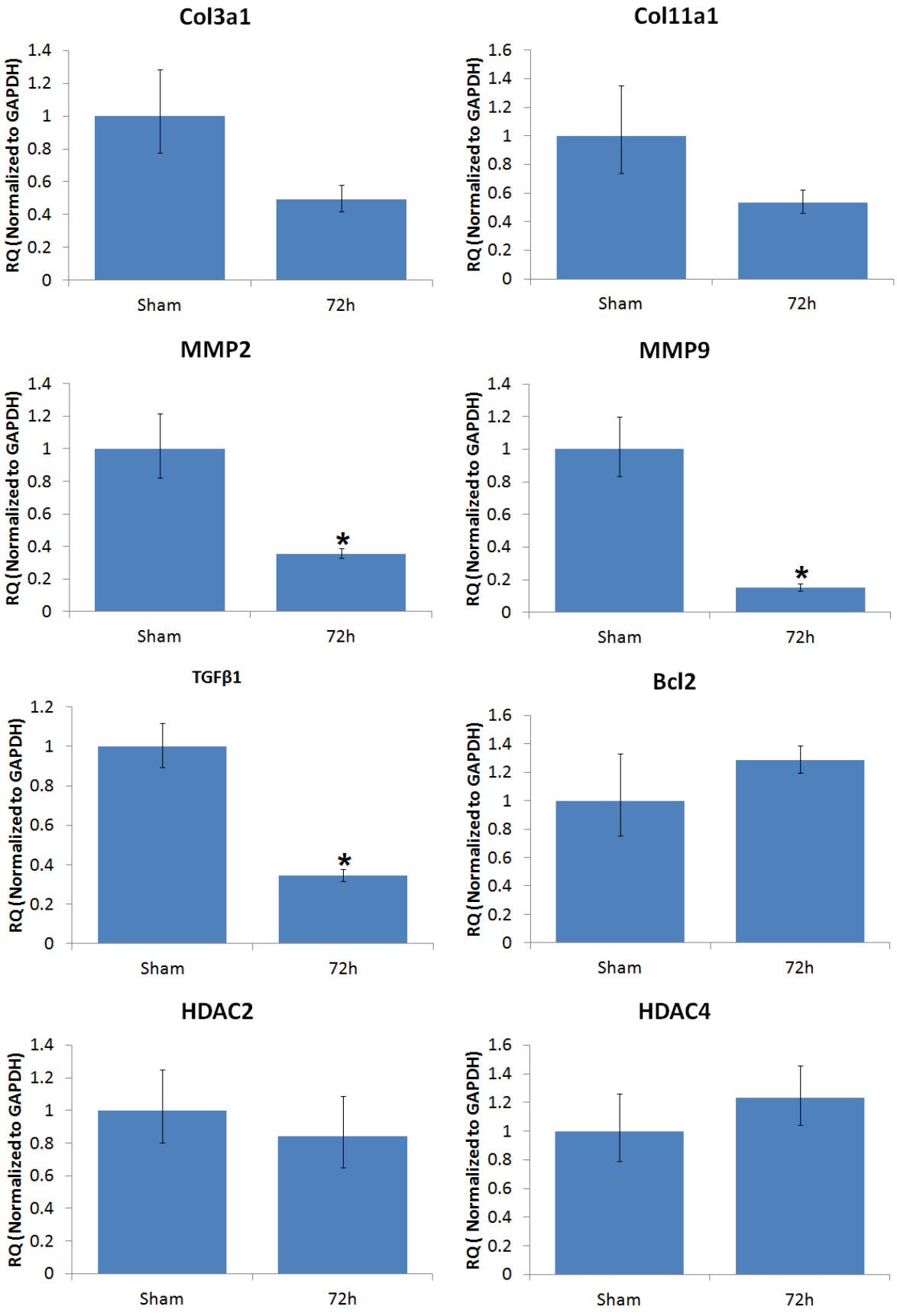

Figure 3.9– Expression of miR-29 targets in rat brain tissue at 72h post stroke - qPCR of miR-29 targets in peri-infarct rat brain tissue at 72h following stroke. All values are presented as RQ (+RQMax,-RQMin) normalized to GAPDH, n=4. (* - $p<0.05$ as determined by two-tailed unpaired Student's t-test)

3.2.7 Inhibition of miR-29b and miR-29c with anti-miRs and Overexpression with miRNA mimics

Modulation of miR-29 species was attempted through the use of commercially available miRNA oligonucleotides transfected into B50 cells which were subsequently subjected to hypoxic challenge. No changes were observed in expression levels of miR-29b or miR-29c at any concentration of negative control transfection. Significant upregulation of miR-29b was observed at all concentrations of miR-29b mimic used, with the transduction being most prominent with a 60-fold increase in expression of miR-29b following administration of 100nM of mimic (Figure 3.10).

miR-29c was significantly upregulated at all concentrations of miR-29c mimic administered, with the upregulation most profound ~20-fold at 100nM (Figure 3.10). These results definitively demonstrate that it is possible to significantly upregulate miR-29b and miR-29c in B50 cells with Ambion miRVANA miRNA mimics.

Transcriptional expression levels of several miR-29 cluster targets was assessed following modulation of these miRNAs. HDAC2, HDAC4, MMP2, Col11A1, Bcl2 expression levels were measured by qPCR however, no difference in expression was observed. MMP9 was found to be increased following transfection with miR-29b but not with miR-29c whilst Col3A1 expression was found to be elevated after transfection with both miR-29b and miR-29c. This is not consistent with what would be expected to be seen if gene regulation was being controlled by the miRNAs of interest in these cells under these conditions (Figure 3.11).

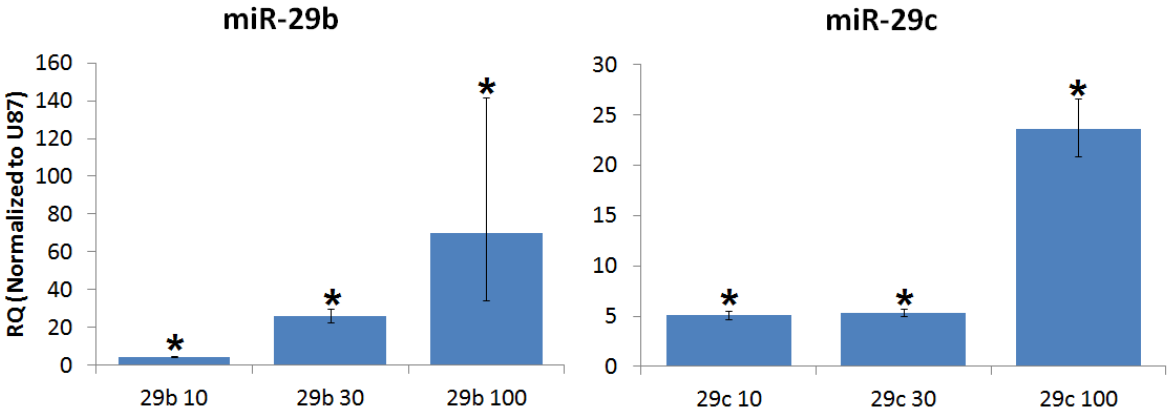

Figure 3.10 - Validation of miR-29 overexpression by pre miR mimics – qPCR was performed in B50 cells which had been transfected using siPORT transfection reagent. Concentrations of miR-29b and miR-29c from 10nM-100mM as well as Cy3-labelled negative control were applied to cells before expression levels were determined. All data normalized to relative Cy3 concentration and presented as mean RQ±RQmax/RQmin, n=3. Significance determined by one way ANOVA and Tukey's post hoc analysis *=p< 0.05.

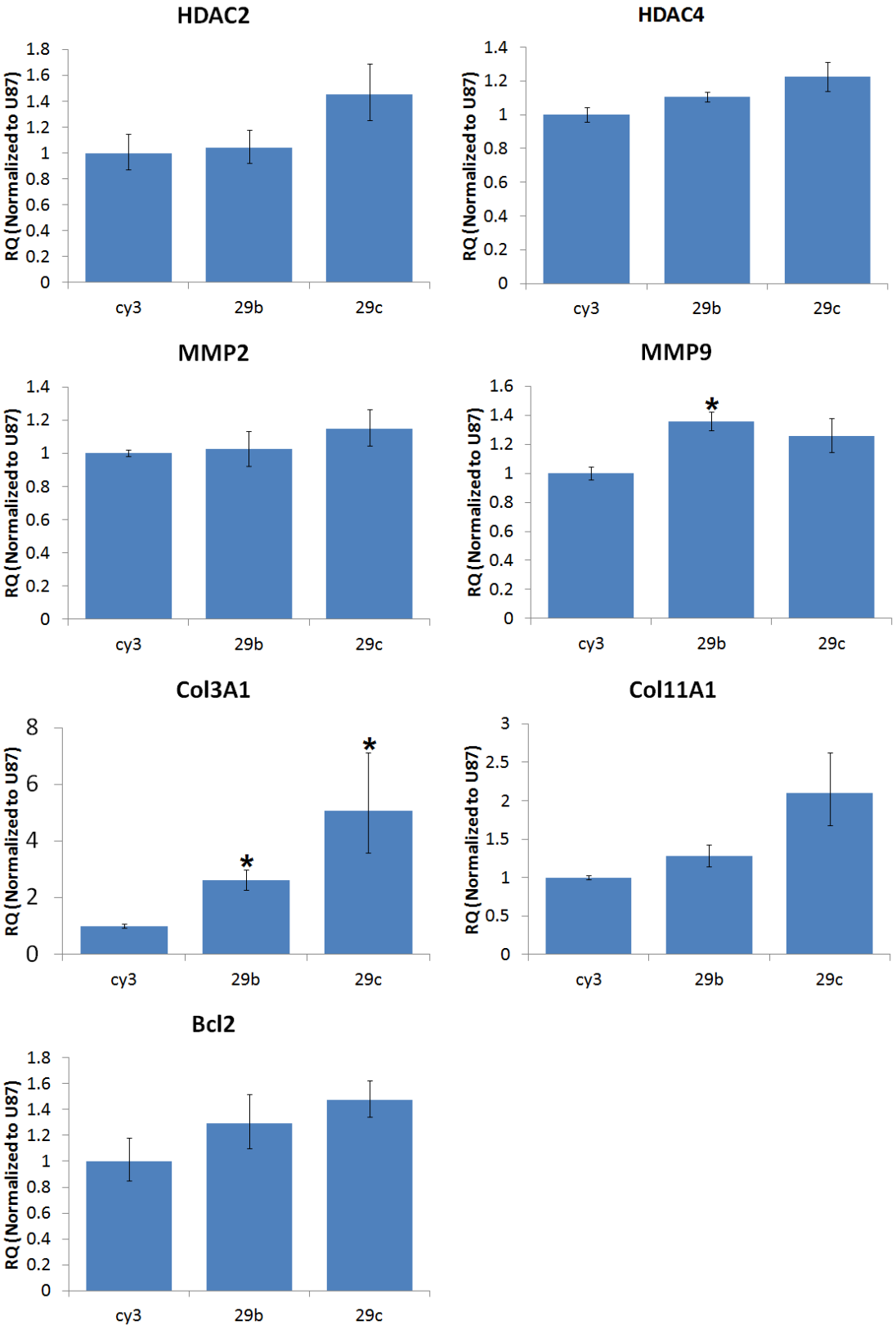

Figure 3.11 – Expression of miR-29 Targets - Transcriptional expression of miR-29 cluster targets in hypoxic B50 cells transfected with 100nm of relevant mimic or concentration matched cy3 negative control. miR-29b transfection corresponded with a significant increase in MMP9 expression whilst Col3A1 expression was determined to be significantly increased following transfection by both miR-29b and miR-29c. n=3 Data are presented as mean RQ±RQMax/Min. Significance determined by one way ANOVA with Tukey's post hoc analysis *=p<0.05

3.3 Discussion

MiR-29 modulation was assessed in several brain regions of spontaneously hypertensive stroke prone rat at two time points following stroke. Specifically miR-29b was upregulated at 72h following stroke and downregulated in infarcted tissue at both 24h and 72h. miR-29c was decreased in border tissue at 24h following stroke and in infarcted tissue at 72h following stroke. Reciprocal changes in expression of target genes: MMP2, MMP9 and TGFβ1 were observed at 72h following stroke. These reflect what would be expected if these genes were under control by miR-29 family members.

Initially, the aim was to clone miR-29b and miR-29c into the pSFFV to produce lentiviral vectors for the purposes of transducing miRNAs in cells used. Considerable difficulties were encountered during cloning which were never fully understood. Viral vectors assessed by cell titration assays were also incompatible with some of the cell types being used. For these reasons, and because reproducible high levels of transduction were attainable using commercially available miRNA mimics subsequent experiments focussed on the use of miRNA mimics. There are advantages and disadvantages to both platforms from a clinical perspective. The greatest advantages of viral vectors is that, when successful, they offer the greatest transduction efficiency and allow the transgene to be expressed only in desired cell types through the use of conditional expressers. Viral vectors suffer from being considerably more expensive to produce, having to comply with a more rigorous set of rules when being considered for human use and having to consider the complexities of the human immune system as well as the disease being studied. miRNA mimics are less expensive, but do not allow for conditional expression and have less transduction efficiency.

It remains to be determined whether the change in miR-29 expression following cerebral ischaemia is damaging or beneficial. Recent research has demonstrated that miR-29 species are potent inhibitors of TGF-β mediated fibrosis (Van Rooij et al. 2008). Glial scar formation following stroke is a factor in the loss of cognitive function following stroke. Therefore, inhibiting expression of fibrotic proteins may potentially limit the extent to which pathological glial scar tissue is formed, and may reduce the loss of cognitive

function. TGF-β signaling is of importance for neuronal cell survival (Silver and Miller 2004; Badan et al. 2003). Recently it has been suggested that *in vitro* upregulation of miR-29 promotes cell death following hypoxic challenge (Shi et al. 2012; Khanna et al. 2013). However, this group also suggests that miR-29b is upregulated *in vivo* following transient middle cerebral artery occlusion which is antithetical to the rest of the scientific literature in this respect (Shi et al. 2012).

3.3.1 miR-29 expression in vivo

For miR-29b no change in expression was observed at the border region at the 24h and 72h time points. A significant upregulation of miR-29b was present in peri-infarct tissue at 72h following stroke, whilst miR-29b was downregulated within the infarct at both times.

For miR-29c a significant downregulation was observed in the border zone at 24h following MCA occlusion. No differences were observed for miR-29c expression at either time point relevant to sham in the peri-infarct region. However, there was a significant decrease in miR-29c expression within the infarct at 72h following stroke.

In order to identify associations with these miRNAs and their targets, qPCR was also performed on several coding genes. These were selected based upon candidates assessed elsewhere in the literature. MMP2, MMP9 and TGFβ1 were all found to be significantly downregulated at the transcriptomic level in the peri-infarct region at 72h following stoke. This implies that MMP2, MMP9 and TGFβ1 may be repressed by miR-29b during stroke. It is likely that these changes would be beneficial in the context of this disease (Krupinski et al. 1996; Zhao et al. 2010).

Lower levels of MMPs in the acute phase following stroke will result in less blood-brain barrier breakdown and act to limit evolution of the infarct (Asahi et al. 2001; Rosenberg, Estrada, and Dencoff 1998). TGFBβ1 signaling plays an important role in glial scar formation and the reduction here may also correspond to a reduction in the size of ischemic lesion. It should be noted that MMPs expressed in the more chronic phase of stroke aids angiogenesis and other repair mechanisms meaning that suppression of MMPs in the longer term may have negative effects on recovery (Arai et al. 2009). As this study only assessed outcomes up to 72h following stroke, only the acute role of MMPs has been considered however, it goes without saying that the biphasic nature of these

enzymes in stroke would need to be considered when targeting this pathway therapeutically in the clinical setting.

The evidence presented here shows correlation between directional change in miRNAs and corresponding changes in target transcripts. This is suggests miR-29 may be modulating these genes, but direct interaction between the miRNA seed sequence and target transcripts would have to be demonstrated to state this conclusively. Further experiments would be required to demonstrate definitively that these genes are interacting, and it will be important to demonstrate that changes in gene expression are reflected at the protein level also.

The fold changes which have been found to occur in miR-29b/c expression following stroke are relatively modest and it is unclear whether this would be sufficient to achieve physiological effects due to the modulation of downstream genes. It is very challenging to ascertain the fold miRNA changes in human brain tissue due to stroke as biological samples for this type of experiment are not readily variable. Several miR-29 targets were assessed, and some of these were found to be altered in a manner consistent with miRNA regulation, although 3'UTR mutation experiments would be necessary on follow up to determine conclusively that these miRNAs are responsible for this modulation. It may have been preferable to conduct a screen experiment to assess the whole of the transcriptome as it is quite likely that many miR-29 targets which are modulated following stroke have been missed in this study, however there are considerable challenges associated with such transcriptomic experiments. Another aspect of this investigation that would benefit from follow up would be assessment of miRNA gene targets at the protein level to confirm than changes in transcriptional expression is reflected at the protein level.

3.3.2 miR-29 modulation in vitro

I have conclusively demonstrated that transfection of both miR-29b and miR-29c can be achieved via use of the pcDNA3.1(+) cloned constructs. Following this considerable difficulties were encountered when cloning SFFV-miR-29b constructs. I have noted that there is a region of TTTTTT in the miR-29b sequence and suspect that this may result in some unexpected secondary structure which results in some unusual enzymatic activity.

Although, I was able to produce lentivirus containing both miR-29b and miR-29c eventually, I was unable to observe any transduction in infected cells. Taking into consideration these technical issues and given the availability of commercially produced miRNA mimics which have been found to substantially upregulate expression of miRNAs in vivo, it was decided that the miRNA mimic approach would be used in subsequent in vitro experimentation. Advantages here consist of improved reproducibility and reduced preparation time (as viruses do not have to be generated & titred). The disadvantages of the miRNA mimic strategy are that it renders us unable to take advantage of the sophisticated viral machinery which can be used to ensure specificity and high efficacy of miRNA treatment. Nevertheless, upon transfection with both miR-29b and miR-29c mimics a marked increase in expression was observed in transfected cells. There were also significant differences in some of the miR-29 cluster targets, but they were being modulated in the opposite direction of what would be expected given that they are under regulation by these miRNAs under these conditions. It is possible that some counter-regulatory mechanisms are in place which results in the differences observed. Another consideration which should be made is that the single time point approach offers only a 'snapshot' and effects may be present at other time points. It would have been preferable to look at multiple time points to obtain a more detailed understanding of longitudinal changes in gene expression following stroke, but due to limitations in resources, these were the only time points assessed. Another caveat in these experiments is that only a small number of all potential targets of the miRNA of interest have been investigated. A transcriptomic approach may provide a path of least resistance towards identifying mechanisms, although, there are considerable technical issues associated with such an endeavour. Only one dose of miRNA mimic was attempted in this study when assessing changes in target transcripts. The dose of 30nm was selected as it achieved maximal modulation, it could be suggested that the lack of expected changes in target genes may be due to counter regulatory mechanisms which are responding to modulation of miR-29 beyond normal physiological parameters. This was considered during planning of the experiment, and it was determined that simulating a physiological change might not have sufficiently perturbed the system enough to effectively modulate target genes. It is possible that other miR-29 targets have been modulated, but the strategy of selecting candidate targets has failed to identify them in this study.

Following completion of my miR-29 experiments, two groups have published research on the role of miR-29 species in the context of stroke. It will be useful to consider their results and how they relate to observations made in my study. Loss of miR-29b following acute ischemic stroke has been shown to contribute to neural cell death and infarct size (Khanna et al. 2013). Assessment of their in vivo data shows that a comparison has been made between the infarcted region and non-infarcted region in mice to observe this downregulation in vivo. This is consistent with my observations for rat infarct. However, this study makes no attempt to identify changes in the peri-infarct region. It is possible that by the time these researchers have measured miRNA expression in the infarcted region post-mortality RNA degradation is already underway. In order to determine the effects of miR-29b in vivo, they pre-treated the mice with a miR-29b mimic and found that this significantly reduced infarct size.

This demonstrates the potential therapeutic applications of this miRNA in the context of stroke and suggests that increase in miR-29b expression in the peri-infarct region that I have observed at 72h following stroke may be part of the organism's intrinsic response to protect itself from injury. Again, in keeping with the STAIR guidelines, Khanna *et al.* have also attempted to address differences in the sensorimotor function in mice treated with the miR-29b mimic using the open field test (Khanna et al. 2013). A marked improvement was observed in the behaviour of the mice at 48h following stroke for the treated group versus control. Despite the accumulating data regarding the role of miR-29 cluster members in stroke, the mechanisms whereby these microRNAs are having an effect remain poorly understood. Much of this opacity relates to the complexity of the disease and technical limitations faced when interrogating our disease models. Perhaps success in the marketplace for other miRNA based therapeutics will increase industrial interest in these treatments. Further experimentation will result in improved understanding of the role of miR-29 in stroke and we may one day see a treatment based on this miRNA enter the clinic.

Chapter 4 – Modulation of serum miRNA levels in response to ischaemic stroke.

4.1 Introduction

Stroke must be accurately diagnosed swiftly following onset of symptoms and this is the reasons for many of the outstanding challenges in treating it. Such diagnosis is often clinically challenging despite ongoing advances in improved practices in stroke medicine. A secondary, ongoing challenge that is faced following the resolution for stroke is that it is impossible for clinicians to predict the extent of patient recovery, which patients will respond to the administered treatment, and knowledge of patient's quality of life following recovery.

4.1.1 miRNAs as novel biomarkers for Stroke?

In order to address at challenges associated with stroke, the development of novel biomarkers of ischaemic stroke will be helpful. A biomarker, or biological marker for a disease is a clinically measurable indicator of some aspect of a disease. The most widely used biomarkers for stroke in the clinic are the presence of risk factors such as hypertension or obesity, whilst these provide essential information to clinicians they are imprecise and unable to accurately predict stroke onset as well as predict disease progression. Recently there has been increasing interest in the identification of novel molecular biomarkers for cardiovascular diseases with C-reactive protein being arguably the most successful candidate so far. Most miRNA research to date has focussed on the role of intracellular RNAi, although there is increasing interest in the biology of circulating serum miRNA (Clancy et al. 2014; Qing et al. 2014). Whilst the discovery of the serum component of the miRNAome has functional implications for the role of miRNA-mediated intercellular signalling; it may also have diagnostic use as a clinical blood based biomarker. Ideally miRNA biomarkers will be successfully able to aid in earlier detection of stroke risk, as well as allowing for risk stratification and identification of patients who will benefit most from interventions.

Although only a relatively new contender in the arena of modern medicine, it has been demonstrated that there are abundant levels of many miRNAs circulating in the human blood (Gilad et al. 2008). It doesn't take long for one to reach the conclusion that if these molecules are present in the circulation, and important in the onset and development of disease, that they may also be able to inform clinical practice as biomarkers. Currently there is no common clinically implemented miRNA based biomarker for any disease. That

said, there is a great interest in the development of such a biomarker in many different diseases.

The first studies which demonstrated that circulating miRNAs were indicative of disease states occurred in 2008. One such study demonstrated that miR-21 expression levels were elevated in patients with B-cell lymphoma. Subsequently, several studies were conducted which developed miRNA expression levels for many different kinds of cancer (Lawrie et al. 2008).

One of the first studies examining differential expression of microRNAs in response to stroke was a study conducted on Sprague Dawley rats which were subjected to transient middle cerebral artery occlusion (tMCAO) and allowed to recover for 24 or 48h. Whole brain lysates as well as serum were subjected to microRNA screen in order to identify miRNAs which were differentially regulated (Jeyaseelan et al. 2008).

Subsequently, a similar though more sophisticated study followed up on this by assessing miRNA expression levels at several time points following stroke resulting in a more detailed description of changes which may occur following ischaemia (Dharap and Bowen 2009). Spontaneously hypertensive rats were subjected to tMCAO and allowed to recover for a range of time points from 3h to 3 days. However, only brain tissue was assessed in this study. Again, several microRNAs were found to be differentially regulated post-ischaemia. There is considerable overlap between brain microRNA modulation from both the Dharap and Jeyaseelan studies (Dharap and Bowen 2009; Jeyaseelan et al. 2008a), although slightly different models were used in each case. Dharap et al. identify several potential targets of the microRNAs modulated, many of these candidates are proteins known to be of functional importance in the pathophysiology of stroke. However, none of these targets were assessed in this study (Dharap and Bowen 2009). This study was followed up by another assessing the differential expression of microRNAs in response to sub lethal ischaemic preconditioning (Dharap and Vemuganti 2010). These animal studies provide invaluable information about the pathogenesis of stroke as it allows for sophisticated modelling of acute genetic changes following cerebral ischemia. For translation to the clinic, it is necessary to study microRNA changes in a human population of stroke patients.

The first instance of a group assessing serum miRNA expression levels in a human population occurred in 2009. Specifically this study looked at the miRNAome in young stroke patients (18-49) years compared with age matched healthy controls. Analysis of miRNA expression levels demonstrate that it was possible to identify TOAST classification subtypes of patients and distinguish these from healthy controls. However, this study demonstrated proof of principle that it is possible to use circulating miRNA levels as a biomarker for cerebral ischaemia (Tan et al. 2009). This study was followed up in 2013 by another investigation which sought to identify miRNA expression profiles in low/no-risk stroke patients in order to differentiate them from patients exhibiting traditional risk factors for stroke. Again, it was demonstrated that it is possible to do so and that molecular mechanisms underlying stroke pathology may differ significantly from more traditional clinical manifestations of the disease (Tan et al. 2013). One study sought to assess dysregulation in serum miRNAs in the context of intracerebral haemorrhagic (ICH) stroke. Comparisons were made between 16 ischaemic stroke patients, 15 ICH patients and 8 healthy controls and 30 miRNAs were determined to be significantly dysregulated. Subsequent replication of these findings in a validation population support the validity of the original observations (Guo et al. 2013). Additional evidence supporting the hypothesis that miRNAs are modulated in serum following stroke comes from a clinical study in which plasma miRNA expression levels were measured at 24h, 1 week, 4 weeks, 24 weeks and 48 weeks following symptom onset compared with 50 healthy controls. In this study, both miR-30a and miR-126 were found to be significantly downregulated at all time points following stroke until 24 weeks, whilst let-7b was lower in patients with large-vessel atherosclerosis than healthy volunteers and higher in patients with other forms of stroke until 24 weeks (Long et al. 2013).

4.1.2 Exosomal miRNAs

Recently there has been increasing interest in the functional role of circulating miRNAs (Hsu et al. 2014; Sun et al. 2014; Grasso et al. 2014). Initially these miRNAs were thought to be a fingerprint generated by RNA released from lysed cells. However, it has been observed that there is active export of miRNAs into vesicles such as microparticles or exosomes. Vesicular RNA is responsible for intercellular communication of miRNA activity (Valadi et al. 2007) and emerging evidence suggests that this may be of essential functional importance in a variety of contexts. Exosomes are small (30-120nm) molecules

comprised of a membrane bilayer which are constructed intracellularly before being released into the extracellular space. These exosomes contain a variety of lipid, protein and genetic molecules which act on a plethora of targets which vary between cells and disease states (van Niel et al. 2006; Simons and Raposo 2009). Exosomes are released from cells via fusion of a multivesicular body with the plasma membrane. Following invagination of an endosome with a myriad of molecules for exportation the fusion occurs and the exosome is released into the extracellular space. Several proteins present on exosomal membranes are commonly found to be characteristic of released exosomes including CD9, CD63, CD81 and HSP70. Currently the functional significance of these proteins remains to be elucidated, but it is likely that these proteins are involved in adherence and incorporation of exosomes into target cells (Rayner and Hennessy 2013). There are a variety of different vesicular molecules other than exosomes. These molecule classes are generally defined based on diameter of cell and include microvesicles, microparticles, ectosomes, shedding vesicles and apoptotic bodies (Figure 4.1) (Raposo and Stoorvogel 2013).

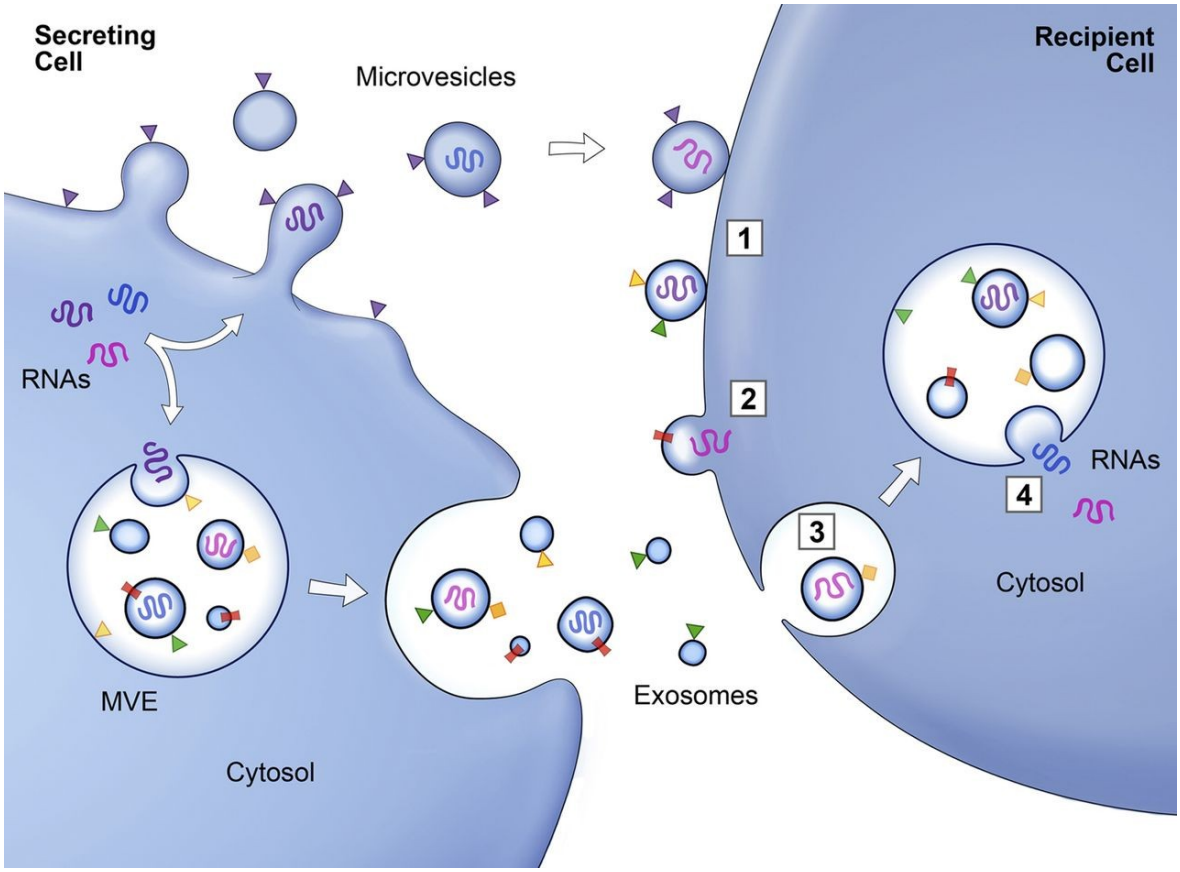

Figure 4.1 - Schematic of protein and RNA transfer of vesicular protein and RNA. Membrane-associated (triangles) and transmembrane proteins (rectangles) and RNAs (curved symbols) are selectively incorporated into the microvesicles and exosomes budding from the plasma membrane (Raposo and Stoorvogel 2013).

To date, little evidence exists which assesses the importance of vesicular RNA in the context of stroke. Isolation of microvesicles from plasma in one stroke study has demonstrated that miRNAs assessed were present solely in microvesicles of plasma samples, however following onset of stroke miRNAs present outside of vesicles in plasma increase. It is possible that expression profiles of miRNAs differ depending on whether they are located within exosomes, or free floating in serum in other cases also. This information may provide a clue as to the role of specific miRNAs in the context of ischemic stroke.

4.1.3 Hypotheses

miRNAs are differentially expressed in serum following stroke. Characteristic miRNA profiles may be used to better phenotype stroke patients

Serum miRNAs are located in excreted exosomes and may play an important functional role following stroke.

4.1.4 Aims

Candidate miRNAs were identified by miRNA Openarray experiment in a cohort of stroke patients and stroke mimic controls.

Candidate miRNAs were assessed for validation in a larger population of patients using qPCR.

miRNA expression levels were assessed in exosomes isolated from serum samples.

4.2 Results

4.2.1 Patient Demographics

In order to identify candidate miRNAs for assessment 75 patients (55 stroke, 20 stroke mimics) were recruited over a period of several months. Stroke mimics were patients who presented at clinic with stroke symptoms, but were determined to have a differential diagnosis following medical imaging and consensus of stroke clinicians. Serum samples were collected initially within 48h of the onset of stroke symptoms, then longitudinal samples were collected from these same patients at 7 days, 1 month and 3 months. In order to minimize the potential for confounding factors relating to differences in demographics between stroke patients and stroke mimic control patients, selection of patient samples for study were matched as closely as possible for age, gender and medical history (Table 4.1) with no statistically significant difference between the groups at baseline.

	Stroke	Non-Stroke	p-value
Numbers (per diagnostic category)	55 (73.33%)	20 (26.67%)	
Age (mean)	65.36 (SD 14.01)	57.41 (SD 14.01)	
Variable (no. Per diagnostic group)			
Male	35	11	0.726
Female	20	9	
Smokers	19	7	0.727
Ex-Smoker	9	4	0.644
Previous TIA	7	4	0.556
Type 1 Diabetes	3	0	
Type 2 Diabetes	7	1	
Hyperlipidaemia	14	2	0.556
Atrial Fibrillation	9	1	
Atrial Fibrillation (on Admission)	8	9	0.213
Family History	9	2	0.508
Hypertension	20	3	0.336
Antiplatlet	17	4	0.277
Anticoagulant	2	1	-
Statin	21	5	0.718
BP Treatment	22	5	0.65
ACE Inhibitor	8	2	
ARB	3	0	
Calcium Channel Blocker	7	2	-
Beta-Blocker	10	3	-
Thaizide	6	1	0.808
Spironolactone	0	0	-
Loop Diuretic	6	1	
Alpha Blocker	1	0	-
Insulin	3	0	
Oral Hypoglycaemic Drugs	4	0	0.494

Table 4.1 –Summary of population demographic information of study population.

4.2.2 miRNA Openarray Experiment to Identify Candidate miRNAs for Validation

miRNA expression levels were determined in the screened population by miRNA Openarray analysis. 16 stroke patients and 14 non-stroke patients were selected from the population for an initial screening experiment to identify candidate miRNAs of interest. Following an initial PCR preamplification run to amplify all RNA present, qPCR was

performed for every miRNA present on the card. Of the 738 genes measured 96 were detected in >70% of samples. Several reference genes were included on the Openarray cards in order to determine an appropriate control for the population of samples being analyzed. However, none of these reference genes was ubiquitously detectable in every patient sample, whilst variation in expression levels was too great between patients for any gene to be assessed. For this reason raw Ct values were analysed. T-test analysis was conducted on raw Ct values between stroke and non-stroke samples and 15 miRNAs candidates were identified for subsequent validation in the total patient population (Table 4.2, Figure 4.2).

miRNA	Non-stroke (Ct value), n=16	Stroke (Ct value) n=14	**p-value**
miR-19b	20 (16-22)	17 (14-17)	0.029
miR-20b	27 (25-29)	25 (23-27)	0.020
miR-21	23 (20-26)	21 (19-24)	0.048
miR-25	24 (21-26)	21 (18-21)	0.028
miR-27a	26 (22-31)	24 (20-25)	0.009
miR-93	25 (23-28)	23 (18-25)	0.004
miR-106a	21 (17-23)	18 (16-23)	0.014
miR-139-5p	25 (22-29)	24 (22-25)	0.029
miR-331	25 (21-30)	23 (19-26)	0.013
miR-374	27 (22-27)	24 (20-26)	0.020
miR-376c	31 (25-33)	28 (14-30)	0.028
miR-532-3p	30 (26-33)	27 (24-31)	0.008
miR-573-3p	25 (23-30)	23 (21-26	0.026
miR-590-5p	26 (23-28)	24 (21-25)	0.017
miR-885-5p	26 (23-30)	24 (11-26)	0.018

Table 4.2 – Most significant hits to come out of the OpenArray miRNA assay experiment at 48h post stroke. The first column indicates the name of the miRNA, followed by mean and range of Ct values for non-stroke and stroke patients. The final column is a p-value from a Student's t-test comparing the two.

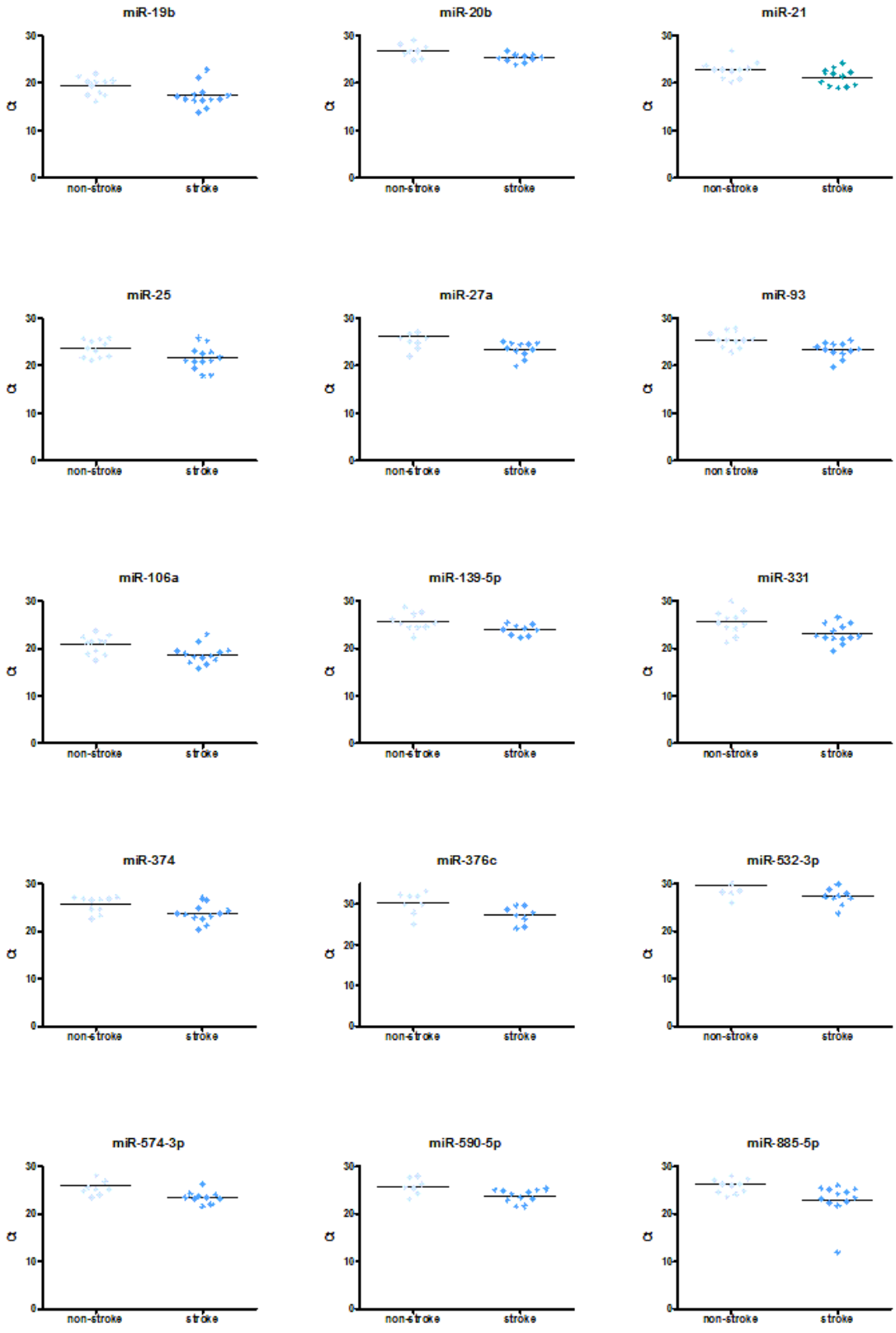

Figure 4.2 – Comparison of miRNA expression levels between stroke and non stroke patients in pilot population at 48h post-stroke – Data are presented as scatter plots indicating raw Ct values for each patient in the pilot study. Student's two-tailed t-test. $p<0.05$. non-stroke n=16, stroke n=14

Due to the fact that the Openarray experiment employed a pre-amplification step, it was deemed necessary to perform a qPCR validation on the OpenArray population (n=30) samples prior to including all validation patient samples in order to ascertain whether the candidate miRNAs were detectable in the absence of a preamplification PCR step. These candidate miRNAs were subjected to qPCR and whilst there was a reduction in the level of the transcript present for all genes in all samples as would be expected without preamplification, the higher Ct values were still within the range of accurate detection by qPCR. As no endogenous control was identified by the Openarray experiment, samples were spiked with a known concentration (5ng) of *c. elegans* miR-39 to act as a reference gene for normalization (Figure 4.3).

qPCR of non-amplified serum samples demonstrated consistent results between the Openarray and qPCR techniques, demonstrating that there was an increase in expression levels for all of the miRNAs identified by the original Openarray experiment in stroke patients versus controls. With the exception of miR-885 and miR-27a all of the top hits from the array were detected as significantly dysregulated (Table 4.3). Of the candidate miRNAs identified, several belonged to the miR-17/92 cluster or associated paralogs (Figure 4.4).

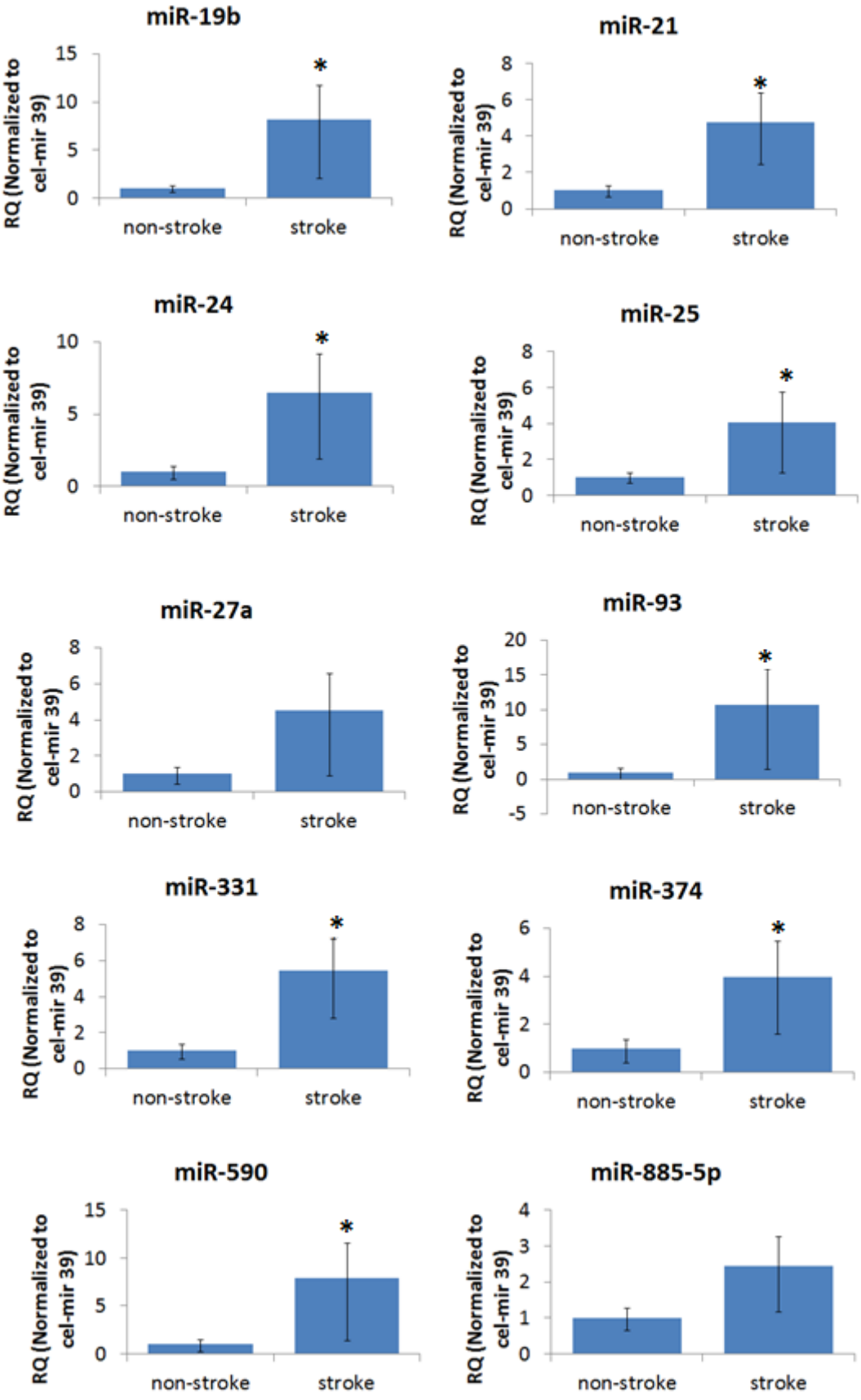

Figure 4.3 – Validation of OpenArray results in non-amplified samples from pilot population at 48h post-stroke - c. elegans miR-39 spiked human serum samples from the pilot patient population were analyzed by qPCR. Upregulation of candidate miRNAs identified by the OpenArray remain significant. (*=p<0.05, Student's unpaired two tailed t-test n=5)

miR	Fold Change (Non-stroke vs. Stroke)	p-value
miR-19b	8.20	0.02
miR-21	4.78	0.0007
miR-24	6.46	0.001
miR-25	4.05	0.009
miR-27a	4.54	0.09
miR-93	10.77	0.03
miR-331	5.43	0.0006
miR-374	3.96	0.02
miR-590	7.91	0.006
miR-885	2.43	0.07

Table 4.3 – Summary of pilot population qPCR normalized to c-elegans spike without preamplification step used in initial OpenArray experiment. – data are presented as fold change between non stroke and stroke patients with p-value as determined by Student's unpaired two-tailed t-test n=5.

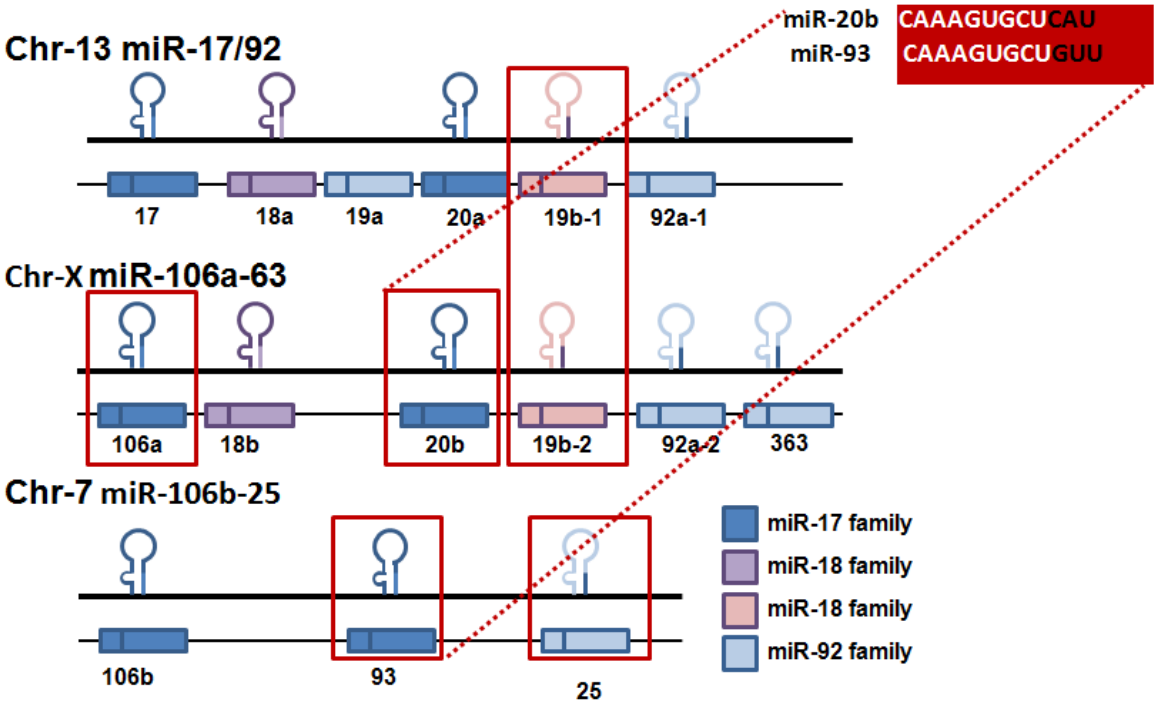

Figure 4.4 - Diagram illustrating the genomic location and identity of miR-17-92 miRNAs and associated paralogs. - miRNAs which were identified by the OpenArray experiment are highlighted in red. miR-17 seed sequence is illustrated for miR-17 family members.(Olive, Jiang, and He 2010)

4.2.3 Validation Population

Patients were recruited over a period of months and serum was harvested. 20 non-stroke subjects and 55 stroke patients were assessed in all. This cohort included the patients used for assessment of potential candidate by the OpenArray experiment described above. Again, care was taken to minimize potential confounding factors relating to differences in patient demographics by ensuring the population was matched so that no significant difference occurred between the two groups at baseline (table 4.1).

Extensive demographic data were obtained about the patients by the clinical researchers (Table 4.1) and there was no significant difference between groups with relation to the sex ratio, or age, medication, blood pressure, body weight and whether patients smoked or not. It is important to assess all of these aspects of patient phenotype as factors such as medication being used, and co morbidity from other diseases may all influence expression levels of miRNAs. The lack of significant difference between patient groups assures us that any difference in miRNA expression that may be observed in this study is as a result of stroke pathology and not any other confounding factor. Intragroup variability regarding differences in patient phenotype, treatment and comorbidity

accounts for much of the variation within a group and this variation necessitates a greater number of patients to achieve sufficient statistical power. In order to ensure that our study was appropriately power, a statistician was consulted and power calculation performed. Thus assured that the population we had access to offered sufficient power to perform our experiment qPCR of the full validation contingent proceeded. No endogenous control was identified by the earlier OpenArray experiment, so samples were post-extraction spiked with a known mass (5ng) of *c. elegans* miR-39. This was used to normalize miRNA Ct values to in order to account for variation from pipetting error etc. No difference was observed at 48h for miR-19b, -20b, -21, -27a, -93, -106a, -139, -331, -374, -532, -590, -885 when assessed by qPCR (Figure 4.5). In most cases there does not appear to be a great deal of variability in expression levels within groups and no significant difference was present between groups.

As mentioned earlier, clinical researchers have indicated the importance of differences in clinical phenotype in the manifestation of stroke, the differences this may make to therapeutic interventions used and also the effect that this will have on severity of patient outcomes. Bearing this in mind it was considered useful to do some post-hoc breakdown of the stroke patient group by TOAST classification stroke subtype (Figure 4.6). This analysis, though by no means definitive, may help to identify relationships that we are currently missing due to heterogeneity of disease subtypes in our stroke population. Upon analysis by TOAST classification subtype, no differences were observed between stroke patients of any subtype and non-stroke controls (Figure 4.7).

Post-hoc analysis of miRNA expression by TOAST classification yielded no new significance, but it did appear that there was a trend towards higher expression in the Large Artery stroke subtype for several miRNAs including the 17-92 cluster members mentioned previously (Figure 4.4). When considering post-hoc analysis it is important to remember that any significance observed here would not be conclusive and require further experimental observation to test novel hypotheses generated by such and analysis, another consideration is that the reduction in group sizes as a result of the subclassification breakdown makes the study presented here considerably underpowered. In order to conclusively determine whether there are differences in miRNA expression between stroke subtypes, a much larger study will need to be conducted.

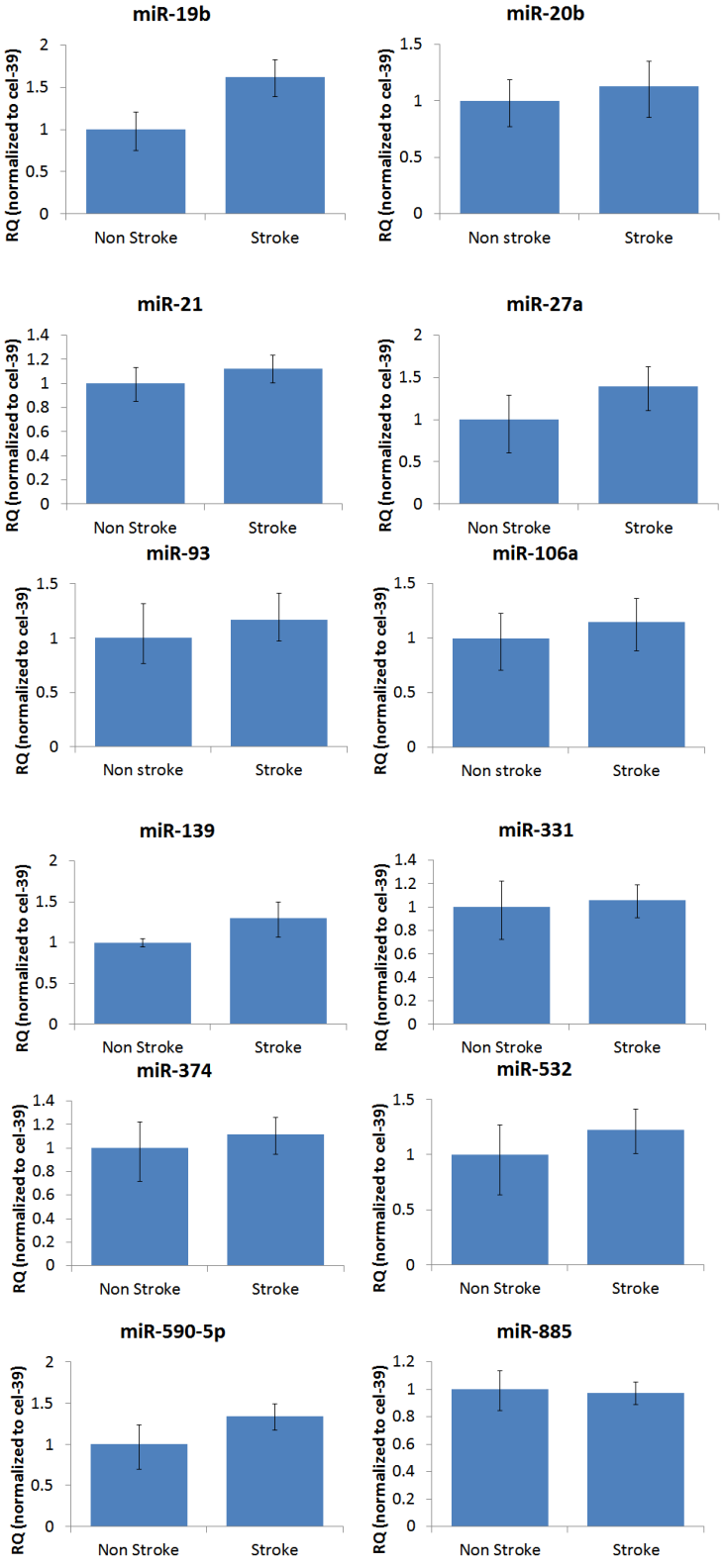

Figure 4.5 – Summary of qPCR validation data for several of the candidate miRNAs assessed. In this graph, expression has been normalized in all samples to the spiked c. elegans miR-39 mimic. – Data are represented as mean RQ (stroke compared to non-stroke) normalized to *c. elegans*miR-39 (cel-39) spike ±RQmax/RQmin. No significant differences were observed. (Non stroke n=20, Stroke n=55)

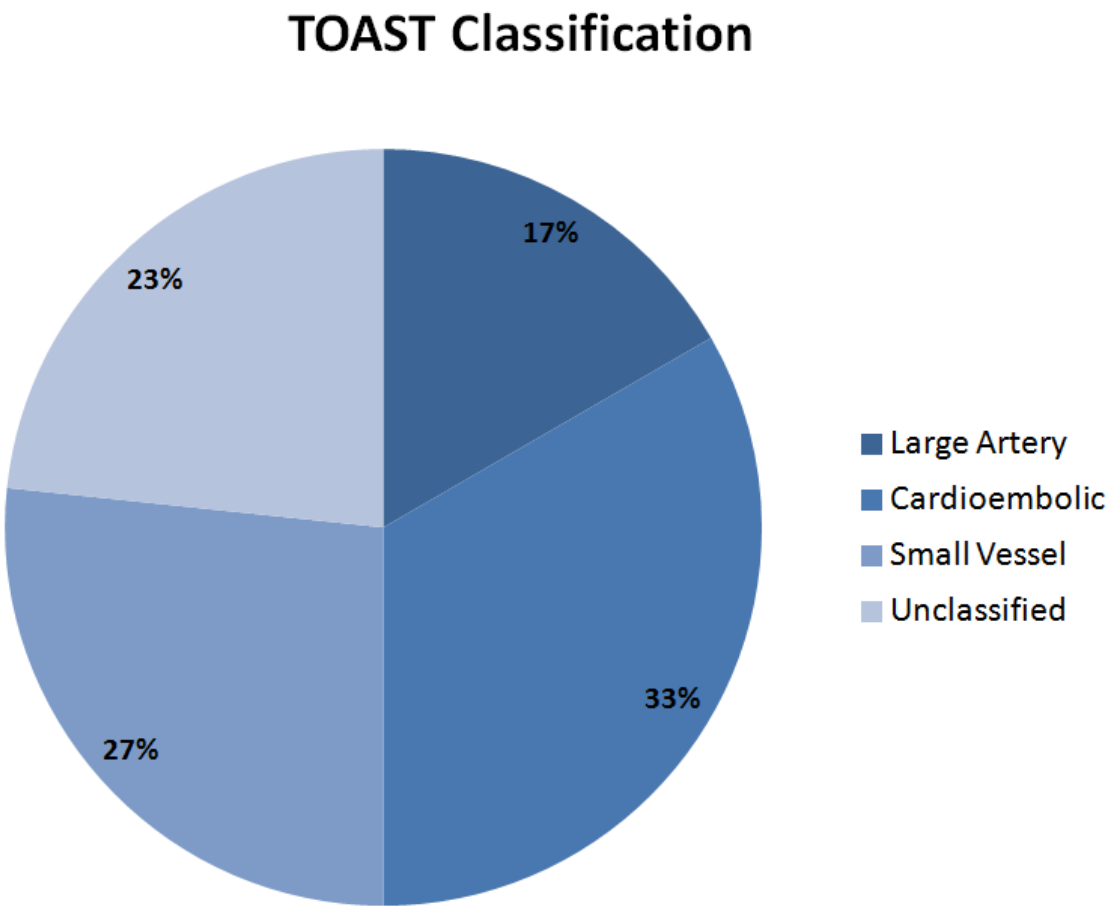

Figure 4.6 - Pie chart illustrating the breakdown of the validation population by stroke subtype.

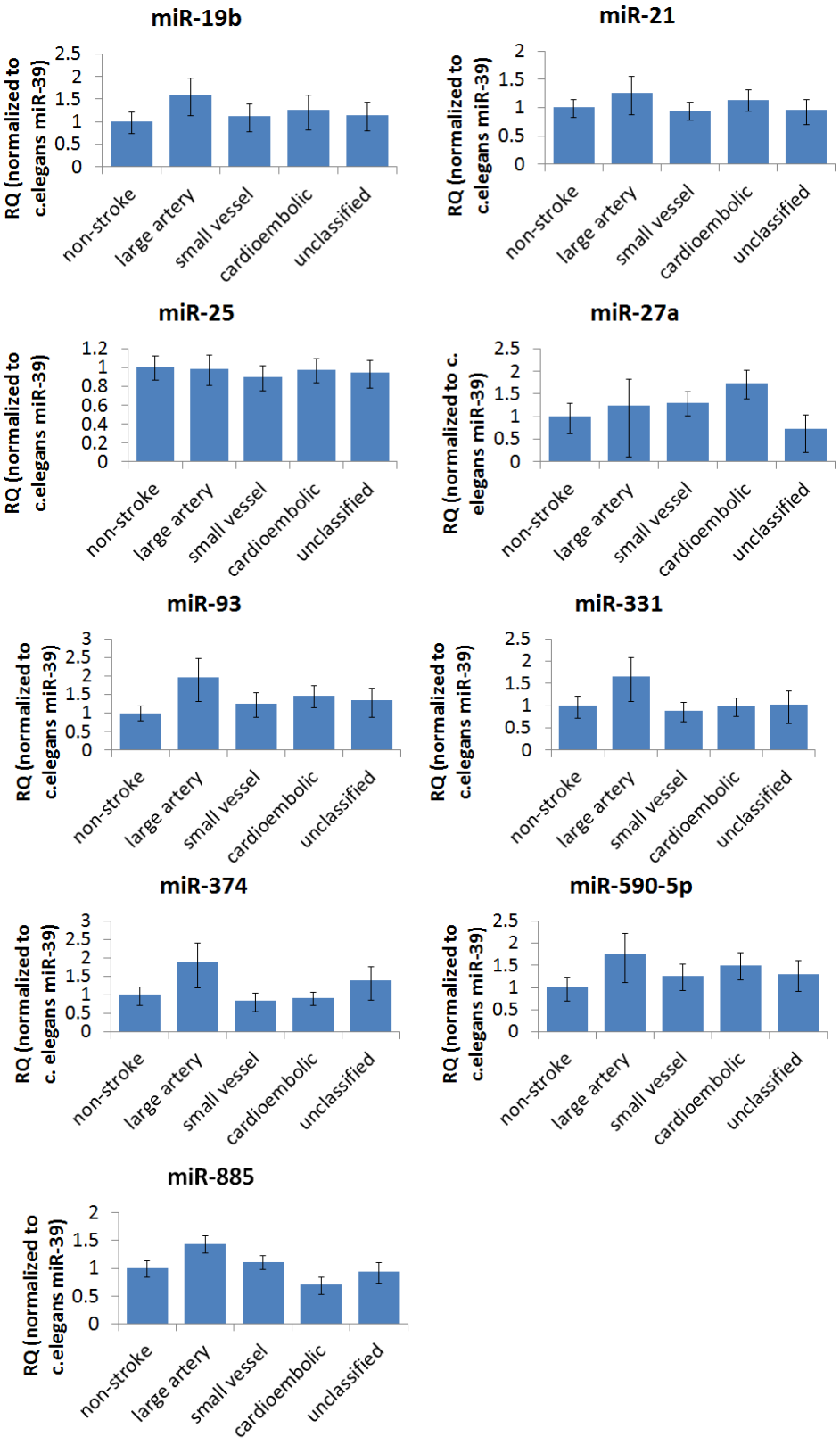

Figure 4.7 – Expression of miRNAs in stroke patients divided by TOAST classification - miRNA expression in validation population measured by qPCR normalized to c. elegans miR-39 spike. – Data are presented as RQ±RQmax/RQmin compared to non-stroke (normalized to *c. elegans*miR-39 spike). (Non stroke n=19, Large Artery n=10, Small vessel n=17, Cardioembolic n=16, Unclassified n=12)

4.2.4 Serum Exosomes

As several recent publications have emphasized the importance of miRNA biology in exosomal mediated transport of RNAi signaling, some preliminary work was conducted in order to identify differences in expression between exosomal expression and total serum expression of miRNAs. If found to be most abundant within a vesicle, credence would be lent to the idea that a particular miRNA may have a functional role.

Using the Nanosight to count number of exosomes (particles from 30-120nm), no significance was observed between non stroke and stroke patients (Figure 4.8).

Preliminary analysis suggested that qPCR analysis on RNA extracted from isolated exosomes yielded better signal than that from total serum RNA. Ideally, it would have been beneficial to assess all of the candidate miRNAs in isolated exosomes from all patients. However, due to limited resources this was not possible. In order to obtain more evidence supporting the importance of exosomal miRNAs in circulating serum several of the candidate miRNAs from the earlier study (section 4.2.3) were assessed by qPCR. miR-19b, -93, -106a and -139 all display a similar trend towards increased expression in stroke patients versus control seen in the serum RNA, but this did not reach significance. (Figure 4.9).

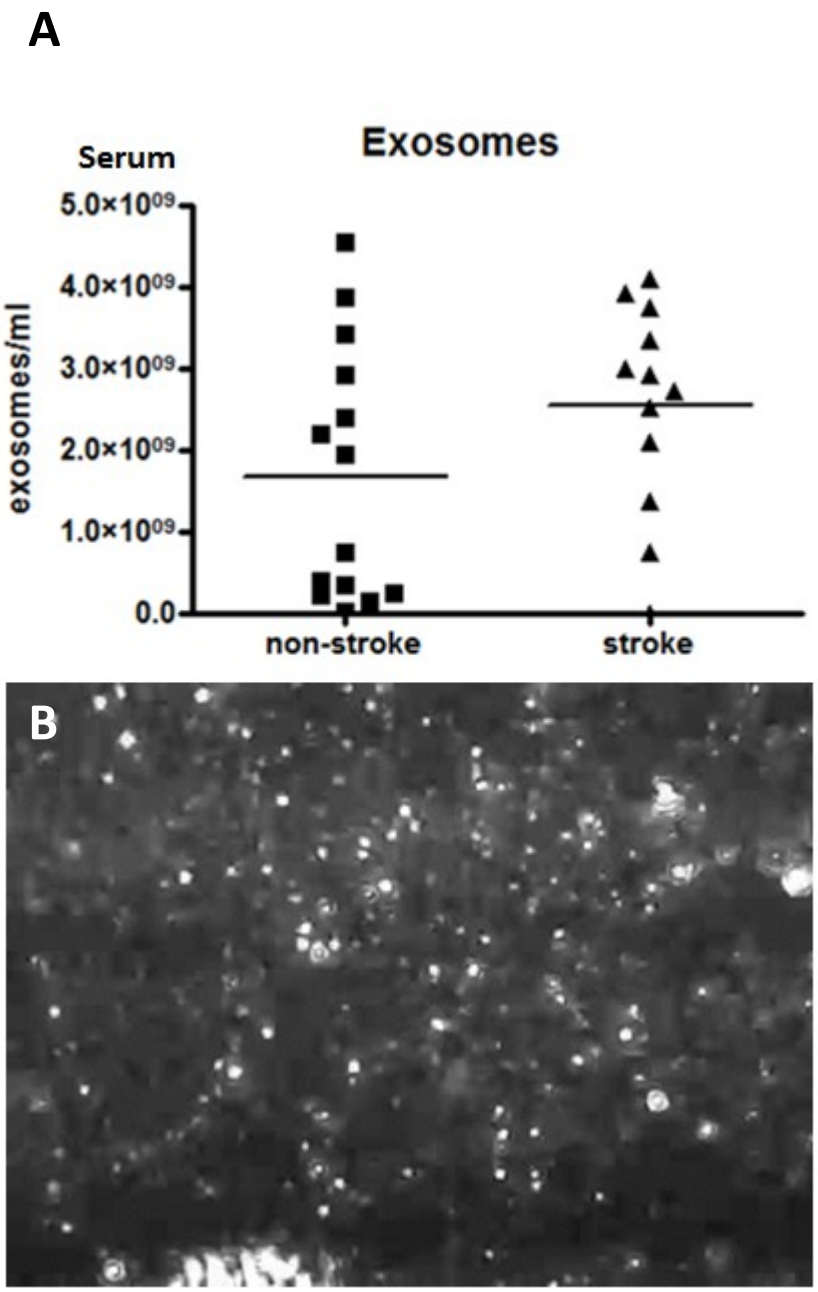

Figure 4.8 -Exosomal analysis – (A) Number of exosomes in each sample was measured by nanosight.. The horizontal bar in the graph represents the mean, whilst the squares/triangles indicate individual samples. **(B)** Image illustrating individual exosomes as viewed on nanosight. Non-stroke n=12, Stroke n=14.

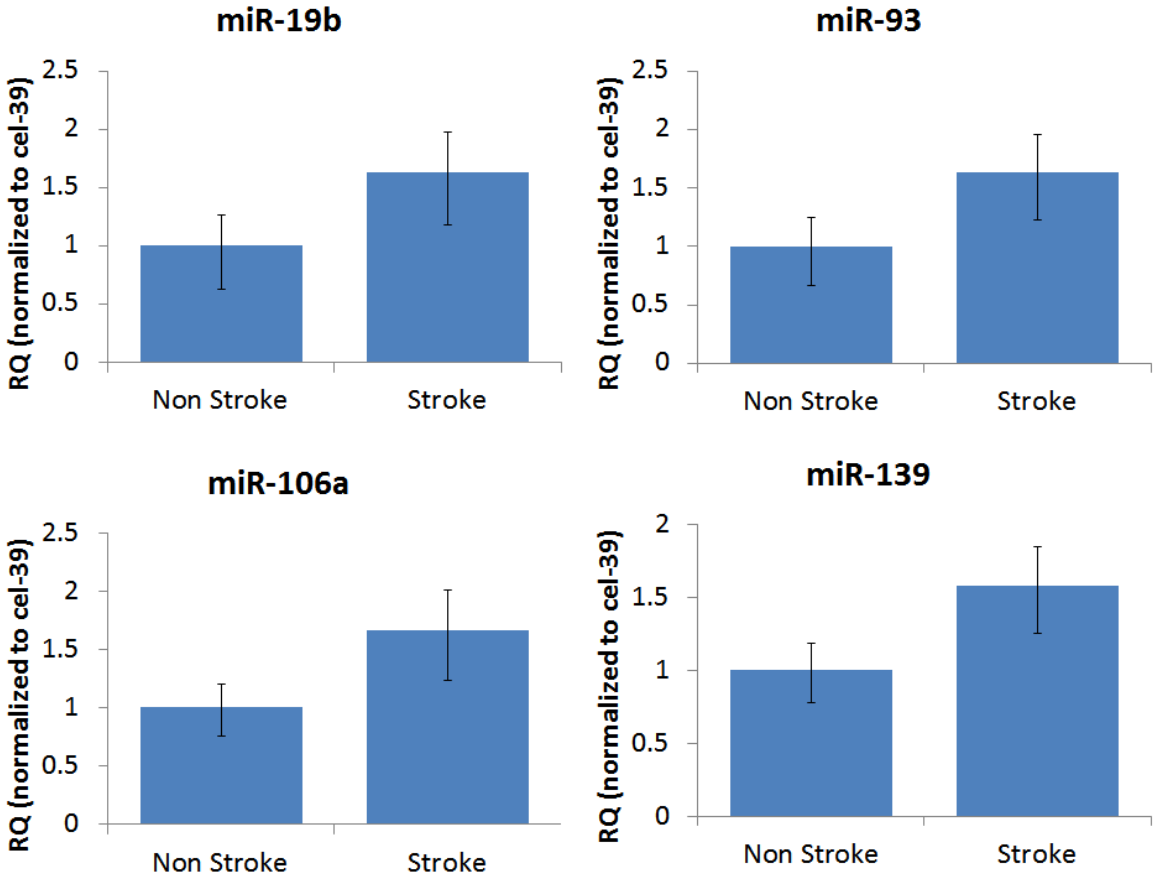

Figure 4.9 – miRNA expression in human serum exosomes - Expression of microRNAs in exosomal RNA measured by qPCR, relative to non stroke normalized to c. elegans miR-39 spike (cel-39). Data are presented RQ±RQmax/RQmin. Non stroke n=12, Stroke n=14 per group. Trends of differential expression for miR-19b, -93, -139 and -106abut the observed increase in expression in the stroke group did not reach statistical significance .

Several of the candidate miRNAs which were identified as being differentially regulated in the clinical population were also assessed in rat serum in order to ascertain whether there were any similarities in expression. Serum obtained from 16-week old SHRSP rats at 72h post tMCAO was compared to matched sham animals. A significant increase was observed following experimental stroke for many of these miRNAs consistent with what was observed in the human population. miR-19b demonstrated a significant ~7-fold increase in serum and a ~5-fold increase in exosomes in samples obtained from stroke animals at 24h versus sham. This increased to ~15-fold in serum at 72h but remained at ~5-fold in exosomes at the corresponding time point. miR-93 demonstrated no significant difference between stroke and sham groups in serum nor exosomes at 24h whilst at 72h there is a non-significant 3-fold increase in the stroke serum ($p=0.076$) versus control and a significant 30-fold increase in exosomes ($p=0.005$) obtained at this time point. No difference was observed in miR-106a serum at either time point, although a significant ~3-fold increase was evident at 24h ($p=0.03$) which increased to ~5-fold by 72h ($p=0.005$). Finally, no significant difference was present at either time point in the exosomal miR-532 levels (at 24h a ~2-fold increase with a p-value of 0.06 is present) whilst in the serum a significant ~4-fold increase is present at 24h ($p=0.05$), rising to ~8-fold by 72h ($p=0.006$) (Figure 4.10).

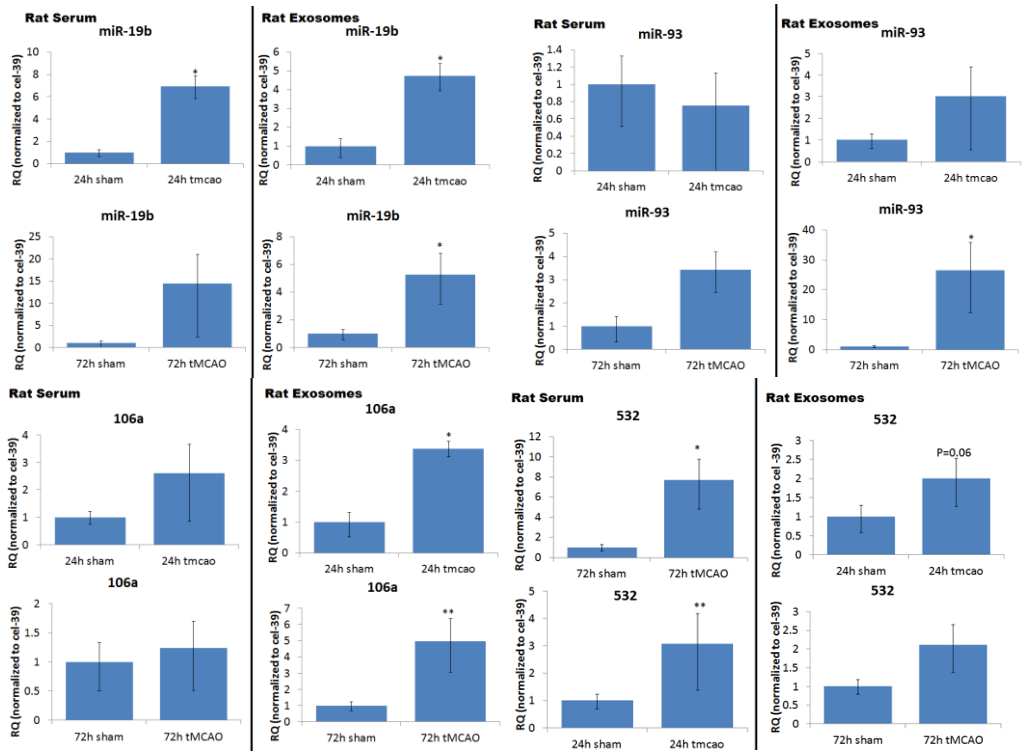

Figure 4.10 -Comparison of expression of miR-19, -93, -106a and -532 in rat serum and exosomes isolated from rat serum. Data are presented as RQ±RQmax/RQmin n=4 per group There was a significant increase in expression for several of the miR-17-92 cluster and associated paralogs, which appears more profound in the isolated exosomes. miR-532was significantly higher in rat serum following stroke, although this significancewas lost when assessing total serum. (*=p<0.05, ** =p<0.005 Students two tailed t-test). n=4

4.3 Discussion

The miRNA OpenArray conducted found 15 miRNAs to be upregulated in a sample population following stroke compared to a stroke mimic group. Initial screening for miRNA biomarkers in our pilot population looked promising but when assessed in a larger population for validation, the candidate miRNAs did not demonstrate significant differences between groups. Post-hoc analysis of candidate miRNA expression levels yielded no significant differences although there was a trend towards differences between TOAST classification subtypes of stroke from non stroke controls. Large artery stroke consistently demonstrated a trend towards increased expression versus non-stroke for miR-19, -21, -93, -331, 374, -590-5p and -885, whilst there was a trend towards higher levels of expression for miR-27a in the cardioembolic stroke patients versus non-stroke controls. No differences were observed in the number of exosomes present in the samples between patients, but assessment of the candidate miRNAs present in validation population exosomes demonstrated that these miRNAs were concentrated in the exosomes in many cases.

A few other groups have started to consider the potential use of circulating miRNA profiles in the context of stroke, and the findings are discussed in context with my own data here;

An early paper on the subject performed a screen on a population of young (18-48) stroke patients in order to identify differences in miRNA expression profile. Serum was collected between 6 and 18 months following stroke. Presence of stroke was confirmed via CT or MRI imaging of the brain. Traditional cardiovascular risk factors were measured, and medical history and demographic information was collected. Patients were also subjected to neurological assessment and subgrouped based on the TOAST classification system. Of the 836 microRNAs evaluated, 157 were differentially regulated across the stroke samples and subtypes. 138 of these microRNAs were highly expressed in stroke, the remaining 19 were downregulated versus healthy controls. Observations in the microarray were validated by qPCR for selected miRNAs. This study has demonstrated in this population that different stroke subtypes can be identified, by characteristic miRNA expression profiles. Principal component analysis (PCA) resulted in the identification of discrete populations of patients in each of the stroke subtypes. Stroke outcome was assessed

using the modified Rankin Scale (mRS). A threshold score of >2 was defined as a patient with good “outcome”, whilst <2 was designated as having “poor outcome”. Again PCA analysis resulted in the classification of discrete populations of good and poor outcome, it is also possible to identify stroke subtypes based on miRNA profiles. This demonstrates the first evidence that microRNAs has the potential to be used as a biomarker for the assessment of stroke in a human population. The biggest caveat in this paper is the small sample size of patients used, and the lack of information regarding statistical methodology (Significance is listed as being determined by one way ANOVA, although no mention of false discovery rate is mentioned). Interestingly, in this study several members of the 17-92 cluster are observed as being differentially expressed (miR-17, -19a, -19b, -20a, -106b). In their next study none of the 17-92 cluster were indicated as differentiating low risk stroke patients from those who presented with traditional risk factors (Tan et al. 2013). It is possible that the lack of significance observed in my study with respect to these candidates may be due to greater than expected underlying variation in the human population used. Emerging preclinical evidence suggests that this family of miRNAs may be a potential therapeutic target in the field of therapeutic neuroregeneration (Li et al. 2012), therefore these candidates warrant further investigation.

The expression levels of miR-21, miR-221 and miR-145 (all previously implicated in cardiovascular disease) were assessed in the serum of a population of 167 stroke patients, and 66 patients with carotid atherosclerosis in order to determine whether these miRNAs could act as biomarkers of stroke. Whilst miR-145 was not present in sufficiently detectable levels, both miR-21 and miR-221 were significantly upregulated in stroke patients compared to atherosclerotic patients. Each of these miRNAs was determined to be an independent predictor of stroke. miR-21is one of the candidate miRNA assessed in this study. Whilst expression was significantly higher in the stroke patients of the original pilot population this was not maintained when assessed in the larger validation population. The Tsai study had a larger group size than my study imparting greater statistical power, and the use of healthy controls in this study can be expected to account for a larger difference between stroke and non-stroke groups (Tsai et al. 2013).

One group interested in serum miRNA biomarkers for stroke assessed expression of miR-30a, miR-126 and let-7b in a population of stroke patients (n=97) versus healthy controls (n=50) (Long et al. 2013). In this study longitudinal samples were collected at 24h, 1 week,

4 weeks, 24 weeks and 48 weeks following onset of symptoms. These miRNAs were selected for study by this group as they had been previously implicated in pre-clinical models of stroke as well as being involved in other vascular diseases (Jeyaseelan et al. 2008b; Tan et al. 2009; Zhao et al. 2013). Both miR-30a and miR-126 were significantly downregulated at 24h versus healthy controls, but by 48 weeks, expression levels had increased to levels seen in healthy controls for all TOAST classification subtypes. Let-7b demonstrates a similar pattern of expression in patients with large artery stroke, but this was different in all other stroke subtypes. For small artery, cardioembolic and undetermined stroke, there was a progressive increase in expression levels which peaked at 1 week before reducing and reaching basal levels by 48 weeks.

I have demonstrated a lack of significant difference between expression levels in the validation population of this study despite robust results in the initial pilot population. Taking the candidates identified in the original population and testing them in a new population is an essential aspect of biomarker development. Tan *et al.* neglected to do this and it is likely that these miRNA biomarkers would not stand up to such rigorous validation (Tan et al. 2009). It is possible that at later time points (7 days, 1 month, 3 months) differences may become apparent.

Another consideration which must be made regarding published studies and my own data is that assessment of miRNA profiles was conducted following stroke. As a result of this it is not possible to determine whether the miRNAs identified have predictive potential in the identification of at risk individuals, pre-ischaemic event. The ideal stroke biomarker experiment would be a prospective longitudinal study where serum samples are taken prior to stroke and then again post-stroke as this would potentially result in the identification of predictive miRNA profiles which could be used to identify at-risk individuals.

Quantification of circulating miRNA levels is still a relatively new technique and no gold standard exists for assessment of circulating levels. There are several different techniques which can be utilized in order to assess miRNA expression levels and ways in which recorded data can be normalized. By far the most commonly used technique for circulating miRNA quantification is quantitative PCR. There are challenges associated with this however. For example, there is no universally accepted reference gene for

normalization. The most effective way to normalized serum miRNA would be to identify miRNA which display minimal variation between samples and to use this as the "housekeeper", although this requires costly experimentation and it may not be possible due to prohibitive expense and limited availability of sample (Jarry et al. 2014).The OpenArray that was conducted in this study contained several genes recommended as being potential reference genes, but none of these was consistently expressed across all samples. An alternative to using an internal control is to employ an external control. RNA from another species is spiked into each sample at a known concentration. The spike can either be added to sample prior to RNA extraction, post RNA extraction or spikes can be added both before and after extraction. There are advantages to each approach. However, due to the low yield from serum RNA extractions and variability in concentration of RNA between samples it was decided that post-extraction spike was the most appropriate approach in this study

Circulating miRNA is potentially more useful than large RNA molecules as a biomarker due to increased robustness. RNA is notoriously sensitive to degradation, which makes it difficult to work with. However, miRNAs remain detectable following several freeze thaw cycles. Several hypotheses existed attempting to explain the reason for miRNA durability, the most popular initially being the association of RNA with DNA protecting the molecules from degradation by RNAse and DNAse. However, in actuality miRNAs are protected by forming complexes with lipid molecules (El-Hefnawy et al. 2004).

Assessment of candidate miRNAs in rat serum demonstrated a significant upregulation was evident for several miRNAs at 24h (miR-19b, miR-532) and 72h (miR-532) following tMCAO whilst significant upregulation of exosomal miRNA expression was observed for several miRNAs at 24h (miR-19b, -106a) and 72h (miR-19b, -93, -106a).miR-19b is in the 18-family whilst miR-93 and miR-106a are in the 17 family (Olive et al. 2010). These miRNAs have been previously implicated in regulation of angiogenesis and expansion of hematopoietic stem cell populations (Li et al. 2012) and it is possible that this is being mediated by these miRNA containing exosomes. Comparisons of miRNA expression levels between serum and exosome samples at a given time point suggests that miR-19b, miR-93 and miR-106a are localized to the exosomes. This is of particular interest as these miRNAs are all related and their presence in the exosomes hints at functional importance as these miRNAs will be effectively transfected into other cells where the miRNAs may

exert post-transcriptional inhibition on target genes. miR-17-92 cluster members have been identified as being upregulated in several forms of cancer and act to promote cell proliferation and inhibit apoptosis (Mendell 2008). In cancer, this is negative and contributes to pathogenesis (O'Donnell et al. 2005) whilst in the context of injury this is likely to contribute to mitigation of deleterious effects of the environmental insult whilst promoting regeneration (Chen et al. 2013). Comparison of serum and exosomal expression levels for miR-532 suggests that this miRNA is predominantly expressed in the serum and is of relatively low abundance in the exosomes. It is difficult to hypothesize the importance of this as all of the current literature investigating miR-532 are associations, (Hall et al. 2014; Qin et al. 2014; Dmitriev et al. 2013; Kanaan et al. 2013; Sasaki et al. 2013; Lu et al. 2005) and to date no publications exist describing functional effects of modulation. One of these studies described miR-532 as being enriched in axonal preparations and fluorescent in situ hybridization identified the localization of this miRNA in granules in distal axons and growth cones indicating that this miRNA may play an essential role in the development and/or function of specific types of neuronal tissue (Sasaki et al. 2013).. This means that this miRNA may be of potential significance in the context of stroke, especially when considered in combination with the rat serum experiments performed in this study

Following the development of accurate miRNA expression profiles relevant to stroke there are many challenges that will face clinicians trying to implement the technology. Perhaps the most notable challenge would be with respect to the use of diagnostic miRNA profiles in the acute phase following stroke. Currently, there is a major time constraint on application of stroke therapy and increased time taken for diagnosis will increase time until treatment meaning that the more diagnostics that are conducted will potentially reduce the number of patients who benefit from therapeutic intervention. Recombinant tPA therapy which is used for thrombolysis of stroke causing clots and recanalization of the patient must be administered within 4.5h of stroke symptom onset (Wahlgren et al. 2008), the time window for thrombectomy is of a similar duration (Castaño et al. 2010). These are the only therapies which have been shown to have a significant improvement on clinical outcomes in acute ischaemic stroke treatment. Currently the technology used for quantification of miRNAs is PCR based. In practice this

means that RNA has to be extracted from serum samples following collection from the patient (this takes approximately 1-2 hours) followed by a qPCR reaction (this takes approximately 1 hour to set up and 2 hours to run, followed by time for analysis which is also a time consuming procedure). The logistics of miRNA analysis means that results cannot be obtained from a patient until several hours from presentation at clinic; this means that miRNA expression profiles are unlikely to be implemented in clinical practice for diagnosis during the acute phase of stroke. Currently, several groups are working on developing non-PCR based methods for analysis of circulating miRNAs which may be more useful in the clinic. The ideal platform for point of care analysis of miRNA expression levels would be a device similar to the blood glucose detectors which are widely implemented at the moment. Luis Vaca's group is developing one such device which used isothermal PCR in order to detect miRNA expression. Isothermal PCR uses an enzyme which has DNA displacement properties, removing the need for temperature cycling and thus reducing energy requirements of the device increasing portability. It is also theoretically possible to use microarrays to measure miRNA expression levels. This technology utilizes hybridization of RNA sequences to fluorescently labeled RNA sequences immobilized on a surface; this technique is an effective though expensive way to analyze large numbers of genes in a single reaction. However, it is not possible to detect miRNA expression levels in serum samples without prior amplification(Vaca 2014).

Another consideration to make regarding the usefulness of serum miRNA as biomarkers is the effect of haemolysis on miRNA content. It was noted during extraction of RNA from the serum samples that varying degrees of haemolysis were present in some of the samples (indicated by pink hue of solution as opposed to expected yellow). As the serum was isolated by centrifugation there was the potential for rupture of erythrocytes, releasing intracellular miRNAs into the extracellular space. Several miRNAs are known to be enriched in erythrocytes including miR-21 and miR-106a which were identified in this study (Kirschner et al. 2011; Kirschner et al. 2013). Differing degrees of haemolysis between samples will result in increased intra group variability and may partially account for the lack of significance in the stroke study population compared to the non-stroke group.

Perhaps the greatest setback faced in this study was the inability to obtain the number of patient samples required according to power calculation. Utilization of the stroke mimic

group as opposed to a healthy control was a strength of the study as it ensured that the phenotype of the patient presenting at clinic in our control group was closer to that of a stroke patient. However, it also meant that the number of the stroke mimics who presented at the clinic became a limiting factor in terms of the numbers in the study.

Although there are several technical challenges to be overcome before circulating miRNAs become utilized in clinical practice, the benefits from them will be great. Specifically these profiles may allow prediction of clinical outcomes following stroke, or identifying individuals who may benefit from particular treatments. Use of exosomes as a vector for miRNA based gene therapy also may be of great benefit in the future.

Chapter 5 – the role of miR-21 in Stroke

5.1 Introduction

5.1.1 miR-21 Background

miRNA-21 is one of the first discovered microRNAs and as a result, one of the most widely studied miRNAs. It has been implicated with importance in a variety of different pathological states including cancer and cardiovascular disease (Krichevsky and Gabriely 2009). MiR-21 is a highly conserved miRNA reflecting its fundamental importance in mammalian biology, it is located in the intronic region of the TMEM49 gene located on the plus strand of chromosome 17q23.2 where it displays some overlap with the coding region of the gene VMP01 (Fujita et al. 2008). Despite this overlap the pri-miR-21 transcript is transcribed independently from an intronic promoter region. Emerging evidence suggests a protective role for miR-21 in neuronal cultures in *in vitro* models of stroke (Ziu et al. 2011; Buller et al. 2010), whilst primary cerebral endothelial cells benefit from miR-21 overexpression *in vitro* by promotion of angiogenesis (Guduric-Fuchs et al. 2012).

miR-21 is abundantly expressed in several tissues of different organ systems including the heart, spleen, small intestine and colon (Lagos-Quintana et al. 2002). Initial research into the role of miR-21 in disease states focussed on cancer biology, these studies demonstrating that miR-21 has pro-oncogenic effects leading to it being defined as an 'oncomir'. The potential oncogenic role of miR-21 was identified following observation of elevated levels in high grade glioma samples which was demonstrated to inhibit apoptosis in vitro (Chan et al. 2005). It has been suggested that there are complex mechanisms underlying the pathological effects of miR-21 upregulation whereby the net effect is to inhibit apoptosis and promote proliferation via the inhibition of multiple gene targets (Krichevsky and Gabriely 2009).

5.1.2 miR-21 and Cardiovascular Disease

In addition to the considerable volume of cancer research pertaining to miR-21 there has recently been increasing interest in the role that it may play in cardiovascular disease. In theory, inhibition of apoptosis and promotion of proliferation could be desirable for the administration of regenerative medicine to the cardiovascular system assuming that the right conditions can be created in order to promote the restoration of damaged or

diseased tissue to a stable healthy state (Han et al. 2011). Several miRNAs have been identified as being upregulated during cardiac hypertrophy, with miR-21 consistently implicated across research produced by several groups (Sayed et al. 2007; van Rooij et al. 2006; Cheng et al. 2007; Tatsuguchi et al. 2007).The role that miR-21 plays in this condition remains elusive as miR-21 transgenic animals display no apparent differences in cardiac morphology, and both display similar responses when subjected to pressure overload hypertrophy (Sayed et al. 2010; Thum et al. 2008). Though the upregulation of miR-21 is global during cardiac hypertrophy, the increase is most profound in myofibroblasts. This is at least partially mediated through targeting and suppression of sprouty1, enhancing erk1/2 phosphorylation and myofibroblast survival (Thum et al. 2008). It is likely that changes in these genes contribute to fibrosis generally observed during cardiac hypertrophy. However, studies which have attempted to modulate miR-21 activity via the use of genomic ablation of miR-21 in transgenic mice or knockdown by LNA miRNA inhibitors had no impact on cardiac hypertrophy resulting from pressure overload, Angiotensin II, Calcineurin or infarction(Patrick et al. 2010).

5.1.3 Evidence implicating the importance of miR-21 in Stroke

Initial evidence supporting the importance of miRNA activity in relation to stroke was obtained from experimental stroke models. Whilst several miRNAs were observed to be differentially regulated in response to tMCAO *in vivo*, miR-21 was not reported as being altered (Jeyaseelan et al. 2008a; Dharap and Bowen 2009). Although initial studies did not implicate miR-21 in stroke, induction of stroke in Wistar Kyoto rats by embolic model resulted in a marked upregulation of miR-21 as measured by *in situ* hybridization and qPCR (Buller et al. 2010). Primary neuronal cultures were derived from these animals and in vitro analysis demonstrated that the miR-21 upregulation was neuroprotective and that this was at least partially mediated by inhibition of an apoptosis promoting tumour necrosis-α family member, FASLG (Buller et al. 2010). Other evidence supporting the importance of miR-21 in neurological injury, as well as suggestion of its therapeutic potential was demonstrated in a model of spinal cord injury in mice. Following induction of injury, an upregulation of miR-21 in the central nervous system was observed displaying a similar pattern to what was observed in response to embolic stroke. Modulation of miR-21 expression in astrocytes via the use of transgenic animals demonstrated that miR-21 expression was protective following spinal cord injury through

its interaction with fibrotic targets in the cell signalling pathway promoting optimal resolution of glial scar (Bhalala et al. 2012).

Additional support for the potential importance of miR-21 in relation to stroke comes from data present in the clinical literature. miR-21 was one of several miRNAs to be upgregulated in the serum of young stroke patients versus healthy controls and was identified via pathway analysis to play an important role in vascular remodelling as well as having been previously demonstrated to show aberrant expression in cardiac diseases (K. S. Tan et al. 2009). This observation has been replicated by other researchers in other populations of stroke patients supporting the validity of its use as a potential biomarker as well as providing rationale for studying its functional role in the pathophysiology of stroke (Tsai et al. 2013).

5.1.4 Hypothesis

miR-21 plays a functional role in stroke pathology and modulation of this miRNA may be of therapeutic benefit in models of stroke.

5.1.5 Aims

Characterization of miR-21 post-stroke in SHRSP rats by qPCR and in situ hybridization of this miRNA and qPCR analysis of target genes.

Establishment of mouse *in vivo* tMCAO model of stroke and assessment of infarct size by MRI and behavioural testing.

Baseline characterization of miR-21 transgenics and miR-21 knockouts and subsequent assessment of differences between these animals and corresponding controls following stroke.

5.2 Results

5.2.1 Quantification and Characterization of miR-21 expression following stroke

Male SHRSP rats, aged 16-weeks and weighing 290-300g were subjected to 45 minute transient middle cerebral artery occlusion and allowed to recover. MRI was used to identify region of infarct, peri-infarct and remainder prior to RNA being extracted from each region (Figure 3.5).

Earlier, miR-29b and miR-29c were assessed in this tissue and miR-29b was determined to be significantly upregulated in peri-infarct tissue at 72h post stroke (Figure 3.6).

Mir-21 expression levels were quantified by qPCR at 24h and 72h following stroke in the peri-infarct region. No significant difference in expression was observed at 24h in the ipsilateral or contralateral regions of the brain compared to time-matched sham control animals. There was a significant increase in miR-21 expression levels globally at 72h following stroke ($p<0.05$, Student's two-tailed t-test)(Figure 5.1).

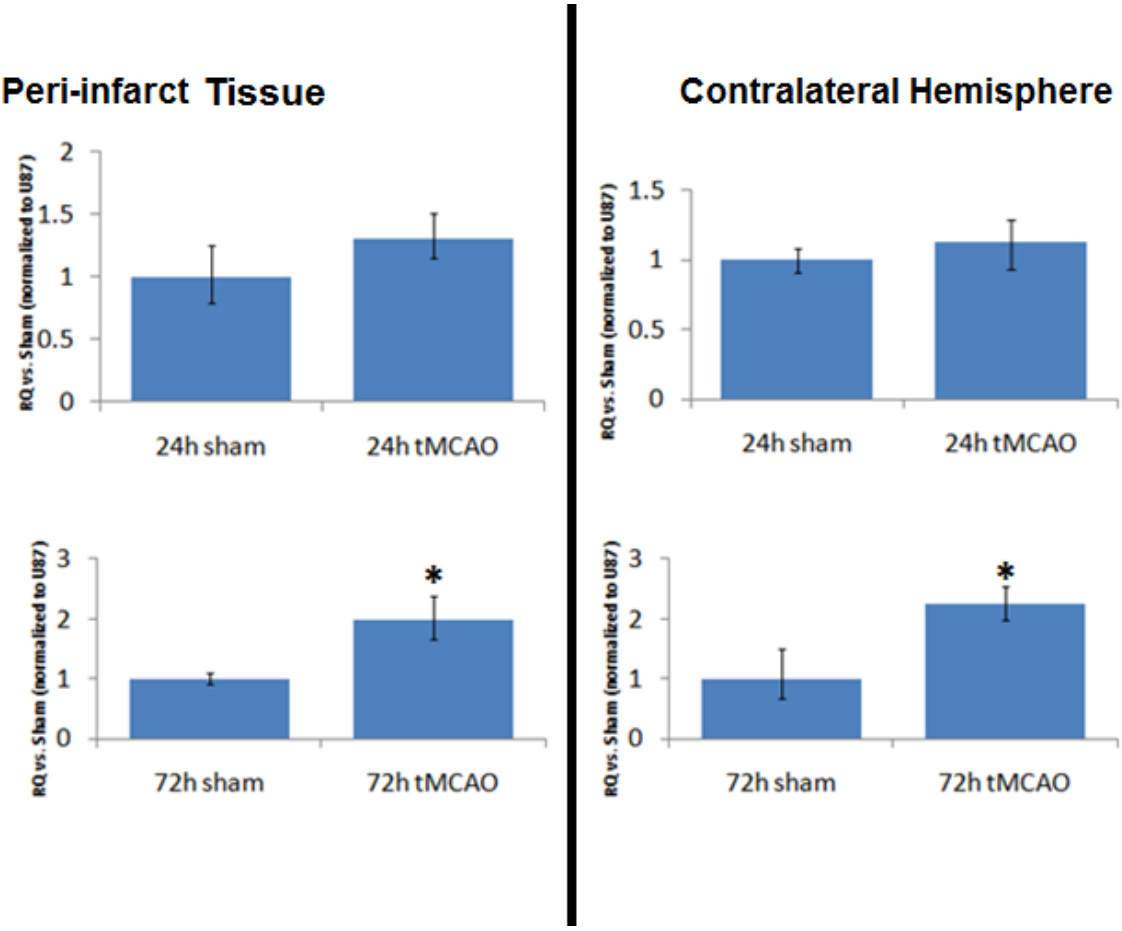

Figure 5.1 – MiR-21 Expression is increased in peri-infarct tissue - qPCR of lysates for miR-21 expression in the peri-infarct region and corresponding contralateral region. Data presented as RQ compared to time-matched sham. Expression normalized to U87 (Data are presented as mean RQ ±RQmax/RQmin, n=6 ,*=p<0.05, student's two-tailed t-test).

5.2.2 Mouse tMCAO Pilot Study

In order to determine the variability and infarct size of the mouse tMCAO model of stroke, a pilot study was conducted. Male ICR mice were subjected to stroke (n=8) or sham surgery (n=3). Stroked animals were assessed by T_2-weighted MRI scan at 72h post-stroke (Figure 5.2). A significant behavioural deficit was measured by Garcia neuroscore and Ladder test at all time points longitudinally following stroke. No deficit was observed in the sham controls (Figure 5.3).

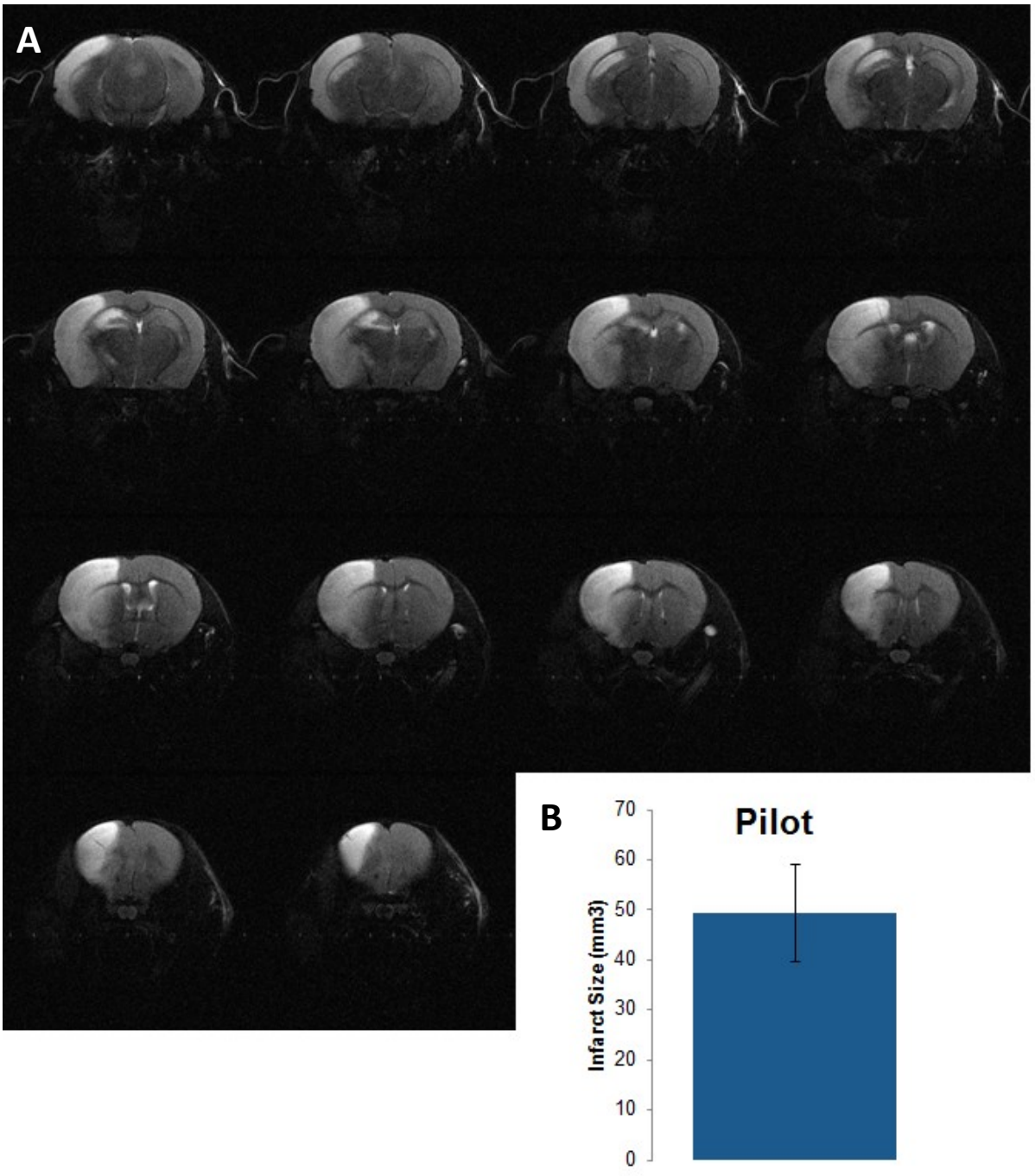

Figure 5.2 – Infarct Volume in Pilot Study - (A) Representative stack images of T_2-weighted scan of stroked mouse at 72h post-stroke with infarct identified as region of hyperintensity.**(B)** Volume of infarct determined using ImageJ (n=8). Data are presented as mean ±SEM

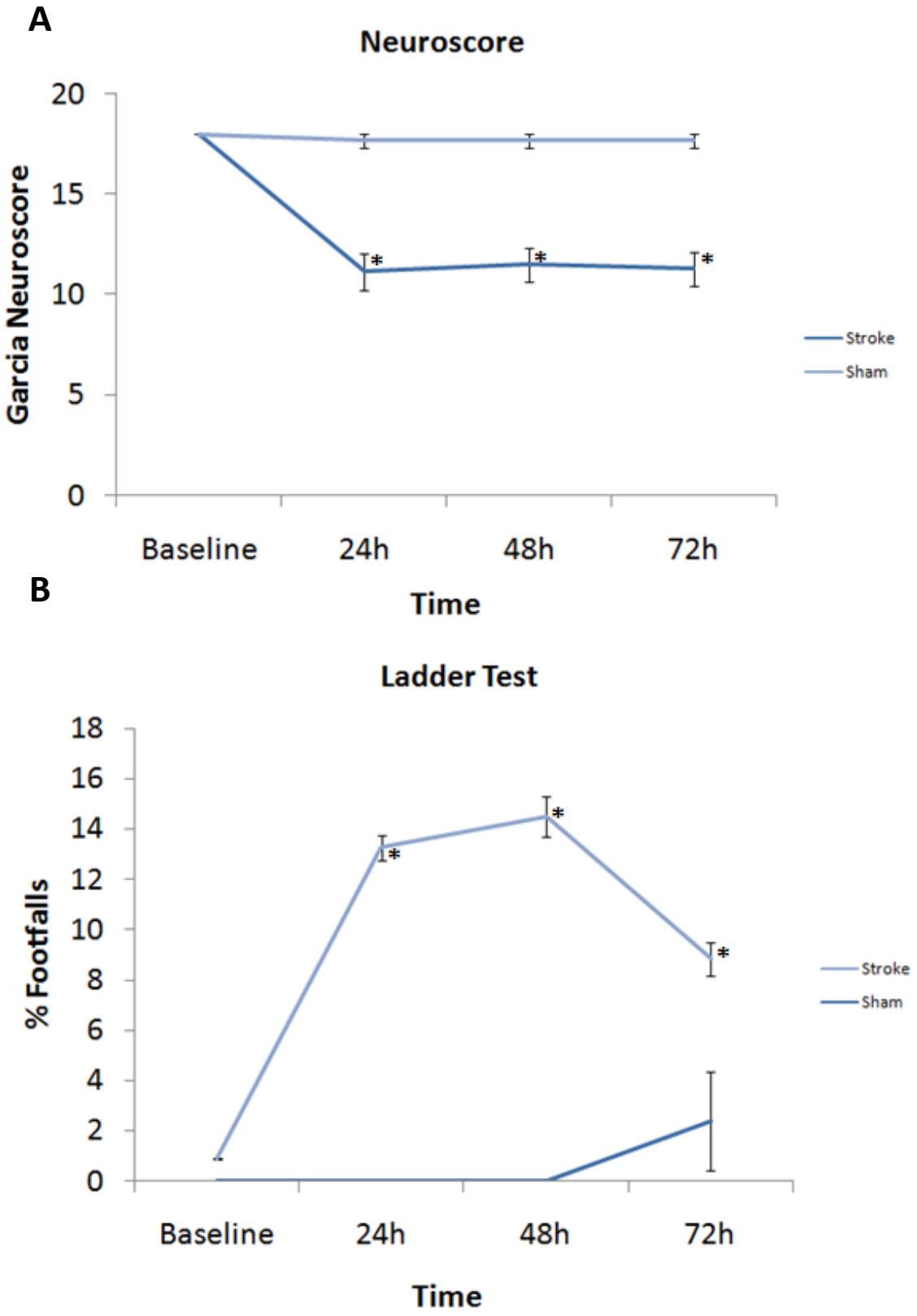

Figure 5.3 – Induction of stroke results in a measurable cognitive deficit - Behavioural measures assessed longitudinally demonstrate a significant deficit following stroke in (A) Garcia Neuroscore and (B) Footfalls during Ladder test (Data are displayed at mean ±SEM, Stroke n=8, Sham n=3, $p<0.05$ repeated measures ANOVA was used with Bonferroni's post hoc test).

5.2.3 Memphis CAG-MiR-21 Mouse Study

miR-21 expression was measured in brains from the Memphis CAG-miR-21 mouse colony to determine basal expression levels. A significant ~6-fold increase was present in whole brain lysates of heterozygous mice versus wild type ($p<0.05$, t-test)(Figure 5.4). No diference was observed in blood pressure at baseline between wild type and transgenic mice as determined by plethysmography (Figure 5.5). There are considerable limitations associated with the use of tail cuff plethysmography, specifically the blood pressures observed in this study are higher than would be expected under normal physiological conditions. This is due to the mice becoming stressed whilst they are restrained. Attempts were made to habituate mice to the tail cuff procedure, but no reduction in blood pressure was observed. Further assessment of blood pressure would have to be performed by more sophisticated measures such as radiotelemetry to determine the blood pressure accurately. This study was looking to see whether a difference existed between groups to account for any changes in infarct volume after stroke. Radiotelemetry would also allow for post-stroke measurement of blood pressure which would arguably be the more important time to assess this. In order to ascertain whether any changes in the gross anatomy of the cerebrovasculature were present animals were perfused with gelatin/indian ink solution in order to visualize the communicating branches of the circle of Willis. No difference was observed between wild type and heterozygous mice indicating that there are no major differences in the cerebrovasculature as both groups displayed a complete circle of Willis (n=4) (Figure 5.6, 3.11). Mouse body mass was significantly lower in wild type mice versus transgenics at all time points during procedure (Figure 5.7), there was no difference in percentage weight lost between groups indicating that the difference in weight is indicative only of mouse mass at commencement of procedure and not related to differences in animal welfare.

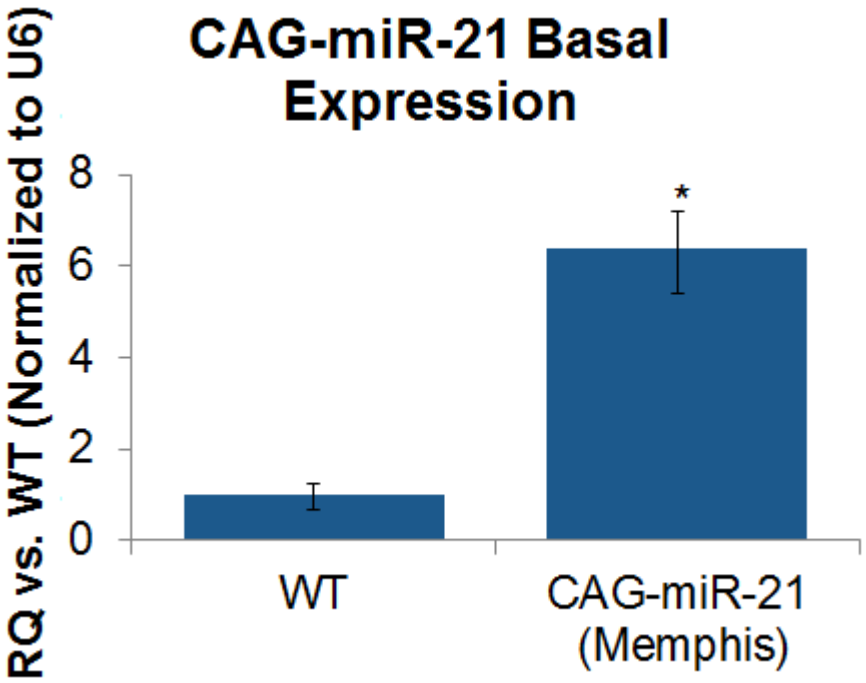

Figure 5.4 – Basal miR-21 levels in transgenic mice - qPCR was performed on mouse whole brain lysates to identify the difference of miR-21 expression between wild type and heterozygous Memphis CAG-miR-21 mice. Expression is presented as RQ relative to wild type and was normalized to U6. (data are presented as mean RQ ±RQmax/RQmin, n=3, $p<0.05$, Student's two-tailed t-test).

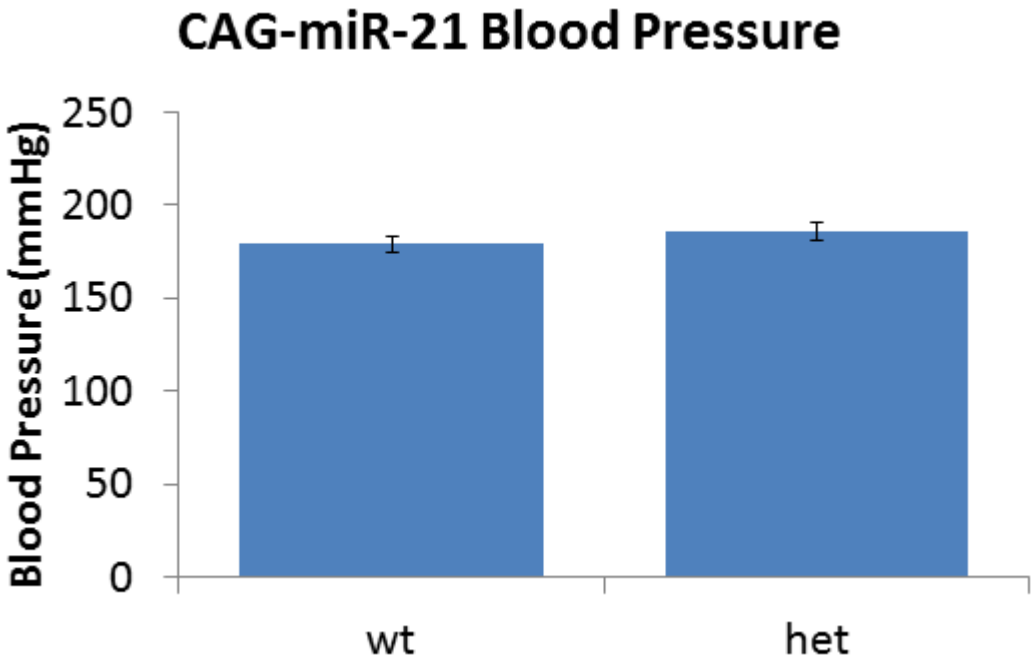

Figure 5.5- Basal Blood Pressure –Blood pressure was observed in wild-type and heterozygous mice in the CAG-miR-21 colony was measured by tail cuff plethysmography. Data are presented as mean systolic blood pressure ±sem.

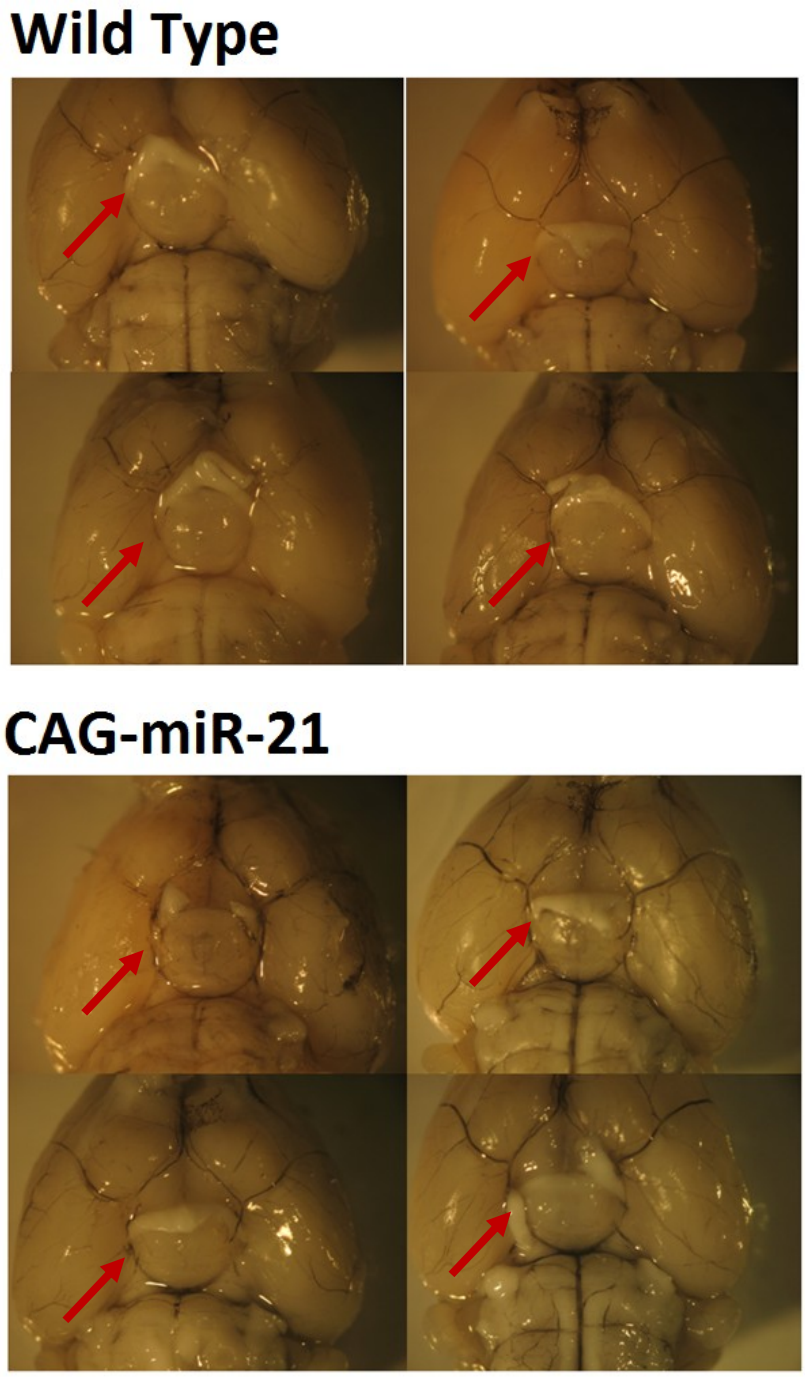

Figure 5.6 - miR-21 Memphis mice display normal circle of Willis anatomy - Indian ink/Gelatine perfusion of mouse brains used to identify branches of the circle of Willis comparison of MiR 21 Memphis wild type and heterozygous animals. Circle of Willis indicated by red arrow.

Stroke was induced using the same conditions and primary outcome measurements as the pilot study in heterozygous and wild type mice. Infarct size was quantified by T_2-weighted MRI scan at 72h following stroke. No difference was observed in infarct volume between groups (Figure 5.8). No difference was observed in neuroscore or foot faults on the ladder test between groups (Figure 5.9). A trend towards improved survival was present in the heterozygous mice versus Memphis mice, but this was non-significant (Figure 5.10).

Following stroke miR-21 expression levels were measured to ensure that the overexpression of miR-21 observed at basal levels (Figure 5.11) persisted following tMCAO. A significant ~6-fold upregulation versus wild type animals was observed. miR-21 raw Ct values were plotted against infarct size for each animal in order to determine whether there was any relationship between infarct size and miR-21 expression levels. No correlation was observed between infarct size and miR-21 expression levels. Not all animals assessed by MRI/neuroscore are presented in this figure as some animals were fixed for histological analysis and therefore tissue was not available for qPCR (Figure 5.11).

Two wild type mice and three transgenic mice were lost on the table, either due to susceptibility to anaesthetic, or because damage in vascular integrity as a result of surgery that could lead to bleeds post-recovery necessitated sacrifice instead of recovery. Post-surgery 3 wild type animals died due to large infarct at ~24h post recovery.

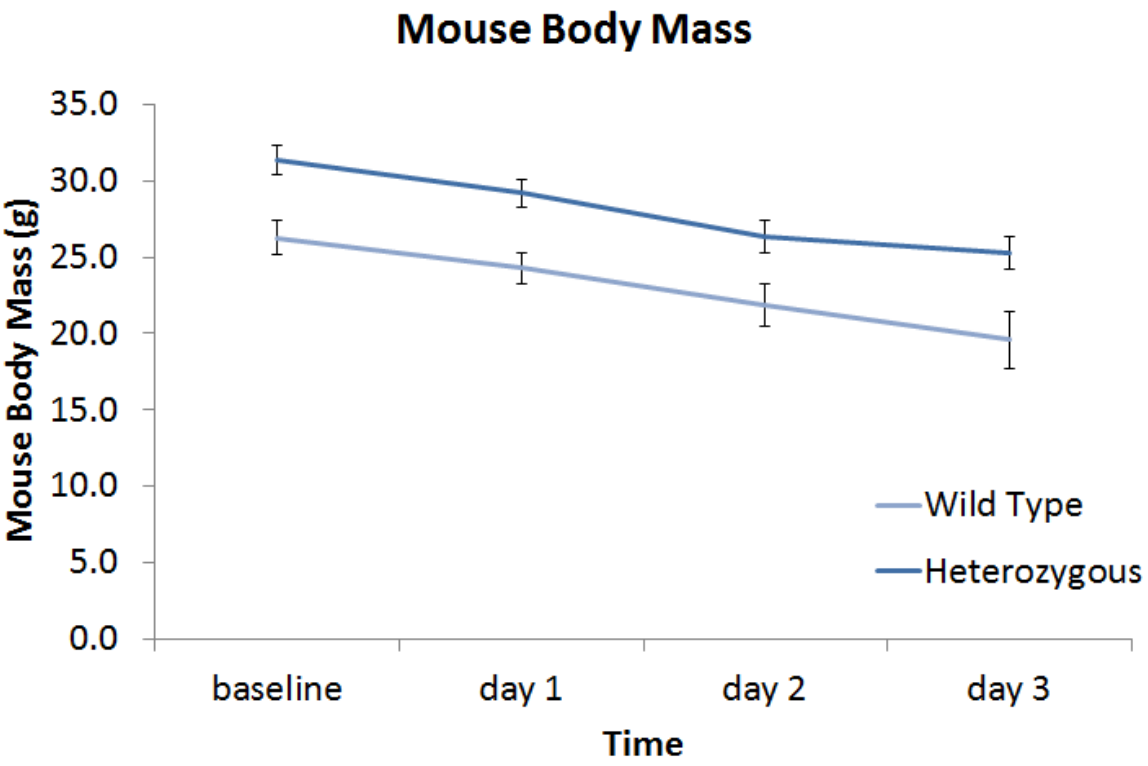

Figure 5.7– Mouse Body Mass During Procedure – Body mass was measured daily whilst animals were on procedure. ($p<0.05$, two way ANOVA with Bonferroni post hoc test, wild type n=6, transgenic n=8.

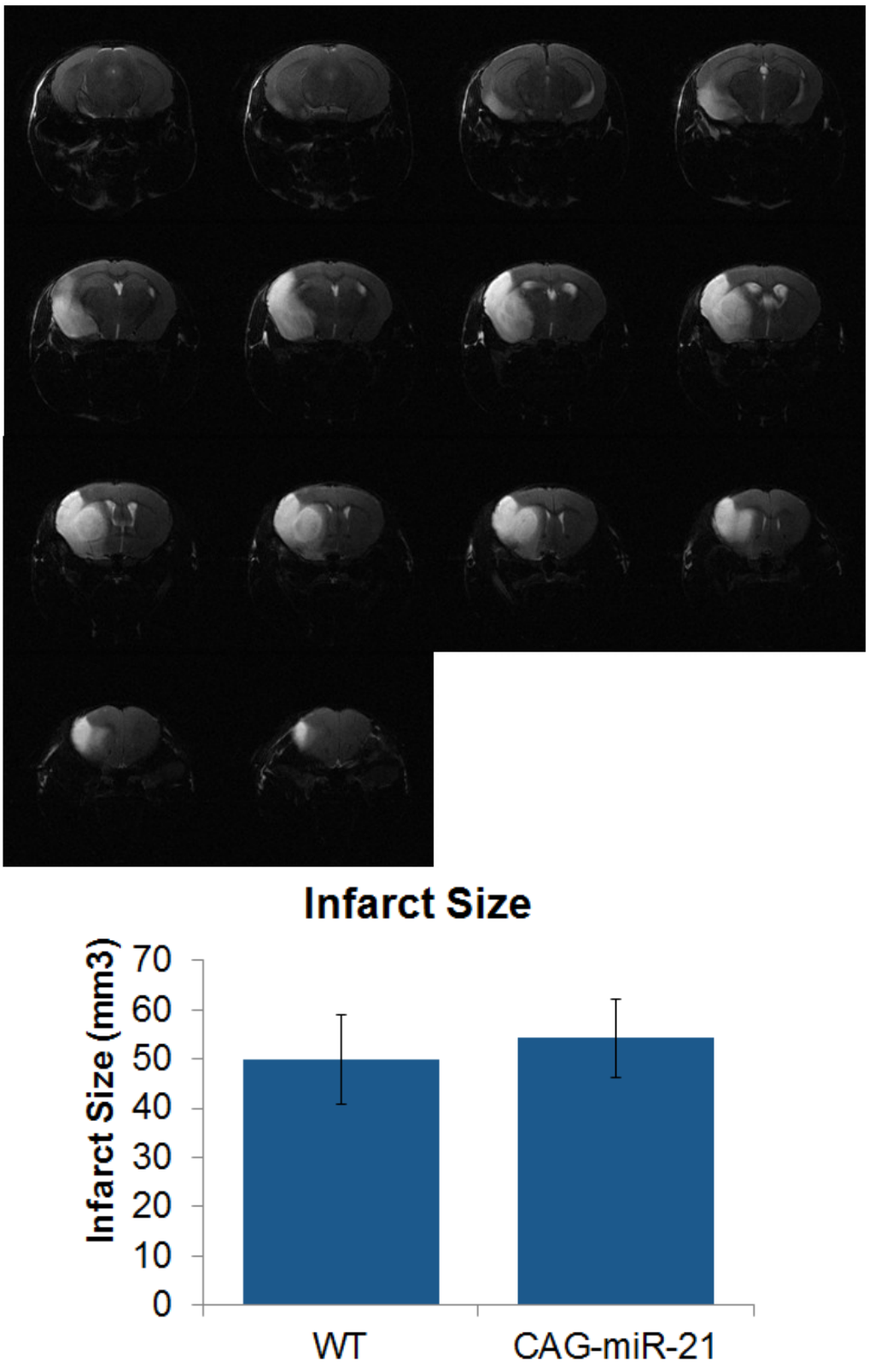

Figure 5.8 – Comparison of infarct size between wild type and miR-21 Memphis mice - (A) Representative stack of infarct from miR-21 Memphis mouse determined by T_2-weighted image at 72h post-stroke **(B)** Infarct size measured from MRI comparison of wild type (n=6) to transgenic (n=8). Data are displayed as mean infarct size corrected for oedema ±SEM)

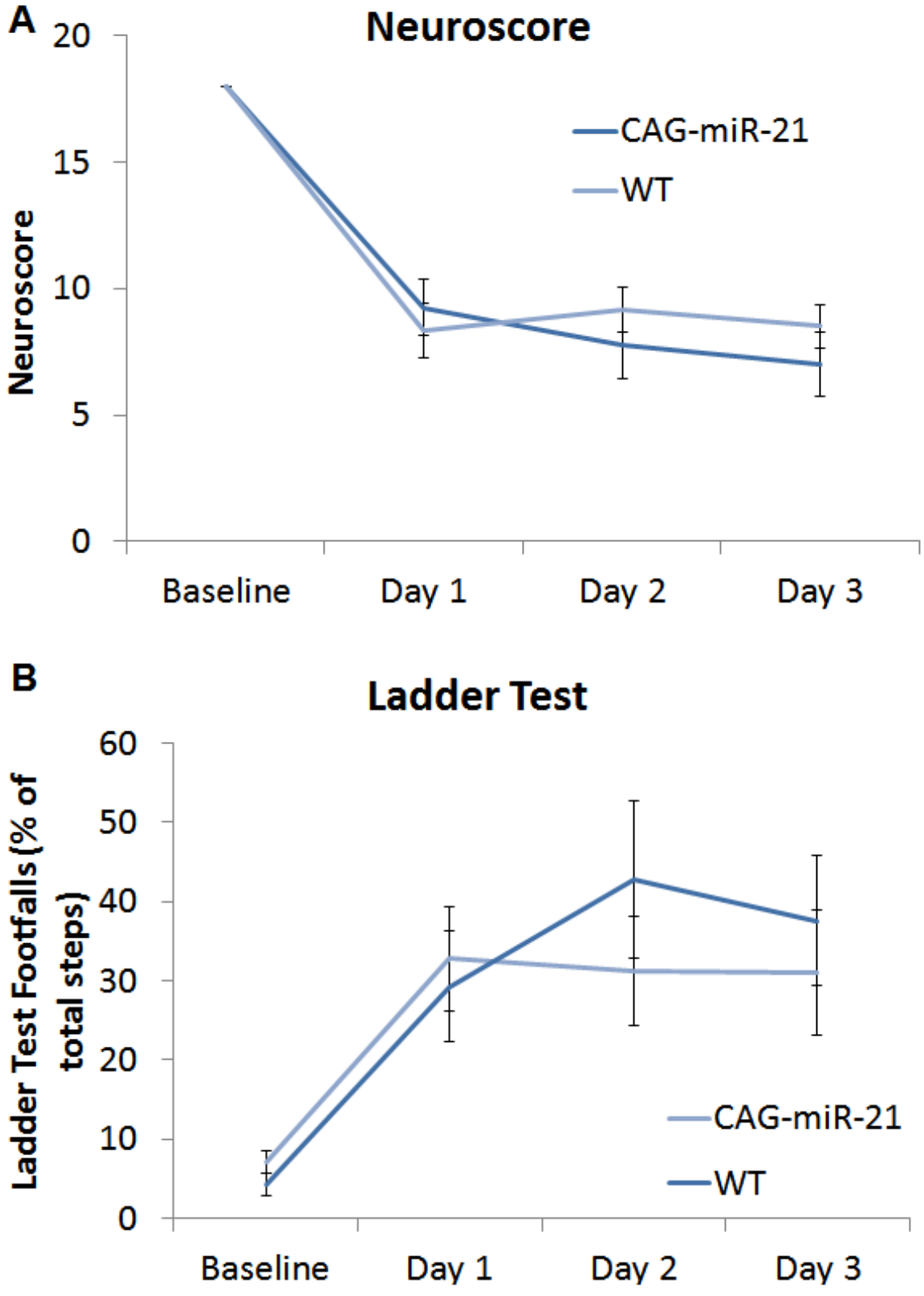

Figure 5.9 - Comparison of behavioural deficit between miR-21 memphis and wild type mice - Behavioural measures assessed longitudinally between CAG-miR-21 and wild type mice by (A) Garcia neuroscore following stroke and (B) Ladder Test. Data are displayed as mean score ±SEM, WT n=6. CAG-miR-21 n=8.

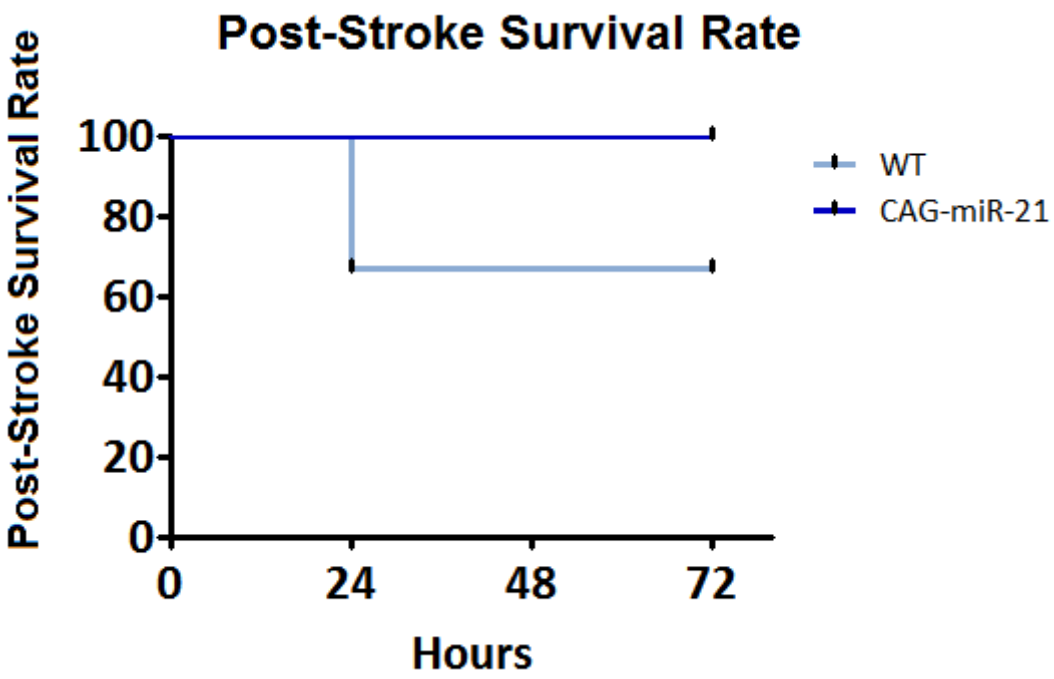

Figure 5.10 –Mortality rate of miR-21 Memphis and wild type mice – Mortality rate illustrated by Kaplan-Meier Survival following experimental stroke. WT n=9, CAG-miR-21 n=8

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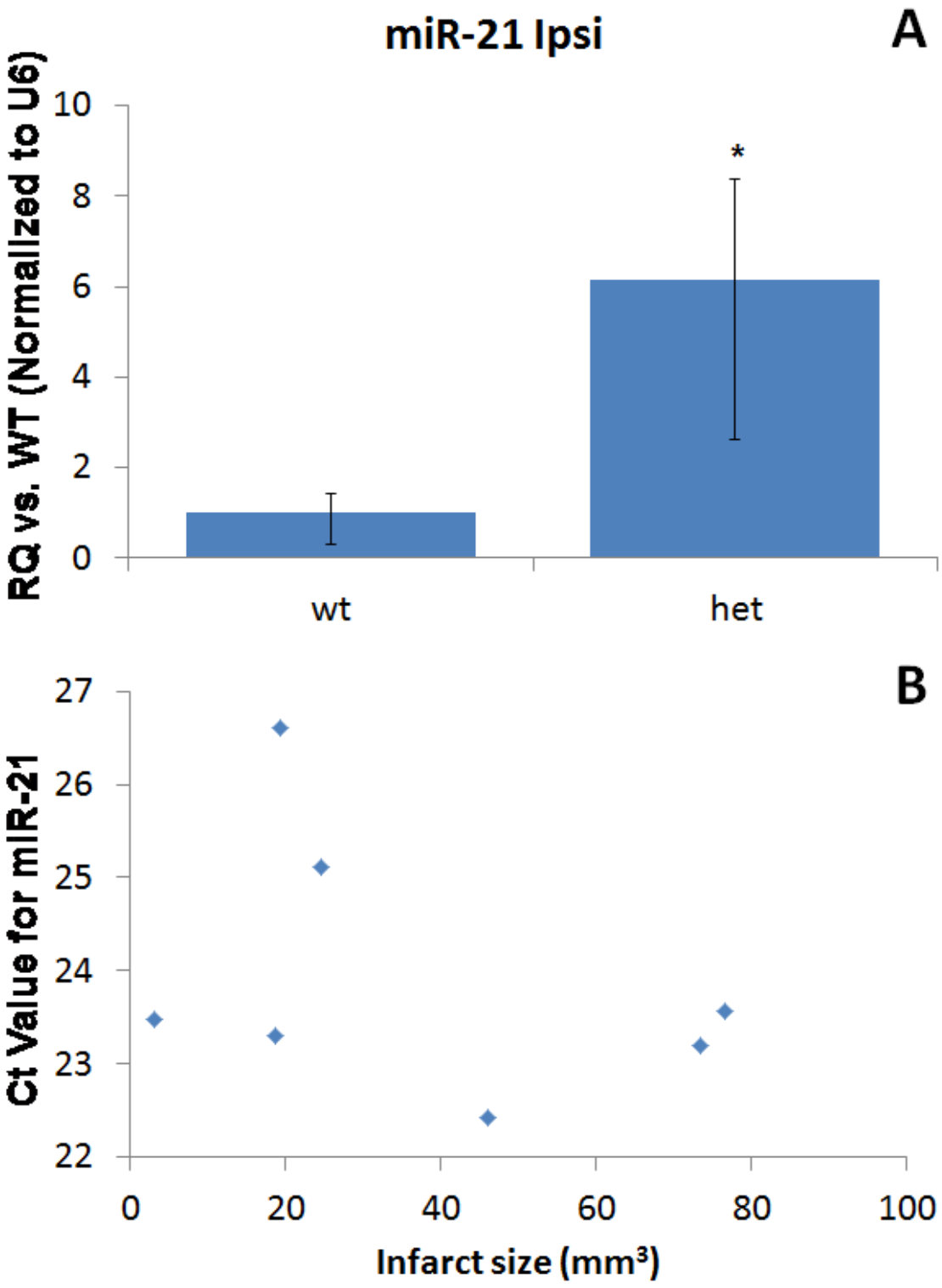

Figure 5.11– Assessment of miR-21 expression with relation to infarct size -(A) miR-21 expression in ipsilateral hemispheric brain lysates at 72h following stroke. Expression was normalized to U6. (data are presented as mean RQ ±RQmax/RQmin, $p<0.05$, Student's two-tailed t-test). (B) Raw Ct values for miR-21 correlated against infarct volume. WT n=3, het n=4

5.2.4 MiR-21 KO Study

In order to determine the effects of miR-21 knockout on infarct size of the mouse tMCAO model of stroke. Male miR-21KO or wild type controls mice were subjected to stroke. Stroked animals were assessed by T_2-weighted MRI scan at 72h post-stroke. This study had to be terminated early due to an unacceptably high mortality rate which occurred within 24h. No evidence of haemorrhage was observed.

Kaplan-Meier Survival analysis conducted after the study was terminated demonstrated there was a significant increase in mortality of miR-21 homozygous KO versus wild type (Figure 5.12).

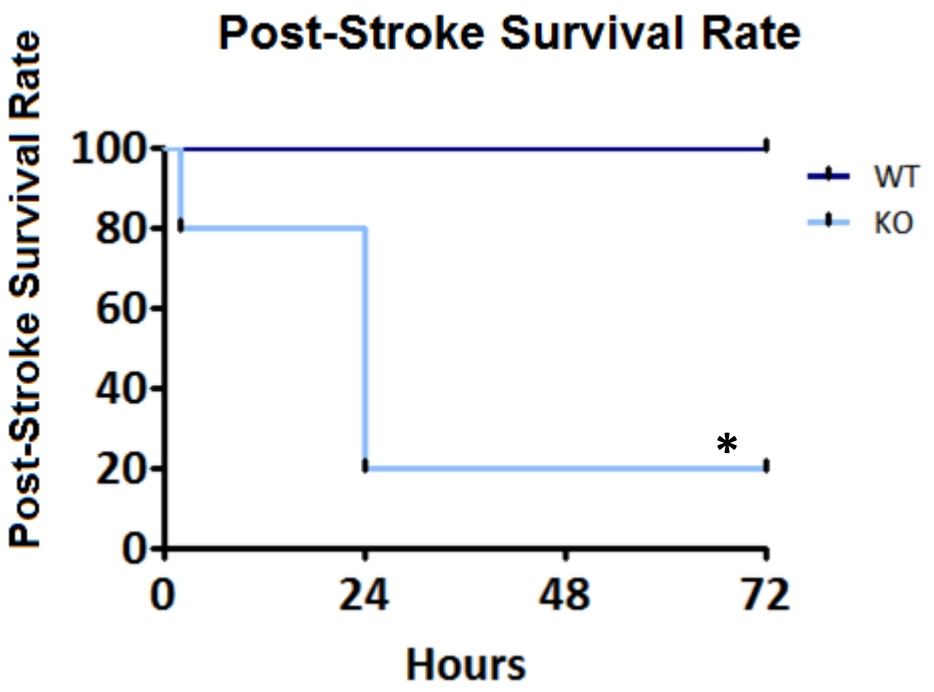

Figure 5.12 – Mortality rate of miR-21 KO and wild type mice - A significant increase in post-stroke mortality was observed in themiR-21 KO group versus wild type (WT n=4, KO n=5 $p<0.05$ when assessed by Log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test and fisher's exact test).

Following cessation of the 72h miR-21 KO mouse study a subsequent study was conducted taking the endpoint as 4 hours. The increased mortality rate suggested that there was physiological effects of miR-21 KO in stroke. Since all mortality occurred within 24h of procedure it was determined that it may be beneficial to look for differences in ischaemic damage acutely at 4h following stroke.

DWI scans were taken to measure ischaemic damage as T_2 scans are unable to detect damage at this time point. Technical problems with the scan meant that there was not enough contrast between the ischaemic damage region and surrounding tissue to accurately estimate size of ischaemic region (Figure 5.13).

In order to assess any differences in the blood-brain barrier integrity following ischaemic stroke gadolinium contrast agent was administered via a tail vein cannula and mouse was scanned to determine whether gadolinium entered the cerebral parenchyma. Technical difficulties successfully administering contrast agent meant that sufficient data was not obtained to conduct a quantitative analysis of this (Figure 5.14, Figure 5.15).

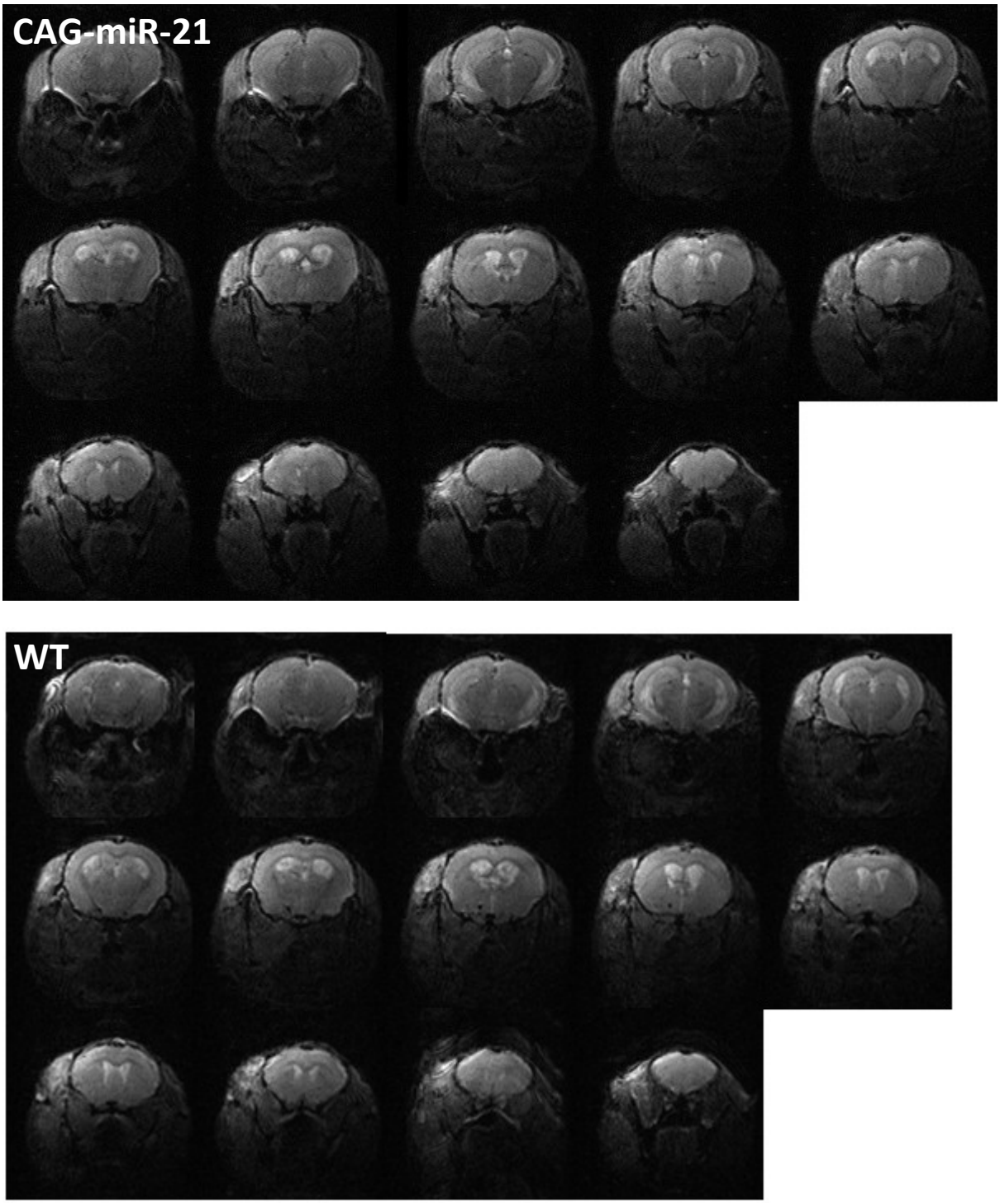

Figure 5.13 – Infarct at 4h post-stroke as measured by DWI MRI scan - Representative images from one animal demonstrating the data obtained by DWI scan.

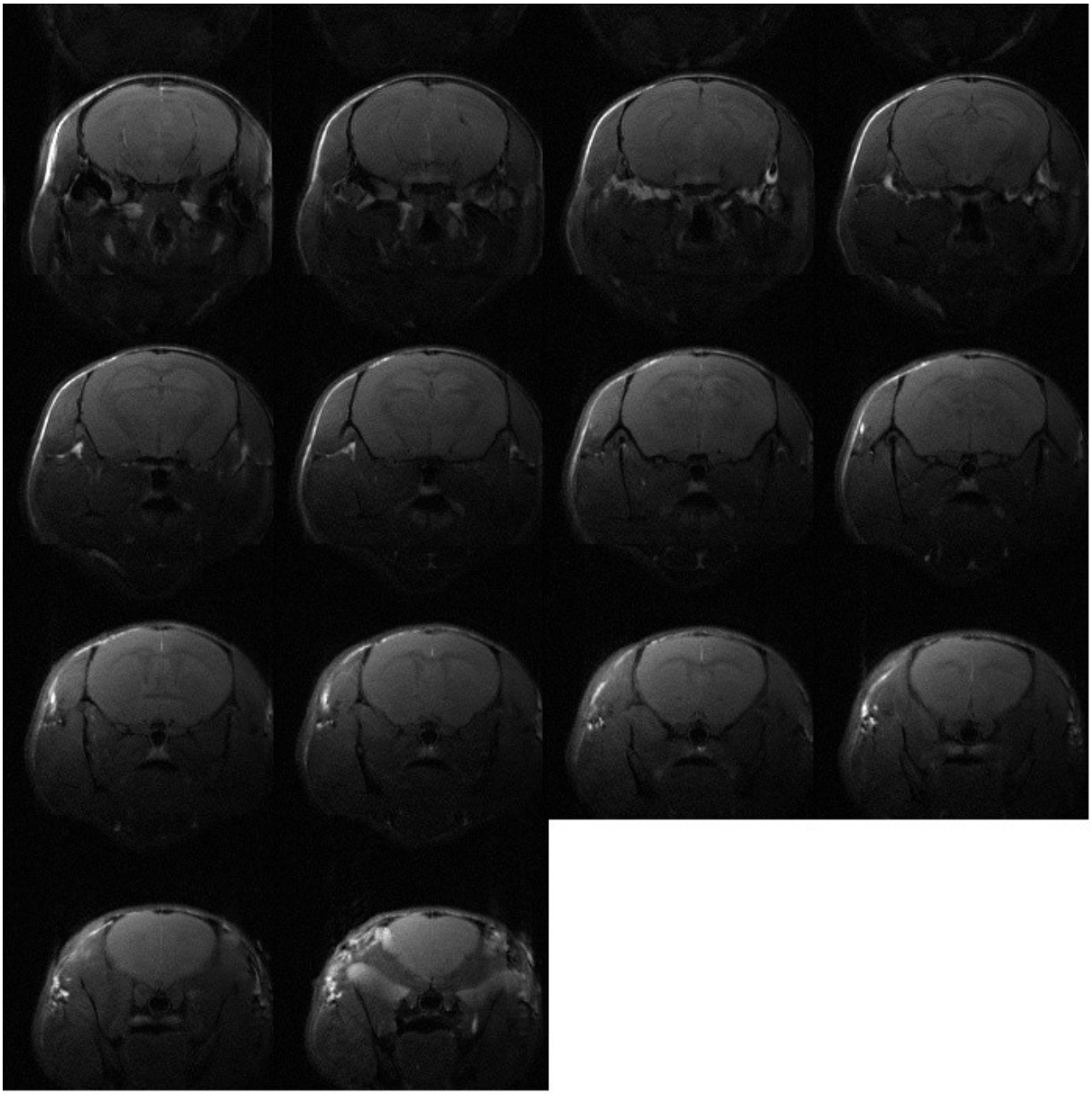

Figure 5.14 - MRI scan to assess blood-brain barrier integrity – Pre-administration of Gadolinium contrast via the tail vein and imaged by MRI in order to obtain a baseline image for analysis. identify whether breakdown of the blood-brain barrier was present.

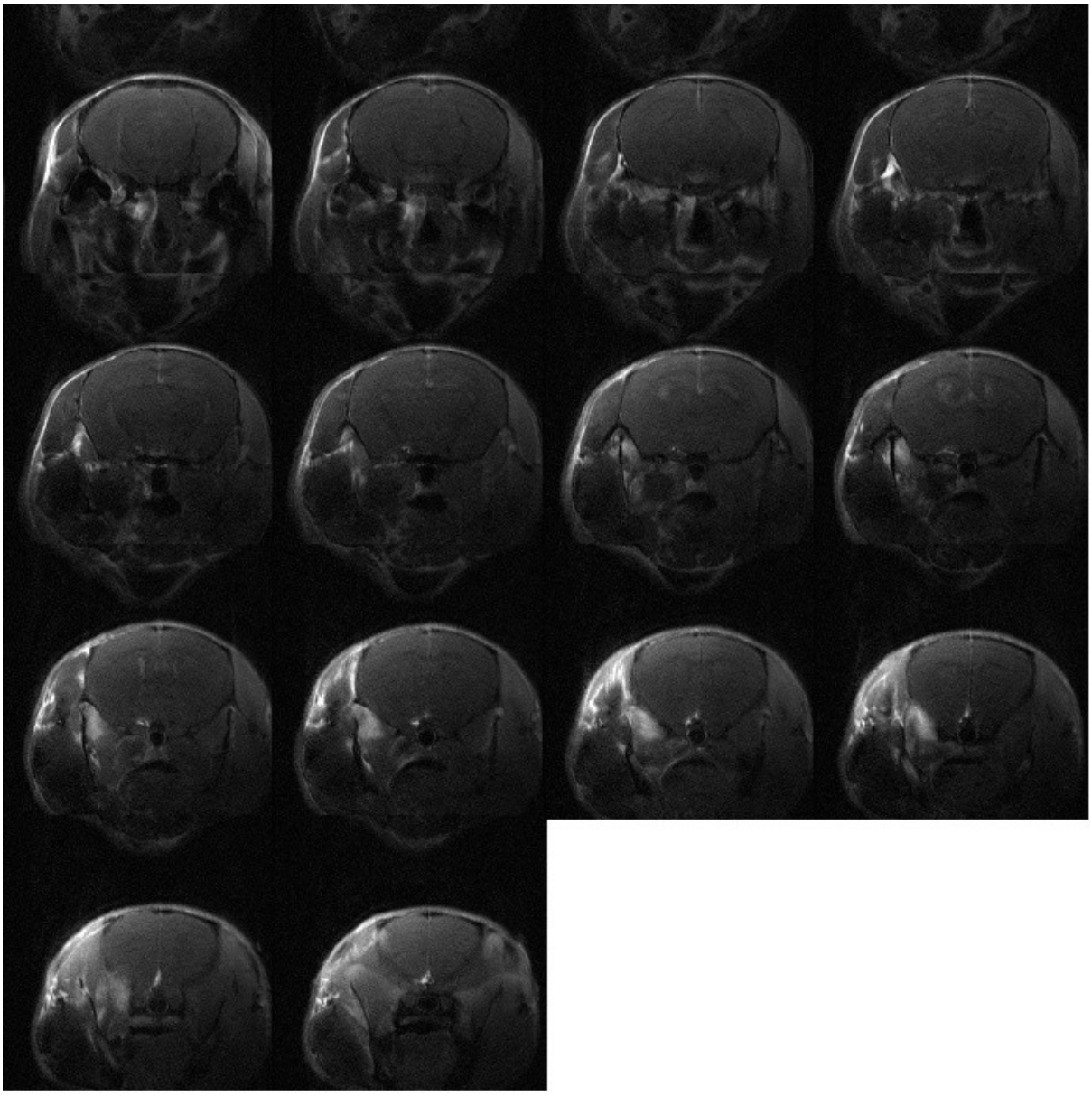

Figure 5.15 –MRI scan of brain following contrast agent administration – Gadolinium contrast agent was administered via the tail vein and brain imaged by MRI in order to identify whether breakdown of the blood-brain barrier was present. Gadolinium shows up on scan as intense white.

5.3 Discussion

In order to test the hypothesis regarding the function of miR-21 in ischaemic stroke a number of experiments were conducted. miR-21 was demonstrated to be upregulated after stroke by performing qPCR on peri-infarct tissue isolated from aged SHRSP rats subjected to tMCAO stroke. *In situ* hybridization data supported this observation and demonstrated that there was a characteristic change in localization of miR-21 expression. Subsequent studies sought to investigate the effects of modulating miR-21 assessing change in both directions via the use of overexpressing and knockout transgenic mice. Measurement of blood pressure and characterization of cerebrovascular anatomy demonstrated no basal differences between transgenic and wild type animals other than the differential expression of the miRNA of interest. Following the commencement of the transgenic overexpression study, no difference was observed in infarct size or behavioural measurements between overexpression and wild type controls. Subsequent experimental work on the miR-21 KO strain had to be terminated early due to a high mortality rate. Mortality in the miR-21 KO experiment occurred exclusively in the KO group and was significant, suggesting that miR-21 KO worsens pathology of stroke.

Alteration in miR-21 expression and localization in SHRSP rats following stroke displays characteristic differences. Immunohistological evidence was generated which suggests that expression of miR-21 is located in neuronal nuclei of pyramidal neurons in the non-infarcted brain tissue whilst there is an apparent localization of miR-21 to invading astrocytes in infarcted tissue. Although evidence exists demonstrating an upregulation of miR-21 following stroke in rats, this is the first time that a significant upregulation of miR-21 has been described in peri-infarct tissue following ischaemic stroke in co-morbid rats (Buller et al. 2010). Taken together, the combination of the immunohistochemistry and *in situ* data analysed with consideration to the global increase in miR-21 expression observed by TaqMan does seem to indicate that the increased levels of miR-21 are due to invasion by infiltrating cell populations. Other cells that will be present at 72 hours following stroke such as macrophages (Chiba and Umegaki 2013) were not assessed in this study and it is possible that these cell types will also express miR-21. Interestingly similar changes have been described by others in mice following spinal cord injury and

overexpression of miR-21 by astrocytes was determined to be protective in the prevention of the breakdown of the blood-brain barrier (Bhalala et al. 2012). It has also been demonstrated that there is a significant upregulation of miR-21 expression following traumatic brain injury in mice (Redell, Zhao, and Dash 2011).Although this diseases and models are considerably different from stroke, one might expect that these fundamental cell pathways which regulate response to cellular injury in the central nervous system could exhibit a great deal of mechanistic overlap at the molecular level. Ideally, for the co-localization experiments both the in situ and immunohistochemistry would be conducted on the same section instead of serial sections to improve resolution and confidence that the same cell was being assessed in analysis (it would also remove the challenge of trying to locate identical regions of the brain on two different slides under a confocal microscope). However, due to the fact that the *in situ* platform employed used a dye and the immunohistochemistry used fluorophores this was not possible at this time.

To date only one paper exists which has described a difference in MiR-21 expression level stroke (Buller et al. 2010), in situ hybridization of post-stroke brains determined there to be an upregulation of miR-21 following stroke in WKY rats. There are several methodological differences between this study and the one conducted here. The greatest difference is the use of WKY instead of SHRSP rats, SHRSP rats exhibit co-morbidities associated with stroke and are therefore considered to be more clinically relevant. The other major methodological difference is that whilst this study has employed the filament model of stroke, Buller *et al.* have used the embolic model of stroke by administering a blood clot generated *ex vivo*. This model, though not as widely studied as the tMCAO filament model is considered to be more clinically relevant as it is a better representation of what happens in humans (Hashimoto et al. 2010b). Whilst the work described here used the time points 24h and 72h for assessments of phenotype, Buller *et al.* have looked at 48h and 7 days. Despite these considerable differences between these studies, their findings are consistent with regards to expression levels of miR-21.There is also some evidence demonstrating differential expression in serum following stroke. One study suggested that miR-21 expression was higher in serum of stroke patients than healthy controls (Tsai et al. 2013), whilst another demonstrated a downregulation in plasma expression levels (Zhou and Zhang 2014). The contradictory evidence regarding the direction of miR-21 change in these human studies is disconcerting and likely has arisen

as a result of differential underlying genetics in the groups used for the two studies as has been discussed earlier in this thesis.

Differences in localization of miR-21 following stroke as determined by in situ hybridization offers some insight regarding the function of this miRNA in the context of disease. Loss of neuronal expression in the infarcted region is most likely a feature of cell death in this region. Whilst the speckling pattern observed in the parenchyma was probably due to infiltration by invading astrocytic cells and inflammatory cells such as macrophages.

Perhaps the most notable aspect of the differential localization following stroke is the increased expression present in the endothelium of the cerebral microvasculature, this has not been previously described in the context of stroke. Promotion of miR-21 was observed *in vitro* in endothelial cells following induction of shear stress(Weber et al. 2010). Subsequent studies have identified modulation of miR-21 expression in the systemic circulation following vascular injury (Urbich et al. 2008). Further investigation will be necessary to determine the functional significance of the increased endothelial miR-21 in the cerebrovasculature following stroke. However, other evidence assessing the function of miR-21 in primary retinal endothelium following stroke suggests that this miRNA promotes angiogenesis (Guduric-Fuchs et al. 2012).

Following characterization of miR-21 changes in the rat, it was deemed necessary to achieve experimental modulation of miR-21 in models of stroke in order to assess its potential use therapeutically. Initially consideration was given to use of viral vectors or miRNA mimics. However, due to technical challenges with both vector generation and robust modulation of miRNA expression, transgenic animals were decided upon as being the best strategy for manipulation. As miR-21 transgenic rats do not currently exist, it was necessary to translate this research into the mouse model of stroke. MRI imaging of infarct size was decided upon as being the primary measure of outcome and in keeping with the STAIR guidelines (Saver et al. 2009b) behavioural testing was also incorporated. For this experiment the Garcia neuroscore (Garcia et al. 1995) and rung ladder test(Metz and Whishaw 2009b) were used.

Following establishment of the mouse transient middle cerebral artery occlusion in the pilot study there was an assessment of the effect of increasing miR-21 expression in the

transgenic animals in the Memphis colony with relation to stroke. There is a 6 fold upregulation in the basal levels in heterozygotes versus the wild type mice. This is larger than the post-ischaemic 2 fold upregulation observed in the rats and can be expected to exhibit a physiological effect. Ultimately no difference was observed in infarct size between groups. Variability within each group may be a contributing factor to the lack of difference observed. A brief survey of the literature illustrates that variability is different between studies (Casals et al. 2011). Some of this occurs as a result of variation in the genetic background in a colony, some as a result of the specific technique used(Connolly Jr et al. 1996). Every methods section identified which employed tMCAO in mice stated that the monofilament should be advanced 9-10mm (regardless of strain used) meaning that there is immediately a 10% measurement error with regards to the filament alone, before taking into consideration the anatomical variations present in the animals; it was often not possible to advance the filament even 9mm from the bifurcation of the common carotid without risking rupture of the cerebrovasculature so in many cases the filament was advanced until it was met with resistance. Many researchers permanently ligate all branches of the common carotid artery with the exception of the internal carotid (Chiang et al. 2011), but in this study flow through the circle of Willis has been maintained in an attempt to mitigate the severity of the procedure. A recent study demonstrated that permanent occlusion of the pterygopalatine artery in mice (something not usually performed) resulted in a statistically significant reduction in infarct variability (Chen et al. 2008). It is difficult to make direct comparisons between techniques performed by others due to differences in strains used and intrastrain differences between institutes resulting from genetic drift, but it is likely that permanent ligation of common carotid branches may result in less variability. Whilst the permanent ligation of these vessels would likely reduce variability across the group (Chen et al. 2008) it would result in increased severity for each individual animal. Branches of the carotid were all ligated temporarily so that as much normal blood flow as possible could be retained following recovery. This results in less severe phenotype thus complying with the 'Refinement' aspect of the Home Office's 3Rs policy (Russell et al. 1959).

In an ideal world it would have been beneficial to optimize the stroke procedure in the mouse assessing relationship between infarct size and variables such as distance of advancement of filament and occlusion times in order to identify whether it was possible to obtain a smaller, more consistent infarct. The strokes observed here are quite large

and relative to size of brain, much larger than what is observed in humans in clinical practice. It is possible that although no difference in infarct size is seen upon resolution at 72 hours that a difference in sensorimotor deficit may exist between groups. I have assessed behaviour out to 72 hours, but it is possible that the animals are still recovering from anaesthetic etc. and this results in a non-stroke related reduction in sensorimotor function. Ideally, behavioural testing should be conducted at longer time points such as 1 week following stroke. However, it was not possible to take the experiment out to this point due to time constraints, limited resources and concerns regarding mortality/animal welfare.

Additional animals were required to conform to the numbers determined by power calculation. 6 and 8 mice were used in each group instead of the suggested 12 and 12. Some mice were lost as a result of the procedure and before they were replaced by additional animals an interim analysis was conducted on data already generated in order to see whether the addition of these animals would support the hypothesis. However, due to the lack of difference in the mean infarct size between groups it was determined that the hypothesis would not have been supported by the inclusion of these additional animals.

It was considered that the reason for the lack of difference observed between the heterozygous and wild-type Memphis animals was that a potent increase in miR-21 was evident in the transgenic animals at baseline; this may be lost following the transcriptional upregulation which occurs following stroke. In order to determine whether this was the case or not qPCR was performed and the 6-fold higher level of miR-21 expression persisted in the Memphis animals at 72 hours following stroke suggesting that this was not the case.

Baseline blood pressure was also assessed, the hypothesis being that if any differences in infarct size were observed following stroke, it would be necessary to determine that there was no difference in blood pressure or cerebral blood flow, as differences here could account for differential phenotype as the result of traditional physiological risk factors as opposed to anything really interesting at the molecular level involving miRNA biology. Tail cuff plethysmography of mice demonstrated there to be no difference in blood pressure between groups.

Unfortunately, the miR-21 KO study using these conditions had to be terminated early as a result of a mortality rate exceeding that expected within limit of licence, although the mortality occurred exclusively in the KO group. This is promising as it demonstrates that miR-21 plays a role in vivo following stroke. It is possible that the lack of effect seen in the Memphis experiment is due to the miRNA not being expressed highly enough, or counter regulatory effects may be in place negating the effects of overexpression.

MiR-21 has previously been observed as being upregulated in the smooth muscle cell layer of atherosclerotic vessels (Raitoharju et al. 2011; Wang et al. 2011). Investigation into miR-21 function in vascular smooth muscle cells has been conducted *in vivo* by performing vein graft transplantation using in pigs and mice. *in situ* hybridization has demonstrated that there is little no expression of miR-21 in the smooth muscle cell layer of ungrafted saphenic veins, but porcine vein grafts demonstrated expression in the adventitial medial and neointimal layers at both 7 and 28 days post-surgery. Mice demonstrated a similar pattern of expression in vein grafts and co-localization immunohistochemistry experiments demonstrated that the cells expressing miR-21 were smooth muscle and macrophages. Ablation of miR-21 in mice which were subjected to vein grafting demonstrated attenuation of neo-intimal formation suggesting that miR-21 plays a role in post grafting pathology, suggesting that therapeutic modulation of this miRNA may be an effective clinical strategy in the treatment of vascular disease (McDonald et al. 2013). Studies in pulmonary arterial hypertension have demonstrated that miR-21 is expressed abundantly in endothelial cells of the pulmonary circulatory system. In the context of pulmonary arterial hypertension, miR-21 inhibits expression of PDCD4 attenuating caspase 3 mediated apoptosis and mitigating pathology of this disease (White et al. 2014). This is somewhat consistent with observations made in this thesis regarding vascular localization of miR-21 expression, and whilst the evidence presented regarding the therapeutic potential of miR-21 in the context of stroke it remains a candidate for further investigation.

Following the termination of the miR-21 KO study taken to 72h and the indication that knockout did seem to worsen phenotype by increasing mortality, the remaining miR-21 KO mice were assessed with a 4h end point. Whilst this has resulted in a reduction of mortality to well within acceptable limits in order to identify whether differences in infarct size are present DWI scans were utilised. No difference was observed in infarct size

between groups. The numbers of animals used in this study were considerably less than those used in the original miR-21 Memphis study and there are also technical challenges regarding the quality and resolution of the DWI scan versus the T_2-weighted scan. Taking these two factors into consideration alongside the increased mortality in the miR-21 KO animals at 72h following stroke, I am not convinced that the absence of evidence in relation to the primary outcomes hypothesised is evidence of absence of effects of the gene.

Injection of contrast agent in order to identify differences in blood-brain barrier integrity could also be performed in attempt to analyse blood-brain barrier at the physiological level. If any difference could have been demonstrated between miR-21 KO and wild type mice, this could account for the difference in mortality rate observed between the two groups. One would expect that if blood-brain barrier is effected by miR-21 expression in this strain of animals as is described elsewhere (Bhalala et al. 2012) that a reduction in BBB integrity would result in a more severe stroke phenotype including higher rate of mortality. In order to assess this gadolinium contrast agent was administered to anaesthetized mouse and visualised by MRI, this is quite technically challenging. Following the induction of anaesthesia the mouse is heated to ensure vasodilation is present (mice can become quite cold acutely following recovery from stroke surgery and vasodilated vessels are extremely difficult to visualize/cannulate). Location of the tail vein was identified by surface anatomy, this was made more challenging by the brown pigment of the mice used. Following identification of the desired vessels the mouse tail vein was cannulated. Successful cannulation was confirmed by allowing blood to flow back along the cannula. Once confirmed a solution of heparinised saline is added to the cannula to prevent coagulation of blood in the line. The mouse was first scanned by DWI scan as infarct size was the primary outcome measurement being made during this study, and due to the time taken for this as well as the agitation of the mouse as it was transported from theatre to the bore of the scanner the line often became blocked or displaced. It was not apparent whether this was the case or not until the contrast agent was administered and the brain imaged. This technique is performed with much greater success in the rat due to the difference in size, although the reason that this was not performed for my study was due to the inability to modulated miRNA expression in the rat at the time experimental work was performed.

The behavioural assessments used in this study are commonly implemented in stroke literature due to the limited speciality equipment needed to perform them, but they are by no means the best. The Garcia neuroscore is likely to be a highly subjective measure of sensorimotor deficit and if the researcher knows the identity of the experimental groups being used this makes it extremely prone to bias. For this reason it is of great importance that the researcher remains blinded to the identity of the subjects in a study. Great care was taken during this study to ensure that I was blinded to animal genotype until it was time to analyse my data, which increases the integrity of these studies. There were also challenges associated with the use of the ladder test. Prior to commencement of the study each mouse was subjected to multiple trials on the run in order to train them to perform the task effectively. Although, animals did generally perform well from the first trial, following stroke the majority of them really struggled. Several of them required encouragement to traverse the ladder which was not desirable for this experiment. It is unclear whether the lack of ability to independently finish the ladder test post-infarct relates to the severity of the stroke or from the subjects still being under the influence of anaesthetic and/or the effects of acute inflammatory response following stroke.

As I have described, there are many variables which could be modified in this experimental procedure including distance of filament advancement, type of stroke model (tMCAO, Embolic, Temura), time of occlusion, recovery time points, and measurements made. The reason MRI quantification of infarct is more desirable is that it allows for measurements of infarct size to be made. Another major consideration is that only one miRNA was assessed *in vivo* and although preliminary data did appear to support the hypothesis it was by no means conclusive. The support for the hypothesis from the literature largely comes from studies which have assessed different diseases, different models and different tissues/cell types making it difficult to draw parallels effectively. This is indicative of an open question present in miRNA research today: is it better to pick candidate genes and targets which have been validated in the context of other diseases and to study them in the context of your disease of interest? Or is it better to perform screens for miRNAs and targets in your own models in order to identify genes and targets of interest? Each presents its own respective benefits and setbacks. For this *in vivo* study the former option was selected. This reduced the initial costs to identify the genes being studied, but in retrospect, this may not have been the correct miRNA to study in this model. Whilst conducting screens specific to disease models may result in improved

candidate selection, these experiments may be prohibitively expensive and by no means guarantee robust data generation. For example, the signal from abundantly expressed genes may act to mask changes which occur in less abundant transcripts. With regards to miRNA expression and cell signalling it is often the lower abundance genes which are the most interesting, especially when these molecules are at the apex of hierarchical cell-signalling cascades. A target specific approach would identify small changes and low abundance transcripts accurately, whilst they may be passed over as candidates for further analysis by the initial array experiments.

Another improvement that could have been to the study was to have achieved the modulation *in vivo* through use of pharmacological agents. Initially the plan was to generate canine adenovirus containing miRNAs of interest to take advantage of its neurotrophic behaviour. However, this approach was discarded due to cloning problems which created issues with regards to generation of vectors and issues regarding downregulation of the CAR receptor which resulted in the reduction of transduction efficiency of the vectors *in vivo*. Subsequently modulation of miRNAs through the use of mimics was attempted. This is more desirable from a translational perspective as this approach frees the researcher from all of the challenges posed when getting a viral vector to clinical trials as well as bringing a viral vector based therapeutic to market. Initial *in vitro* work was very promising in terms of promoting modulations in miR-21 expression levels. However, no changes were observed in target genes assessed in keeping with what would be expected given that targets were modulated by this miRNA in this model under these conditions. This lack of identified mRNA modulation following miRNA modulation is a problem frequently encountered by those investigating miRNAs, it is possible that other targets not assessed in this study are being modulated and they have been missed. Either way, this demonstrates the importance of looking for changes at the protein level as even in cases where there is no measurable difference in mRNA expression levels following miRNA inhibition, there can still be post-transcriptional inhibition of translation. Initial *in vivo* experiments in rats suggested that conducting these experiments would not be possible with the doses being used. For this reason the *in vivo* aspect of this project was translated into mice due to the availability of transgenics. Use of transgenics has demonstrated that it is possible to get reliable modulation of miRNA expression even following stroke, but it does come with some caveats.

Chapter 6 – General Discussion

6 Conclusions

During this doctoral work, the involvement of miRNAs in stroke has been investigated in several different contexts through the use of *in vitro* and *in vivo* preclinical models, as well as in patient serum samples. This work was conducted with the underlying philosophy of translational medicine which seeks to ensure optimal transmission of findings from basic research for maximal benefit in the practice of clinical medicine.

Despite recent advances in the understanding of underlying stroke pathophysiology as well as promising new developments in the ability of clinicians to effectively treat stroke it remains one of the leading health problems in the western world today. Until recently, the only clinically approved treatment for stroke was thrombolysis by alteplase at 4.5h following stroke (Hacke et al. 2008). Due to the narrow therapeutic window of effectiveness for this treatment a minority of patients benefitted from this treatment. More recently experimental stroke research has been focused on the neuroprotection strategy, the field suffering from some high profile failures, most notably NXY-059 (Diener et al. 2008b). The most recent clinical research demonstrating efficaciousness relies on a combination of mechanical thrombectomy and thrombolysis: The results of the MR CLEAN trial were recently presented at the World Stroke Conference in Istanbul and met with a standing ovation from the audience (Donnan 2015). Whilst the ongoing progression in the advancement of stroke interventions by the refinement of existing treatments as well as the introduction of new ones will improve the lives of many, there is still much to be done before stroke can be effectively treated.

miRNAs are short non-coding RNA molecules which act to post-transcriptionally inhibit expression of a myriad of different proteins. Given that a single miRNA can modulate multiple aspects of a single pathway, miRNAs can act as potent modulators of these pathways. Irrevocable evidence supports the fundamental importance in normal cellular biology, as well as the pathogenic (and potentially therapeutic) effects of miRNA perturbations. There is great interest in the modulation of miRNAs for therapeutic purposes and novel experimental therapeutics are emerging with increasing frequency. Arguably the miR-based treatment closest to common clinical practice is Miravirsen, a LNA inhibitor of miR-122 for the treatment of Hepatitis C virus (Janssen et al. 2013). It will be interesting to see how these treatments fare as they pass through the system of

clinical trials as the success of miRNA based treatments in other diseases can only be propitious for miRNA in stroke research.

Previous research in this group, as well as others have implicated the importance of miR-29 cluster members in stroke after noting that its expression was dysregulated following cerebral ischaemia. In order to verify that the endogenous changes in miR-29 cluster expression levels were reproducible in this lab qPCR was performed on rat brain tissue from SHRSP rats subjected to tMCAO. miR-29b was found to be significantly downregulated in ischaemic core at 24 and 72h, whilst it was significantly upregulated in peri-infarct tissue at 72h only versus time-matched sham control. Whilst miR-29c was downregulated in the remainder tissue at 24h and infarct core at 72h only. Description in temporal changes in miR-29 expression at the transcriptomic level was supported by identification of its spatial location by in situ hybridization. However, no notable changes in localization were observed. The greatest strength of the rat characterization versus other similar research is that the brain studied has been assessed on subdivisions looking at ischaemic core, peri-infarct region and remainder tissue. Much of the brain miRNA expression data to date looks only at whole hemispheric lysates. Molecular regulation of gene expression is not homogenous throughout the brain and different pathways are being modulated in different regions depending on whether cell death has occurred, whether it is a region of evolving infarct, or whether it is remainder tissue. This approach offers greater resolution and thus improved understanding of what is occurring in the brain. Ideally it would be informative to further distinguish miRNA activity based on cell type (neurons, astrocytes, cerebrovascular endothelium etc.) but this is currently too technically challenging to perform effectively. Another improvement that could be made to this study would be the assessment of additional acute time points as this would allow for improved temporal resolution and better understanding of how miRNAs alter with time.

In order to further investigate the potential manner in which the miR-29 cluster is involved in stroke pathophysiology several target transcripts were assessed. The miR-29 family has been relatively well described as a potent modulator of fibrosis in the context of other diseases as such several members of the TGF signaling family were assessed in addition to other genes widely studied in the context of stroke as well as being miR-29 targets. Of those selected and studied, MMP2, MMP9 and TGFβ-1 were found to be

significantly downregulated which would be consistent with an increase in miR-29 family member expression assuming that these genes are under control by miR-29b in this context. In order to definitively confirm the interactions between these genes in this tissue further experimentation (such as co-immunoprecipitation) would have to be performed, or modulation of miR-29b would have t o be implemented in this model in order to see if changes in these target genes could be achieved. It is also quite likely that many miR-29 target transcripts are modulated in this model which have been missed due to the manner in which targets were selected. A transcriptomic analysis would potentially identify additional gene candidates. Ultimately, from a clinical perspective the physiological effects of modulating these miRNAs in the context of stroke will be more informative that descriptive studies of the mechanisms of miRNAs in response to stroke in untreated animals, although in depth understanding of these mechanisms will be essential if such a treatment is to be brought into the clinic. Such studies will have to be conducted after a candidate miRNA has demonstrated improvements in reducing infarct, improving sensorimotor function, promoting synaptic plasticity and/or promoting regeneration.

Following up the miR-29 aspect of this project, *in vivo* hypoxic challenge was implemented in a rat neuronal cell line in which miR-29b and miR-29c were modulated by use of miR mimics and anti-miRs. Whilst there was a failure to demonstrate miR suppression by anti-miR, there was a potent upregulation of both miR-29b and miR-29c. No corresponding downregulation of the miR-29 family member targets assessed was observed. However, MMP9 was significantly upregulated following transfection with miR-29b and Col3A1 was significantly upregulated with transfection of both miR-29b and miR-29c. The lack of gene inhibition at the transcript level is not conclusive, and it is possible that changes not apparent at the transcriptional level may be present at the protein level. Likewise it is possible that the gene targets assessed are not under miR-29 regulation in this model. The upregulation in MMP9 and Col3A1 may be due unknown complex regulatory feedback mechanisms although further experimentation would be necessary to confirm or deny this. The lack of cohesion between these observations and published literature on the subject is most likely down to differences in the models used.

The next aspect of this research was to attempt to develop a serum miRNA biomarker for stroke patients. Identification of candidate miRNAs of interest, serum obtained from 30

(16 non-stroke, 14 stroke) patients was subjected to Openarray miRNA analysis. From this initial study miR-19b, -20b, -21, -25, -27a, -93, -106a, -139-5p, -331, -374, -376c, -532, -573, -590, -885 were selected for further investigation. Validation of these candidates was attempted in serum obtained from 75 patients (20 non-stroke, 55 stroke) however no significance was observed between the two groups. Post-hoc analysis of stroke patients by TOAST subclassification was implemented in order to ascertain whether any difference may be present between subtypes of stroke. Again, no significance was observed. The biggest strength of this study in comparison to the literature was the use of stroke mimics as opposed to healthy controls however, this placed a limitation on the number of controls that were available for recruitment. Another likely source for the lack of significance observed is due to greater inter-subject variability of miRNA expression and smaller magnitude of changes in miR expression than was anticipated when the experiment was designed. Following this, exosomes were isolated from patient serum and subjected to qPCR analysis to determine whether any changes may be present in exosomes, but whilst the data benefited from a reduction in variability no significance was observed. Analysis of expression of miR-19b, -93, -106a and -532 in rat serum and isolated exosomes demonstrated significant differences illustrating that changes here were observable in experimental models used. The greatest strength of this study versus what has been published in the literature is the use of stroke mimics. Frequently, when a molecular biomarker for disease is identified by a study like this it will not remain a valid biomarker when subject to further scrutiny. This is often because the initial studies have used healthy controls and the difference in expression of such a genetic biomarker between an at-risk population and disease population is less than that observed between the disease population and a healthy population. Future work on this is seeking to increase patient numbers to improve the power of the study further and will look specifically assess miRNA expression levels in serum exosomes as the data obtained from such studies is more robust, less variable and more interesting from a functional perspective.

The final aspect of this project looked at the effects of in vivo modulation of miR-21 in the context of stroke via the use of transgenic mice. Modulation of miRNAs in experimental stroke models is most desirable after onset of stroke as this is of most clinical relevance. Modulation of miRNAs post-stroke presents considerable technical issues. For this reason the use of transgenic mice was decided upon in order to identify whether miR modulation

could result in improvements of stroke phenotype in physiological and behavioural measurements. miR-21 was selected due to evidence in the literature which has implicated the importance of miR-21 in the context of several cardiovascular diseases including stroke.

Initial experimental work sought to describe basal expression of miR-21 and differences which occur following experimental stroke. qPCR analysis of miR-21 expression in rat brain tissue demonstrated that there was a significant increase in expression in the peri-infarct region of the brain versus sham control as well as the corresponding contralateral region at 72h following stroke. Localization of miR-21 expression at this time point as determined by in situ hybridization demonstrated that there was a characteristic change in localization associated with the global increase in expression. Of most interest was the induction of miR-21 expression in cerebral microvasculature following injury. Co-localization by immunohistochemistry demonstrated that some of the miR-21 expressing cells which become apparent in infracted tissue may be astrocytic in origin.

After demonstrating that miR-21 expression was altered following stroke, the role was assessed by comparing infarct size as well as behavioural measurements in miR-21 overexpressing CAG-miR-21 mice versus wild type control. In order to ensure that any differences physiology following stroke was not due to underlying phenotype some characterization of the mouse colony was conducted. Anatomical variation of circle of Willis anatomy was determined by histological analysis of the vasculature by India ink staining and found there to be no difference between transgenic and wild type animals. Measurement of blood pressure by tail cuff plethysmography demonstrated no difference. No significant difference in stroke volume or neurological deficit was apparent between the transgenic animals and wild type controls. This may be due to the infarct size in this model being too large to achieve a measurable improvement in lesion size by modulation of genes, or it may also be the case that the increase in miR-21 expression is beneficial for some cell types and detrimental in others, these effects negating each other. It may also be the case that the level of modulation may not be optimal to achieve therapeutic effects. Subsequently a similar study was conducted in homozygous miR-21 KO mice and found there to be a significant increase in mortality rate suggesting that miR-21 KO worsens stroke pathology. This study was terminated early due to a mortality rate beyond what was allowed on license. In order to obtain further clarification regarding the

effects of miR-21 KO on stroke, a final study looked to assess the size of stroke at 4h by DWI scan. However, due to technical issues these data were inconclusive. In order to better assess the effects of miR-21 modulation in these mice it would be beneficial to optimize the tMCAO protocol to reduce infarct size and ensure that it is localized to the cortex as this is more clinically relevant and it may be easier to identify improvements in infarct volume and behaviour with a less severe stroke. Use of an alternative stroke model in these mice might be permissible to achieve this.

The work presented in this thesis, along with the literature on miRNAs and stroke represent a mere scratch on the surface of what remains to be done with respect to our understanding of the role small non-coding RNAs play in cerebral ischaemia. Although the hypotheses put forth in this work have not been supported fully by the data, this work has laid foundations whereupon further research will build moving forward. Ideally assessment of miRNAs in stroke would involve a large multi-center multi-model study which systematically works through a large array of different miRNAs. Primary outcomes measured would be physiological and behavioural measurements of stroke, whilst secondary outcomes would seek to identify underlying mechanisms by which specific miRNAs are having these effects by transcriptomic and proteomic analysis. It may also be the case that a cocktail of multiple miRNAs may have improved effects over individual species, whilst the targeting of miRNA modulation to specific cell types will also be desirable in order to attain more sophisticated targeting of treatments. Further research in this area will serve to improve the options available to patients in the movement towards personalized medicine.

Bibliography

Abbott, Larry F., and Sacha B. Nelson. 2000. “Synaptic Plasticity: Taming the Beast.” *Nature Neuroscience* 3: 1178–83.

Adams, H. P., Birgitte H. Bendixen, L. Jaap Kappelle, Jose Biller, Betsy B. Love, David L. Gordon, and EE3rd Marsh. 1993a. “Classification of Subtype of Acute Ischemic Stroke. Definitions for Use in a Multicenter Clinical Trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment.” *Stroke* 24 (1): 35–41.

———. 1993b. “Classification of Subtype of Acute Ischemic Stroke. Definitions for Use in a Multicenter Clinical Trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment.” *Stroke* 24 (1): 35–41.

Adamson, Joy, Andy Beswick, and Shah Ebrahim. 2004. “Is Stroke the Most Common Cause of Disability?” *Journal of Stroke and Cerebrovascular Diseases* 13 (4): 171–77.

Adeoye, O., R. Hornung, P. Khatri, and D. Kleindorfer. 2011. “Recombinant Tissue-Type Plasminogen Activator Use for Ischemic Stroke in the United States.” *Stroke* 42 (7): 1952–55.

Ago, Tetsuro, Takanari Kitazono, Junya Kuroda, Yasuhiro Kumai, Masahiro Kamouchi, Hiroaki Ooboshi, Masanori Wakisaka, Tsukasa Kawahara, Kazuhito Rokutan, and Setsuro Ibayashi. 2005. “NAD (P) H Oxidases in Rat Basilar Arterial Endothelial Cells.” *Stroke* 36 (5): 1040–46.

Albers, Gregory W., Larry B. Goldstein, David C. Hess, Lawrence R. Wechsler, Karen L. Furie, Philip B. Gorelick, Patty Hurn, David S. Liebeskind, Raul G. Nogueira, and Jeffrey L. Saver. 2011. “Stroke Treatment Academic Industry Roundtable (STAIR) Recommendations for Maximizing the Use of Intravenous Thrombolytics and Expanding Treatment Options with Intra-Arterial and Neuroprotective Therapies.” *Stroke* 42 (9): 2645–50.

Amarenco, P., J. Bogousslavsky, L. R. Caplan, G. A. Donnan, and M. G. Hennerici. 2009. “New Approach to Stroke Subtyping: The ASCO (phenotypic) Classification of Stroke.” *Cerebrovascular Diseases* 27 (5): 502–8.

Appasani, K. 2008. *MicroRNAs: From Basic Science to Disease Biology*. Cambridge University Press. https://books.google.co.uk/books?id=H_W8rqYyc9MC.

Arai, Ken, Guang Jin, Deepti Navaratna, and Eng H. Lo. 2009. “Brain Angiogenesis in Developmental and Pathological Processes: Neurovascular Injury and Angiogenic Recovery after Stroke.” *FEBS Journal* 276 (17): 4644–52.

Ardan, Taras, Janetta Kovačeva, and Jitka Čejková. 2004. “Comparative Histochemical and Immunohistochemical Study on Xanthine Oxidoreductase/xanthine Oxidase in Mammalian Corneal Epithelium.” *Acta Histochemica* 106 (1): 69–75.

Asahi, Minoru, Xiaoying Wang, Tatsuro Mori, Toshihisa Sumii, Jae-Chang Jung, Michael A. Moskowitz, M. Elizabeth Fini, and Eng H. Lo. 2001. “Effects of Matrix Metalloproteinase-9 Gene Knock-out on the Proteolysis of Blood–brain Barrier and White Matter Components after Cerebral Ischemia.” *The Journal of Neuroscience* 21 (19): 7724–32.

Asoh, Sadamitsu, Ikuroh Ohsawa, Takashi Mori, Ken-ichiro Katsura, Tomoharu Hiraide, Yasuo Katayama, Megumi Kimura, Daiya Ozaki, Kumi Yamagata, and Shigeo Ohta. 2002. “Protection against Ischemic Brain Injury by Protein Therapeutics.” *Proceedings of the National Academy of Sciences* 99 (26): 17107–12.

Astrup, Jens, Bo K. Siesjo, and Lindsay Symon. 1981. “Thresholds in Cerebral Ischemia-the Ischemic Penumbra.” *Stroke* 12 (6): 723–25.

Ay, Hakan, Karen L. Furie, Aneesh Singhal, Wade S. Smith, A. Gregory Sorensen, and Walter J. Koroshetz. 2005. “An Evidence-based Causative Classification System for Acute Ischemic Stroke.” *Annals of Neurology* 58 (5): 688–97.

Badan, I., B. Buchhold, A. Hamm, M. Gratz, L. C. Walker, D. Platt, C. H. Kessler, and A. Popa-Wagner. 2003. “Accelerated Glial Reactivity to Stroke in Aged Rats Correlates with Reduced Functional Recovery.” *Journal of Cerebral Blood Flow & Metabolism* 23 (7): 845–54.

Bang, Claudia, Sandor Batkai, Seema Dangwal, Shashi Kumar Gupta, Ariana Foinquinos, Angelika Holzmann, Annette Just, Janet Remke, Karina Zimmer, and Andre Zeug. 2014. “Cardiac Fibroblast–derived microRNA Passenger Strand-Enriched Exosomes Mediate Cardiomyocyte Hypertrophy.” *The Journal of Clinical Investigation* 124 (124 (5)): 0–0.

Bartel, David P. 2009. “MicroRNAs: Target Recognition and Regulatory Functions.” *Cell* 136 (2): 215–33.

Berkhemer, Olvert A., Puck SS Fransen, Debbie Beumer, Lucie A. van den Berg, Hester F. Lingsma, Albert J. Yoo, Wouter J. Schonewille, Jan Albert Vos, Paul J. Nederkoorn, and Marieke JH Wermer. 2015. “A Randomized Trial of Intraarterial Treatment for Acute Ischemic Stroke.” *New England Journal of Medicine* 372 (1): 11–20.

Beurel, Eléonore, and Richard S. Jope. 2006. “The Paradoxical pro-and Anti-Apoptotic Actions of GSK3 in the Intrinsic and Extrinsic Apoptosis Signaling Pathways.” *Progress in Neurobiology* 79 (4): 173–89.

Bhalala, Oneil G., Liuliu Pan, Vibhu Sahni, Tammy L. McGuire, Katherine Gruner, Warren G. Tourtellotte, and John A. Kessler. 2012. “microRNA-21 Regulates Astrocytic Response Following Spinal Cord Injury.” *The Journal of Neuroscience* 32 (50): 17935–47.

Bhatia, Rohit, Michael D. Hill, Nandavar Shobha, Bijoy Menon, Simerpreet Bal, Puneet Kochar, Tim Watson, Mayank Goyal, and Andrew M. Demchuk. 2010. “Low Rates of Acute Recanalization With Intravenous Recombinant Tissue Plasminogen Activator in Ischemic Stroke Real-World Experience and a Call for Action.” *Stroke* 41 (10): 2254–58.

Bohnsack, Markus T., Kevin Czaplinski, and DIRK GÖRLICH. 2004. “Exportin 5 Is a RanGTP-Dependent dsRNA-Binding Protein That Mediates Nuclear Export of Pre-miRNAs.” *Rna* 10 (2): 185–91.

Bokoch, Gary M., and Ulla G. Knaus. 2003. “NADPH Oxidases: Not Just for Leukocytes Anymore!” *Trends in Biochemical Sciences* 28 (9): 502–8.

Bornstein, N. M. 2009. *Stroke: Practical Guide for Clinicians*. Karger. https://books.google.co.uk/books?id=Uw0AQVPlMUQC.

Breasted, James Henry. 1930. *The Edwin Smith Surgical Papyrus: Published in Facsimile and Hieroglyphic Transliteration with Translation and Commentary in Two Volumes*. Vol. 3. Chic. UP.

British Heart Foundation. 2009. “Statistics on Stroke in the UK.”

Brott, Thomas, H. P. Adams, Charles P. Olinger, John R. Marler, William G. Barsan, Jose Biller, Judith Spilker, Renee Holleran, Robert Eberle, and Vicki Hertzberg. 1989. “Measurements of Acute Cerebral Infarction: A Clinical Examination Scale.” *Stroke* 20 (7): 864–70.

Broughton, Brad RS, David C. Reutens, and Christopher G. Sobey. 2009. “Apoptotic Mechanisms after Cerebral Ischemia.” *Stroke* 40 (5): e331–39.

Brown, Brian D., and Luigi Naldini. 2009. “Exploiting and Antagonizing microRNA Regulation for Therapeutic and Experimental Applications.” *Nature Reviews Genetics* 10 (8): 578–85.

Buckwalter, Seanne P., Rose Teo, Mark J. Espy, Lynne M. Sloan, Thomas F. Smith, and Bobbi S. Pritt. 2012. “Real-Time Qualitative PCR for 57 Human Adenovirus Types from Multiple Specimen Sources.” *Journal of Clinical Microbiology* 50 (3): 766–71.

Buller, Ben, Xianshuang Liu, Xinli Wang, Rui L. Zhang, Li Zhang, Ann Hozeska-Solgot, Michael Chopp, and Zheng G. Zhang. 2010. “MicroRNA-21 Protects Neurons from Ischemic Death.” *FEBS Journal* 277 (20): 4299–4307.

Cai, Xuezhong, Curt H. Hagedorn, and Bryan R. Cullen. 2004. “Human microRNAs Are Processed from Capped, Polyadenylated Transcripts That Can Also Function as mRNAs.” *Rna* 10 (12): 1957–66.

Calin, George Adrian, Manuela Ferracin, Amelia Cimmino, Gianpiero Di Leva, Masayoshi Shimizu, Sylwia E. Wojcik, Marilena V. Iorio, Rosa Visone, Nurettin Ilfer Sever, and Muller Fabbri. 2005. “A MicroRNA Signature Associated with Prognosis and Progression in Chronic Lymphocytic Leukemia.” *New England Journal of Medicine* 353 (17): 1793–1801.

Caplan, L. R. 1990. “Charles Foix--the First Modern Stroke Neurologist.” *Stroke* 21 (2): 348–56.

Carmeliet, Peter, and Rakesh K. Jain. 2011. “Molecular Mechanisms and Clinical Applications of Angiogenesis.” *Nature* 473 (7347): 298–307.

Casals, Juliana B., Naira CG Pieri, Matheus LT Feitosa, Anna CM Ercolin, Kelly CS Roballo, Rodrigo SN Barreto, Fabiana F. Bressan, Daniele S. Martins, Maria A. Miglino, and Carlos E. Ambrósio. 2011. "The Use of Animal Models for Stroke Research: A Review." *Comparative Medicine* 61 (4): 305.

Castaño, Carlos, Laura Dorado, Cristina Guerrero, Monica Millán, Meritxell Gomis, Natalia Perez de la Ossa, Mar Castellanos, M. Rosa García, Sira Domenech, and Antoni Dávalos. 2010. "Mechanical Thrombectomy with the Solitaire AB Device in Large Artery Occlusions of the Anterior Circulation A Pilot Study." *Stroke* 41 (8): 1836–40.

Castanotto, Daniela, and John J. Rossi. 2009. "The Promises and Pitfalls of RNA-Interference-Based Therapeutics." *Nature* 457 (7228): 426–33.

Castilla, María Ángeles, Gema Moreno-Bueno, Laura Romero-Pérez, De Vijver, Koen Van, Michele Biscuola, María Ángeles López-García, Jaime Prat, Xavier Matías-Guiu, and Amparo Cano. 2011. "Micro-RNA Signature of the Epithelial–mesenchymal Transition in Endometrial Carcinosarcoma." *The Journal of Pathology* 223 (1): 72–80.

Chambless, Lloyd E., Aaron R. Folsom, Limin X. Clegg, A. Richey Sharrett, Eyal Shahar, F. Javier Nieto, Wayne D. Rosamond, and Greg Evans. 2000. "Carotid Wall Thickness Is Predictive of Incident Clinical Stroke: The Atherosclerosis Risk in Communities (ARIC) Study." *American Journal of Epidemiology* 151 (5): 478–87.

Chan, Jennifer A., Anna M. Krichevsky, and Kenneth S. Kosik. 2005. "MicroRNA-21 Is an Antiapoptotic Factor in Human Glioblastoma Cells." *Cancer Research* 65 (14): 6029–33.

Cheloufi, Sihem, Camila O. Dos Santos, Mark MW Chong, and Gregory J. Hannon. 2010. "A Dicer-Independent miRNA Biogenesis Pathway That Requires Ago Catalysis." *Nature* 465 (7298): 584–89.

Chendrimada, Thimmaiah P., Richard I. Gregory, Easwari Kumaraswamy, Jessica Norman, Neil Cooch, Kazuko Nishikura, and Ramin Shiekhattar. 2005. "TRBP Recruits the Dicer Complex to Ago2 for microRNA Processing and Gene Silencing." *Nature* 436 (7051): 740–44.

Chen, Grace Y., and Gabriel Nuñez. 2010. "Sterile Inflammation: Sensing and Reacting to Damage." *Nature Reviews Immunology* 10 (12): 826–37.

Cheng, Yunhui, Ruirui Ji, Junming Yue, Jian Yang, Xiaojun Liu, He Chen, David B. Dean, and Chunxiang Zhang. 2007. "MicroRNAs Are Aberrantly Expressed in Hypertrophic Heart: Do They Play a Role in Cardiac Hypertrophy?" *The American Journal of Pathology* 170 (6): 1831–40.

Cheng, Yunhui, Ping Zhu, Jian Yang, Xiaojun Liu, Shimin Dong, Xiaobin Wang, Bao Chun, Jian Zhuang, and Chunxiang Zhang. 2010. "Ischaemic Preconditioning-Regulated miR-21 Protects Heart against Ischaemia/reperfusion Injury via Anti-Apoptosis through Its Target PDCD4." *Cardiovascular Research* 87 (3): 431–39.

Chen, Hai, Yun Seon Song, and Pak H. Chan. 2009. "Inhibition of NADPH Oxidase Is Neuroprotective after Ischemia–reperfusion." *Journal of Cerebral Blood Flow & Metabolism* 29 (7): 1262–72.

Chen, Jinghai, Zhan-Peng Huang, Hee Young Seok, Jian Ding, Masaharu Kataoka, Zheng Zhang, Xiaoyun Hu, Gang Wang, Zhiqiang Lin, and Si Wang. 2013. "Mir-17–92 Cluster Is Required for and Sufficient to Induce Cardiomyocyte Proliferation in Postnatal and Adult Hearts." *Circulation Research* 112 (12): 1557–66.

Chen, Pei-Hao, Shan Gao, Yong-Jun Wang, An-Ding Xu, Yan-Sheng Li, and David Wang. 2012. "Classifying Ischemic Stroke, from TOAST to CISS." *CNS Neuroscience & Therapeutics* 18 (6): 452–56.

Chen, Po Yu, Lasse Weinmann, Dimos Gaidatzis, Yi Pei, Mihaela Zavolan, Thomas Tuschl, and Gunter Meister. 2008. "Strand-Specific 5′-O-Methylation of siRNA Duplexes Controls Guide Strand Selection and Targeting Specificity." *Rna* 14 (2): 263–74.

Chen, Qun, Shadi Moghaddas, Charles L. Hoppel, and Edward J. Lesnefsky. 2008. "Ischemic Defects in the Electron Transport Chain Increase the Production of Reactive Oxygen Species from Isolated Rat Heart Mitochondria." *American Journal of Physiology-Cell Physiology* 294 (2): C460–66.

Chen, Yili, Akihiro Ito, Keisuke Takai, and Nobuhito Saito. 2008. “Blocking Pterygopalatine Arterial Blood Flow Decreases Infarct Volume Variability in a Mouse Model of Intraluminal Suture Middle Cerebral Artery Occlusion.” *Journal of Neuroscience Methods* 174 (1): 18–24.

Chen, Yun, and David H. Gorski. 2008. “Regulation of Angiogenesis through a microRNA (miR-130a) That down-Regulates Antiangiogenic Homeobox Genes GAX and HOXA5.” *Blood* 111 (3): 1217–26.

Chiang, Terrance, Robert O. Messing, and Wen-Hai Chou. 2011. “Mouse Model of Middle Cerebral Artery Occlusion.” *Journal of Visualized Experiments: JoVE*, no. 48.

Chiba, Tsuyoshi, and Keizo Umegaki. 2013. “Pivotal Roles of Monocytes/macrophages in Stroke.” *Mediators of Inflammation* 2013.

Choi, Dennis W. 1992. “Excitotoxic Cell Death.” *Journal of Neurobiology* 23 (9): 1261–76.

Choi, Dong-Hee, Ji-Hye Kim, Kyoung-Hee Lee, Hahn-Young Kim, Yoon-Seong Kim, Wahn Soo Choi, and Jongmin Lee. 2015. “Role of Neuronal NADPH Oxidase 1 in the Peri-Infarct Regions after Stroke.” *PloS One* 10 (1).

Chouchani, Edward T., Victoria R. Pell, Edoardo Gaude, Dunja Aksentijević, Stephanie Y. Sundier, Ellen L. Robb, Angela Logan, Sergiy M. Nadtochiy, Emily NJ Ord, and Anthony C. Smith. 2014. “Ischaemic Accumulation of Succinate Controls Reperfusion Injury through Mitochondrial ROS.” *Nature* 515 (7527): 431–35.

Chung, Arthur CK, Xueqing Yu, and Hui Y. Lan. 2013. “MicroRNA and Nephropathy: Emerging Concepts.” *International Journal of Nephrology and Renovascular Disease* 6: 169.

Cifuentes, Daniel, Huiling Xue, David W. Taylor, Heather Patnode, Yuichiro Mishima, Sihem Cheloufi, Enbo Ma, Shrikant Mane, Gregory J. Hannon, and Nathan D. Lawson. 2010. “A Novel miRNA Processing Pathway Independent of Dicer Requires Argonaute2 Catalytic Activity.” *Science* 328 (5986): 1694–98.

Clancy, Cillian, Myles R. Joyce, and Michael J. Kerin. n.d. “The Use of Circulating microRNAs as Diagnostic Biomarkers in Colorectal Cancer.” *Cancer Biomarkers*.

Connolly Jr, E. Sander, Christopher J. Winfree, David M. Stern, Robert A. Solomon, and David J. Pinsky. 1996. “Procedural and Strain-Related Variables Significantly Affect Outcome in a Murine Model of Focal Cerebral Ischemia.” *Neurosurgery* 38 (3): 523–32.

Cook, Douglas J., Lucy Teves, and Michael Tymianski. 2012. “Treatment of Stroke with a PSD-95 Inhibitor in the Gyrencephalic Primate Brain.” *Nature* 483 (7388): 213–17.

Corkill, G., S. Sivalingam, J. A. Reitan, B. A. Gilroy, and M. G. Helphrey. 1978. “Dose Dependency of the Post-Insult Protective Effect of Pentobarbital in the Canine Experimental Stroke Model.” *Stroke* 9 (1): 10–12.

Covidien. 2015. “International Neuro Products | Flow Restoration | Solitaire™ FR Revascularization Device | Covidien.” Accessed February 21. http://www.ev3.net/neuro/intl/flow-restoration/solitaire-fr-revascularization-device.htm.

Crick, Francis. 1970. “Central Dogma of Molecular Biology.” *Nature* 227 (5258): 561–63.

Cushing, L., P. P. Kuang, J. Qian, F. Shao, J. Wu, F. Little, V. J. Thannickal, W. V. Cardoso, and J. Lü. 2011. “miR-29 Is a Major Regulator of Genes Associated with Pulmonary Fibrosis.” *American Journal of Respiratory Cell and Molecular Biology* 45 (2): 287.

Daugas, Eric, SANTOS A. SUSIN, NAOUFAL ZAMZAMI, KARINE F. FERRI, THEANO IRINOPOULOU, NATHANAEL LAROCHETTE, MARIE-CHRISTINE PRÉVOST, BRIAN LEBER, DAVID ANDREWS, and JOSEF PENNINGER. 2000. “Mitochondrio-Nuclear Translocation of AIF in Apoptosis and Necrosis.” *The FASEB Journal* 14 (5): 729–39.

Dávalos, Antoni, Vitor Mendes Pereira, René Chapot, Alain Bonafé, Tommy Andersson, and Jan Gralla. 2012. “Retrospective Multicenter Study of Solitaire FR for Revascularization in the Treatment of Acute Ischemic Stroke.” *Stroke* 43 (10): 2699–2705.

Davis, Stephen M., Geoffrey A. Donnan, Mark W. Parsons, Christopher Levi, Kenneth S. Butcher, Andre Peeters, P. Alan Barber, Christopher Bladin, Deidre A. De Silva, and Graham Byrnes. 2008. “Effects of Alteplase beyond 3 H after Stroke in the Echoplanar Imaging Thrombolytic Evaluation Trial (EPITHET): A Placebo-Controlled Randomised Trial.” *The Lancet Neurology* 7 (4): 299–309.

Del Zoppo, Gregory J., Klaus Poeck, Michael S. Pessin, Samuel M. Wolpert, Anthony J. Furlan, Andreas Ferbert, Mark J. Alberts, Justin A. Zivin, Lawrence Wechsler, and Otto Busse. 1992. “Recombinant Tissue Plasminogen Activator in Acute Thrombotic and Embolic Stroke.” *Annals of Neurology* 32 (1): 78–86.

Del Zoppo, Gregory J., G. W. Schmid-Schönbein, Etsuro Mori, Brian R. Copeland, and Cheng-Ming Chang. 1991. “Polymorphonuclear Leukocytes Occlude Capillaries Following Middle Cerebral Artery Occlusion and Reperfusion in Baboons.” *Stroke* 22 (10): 1276–83.

DeMers, Gerard, William J. Meurer, Richard Shih, Steve Rosenbaum, and Gary M. Vilke. 2012. “Tissue Plasminogen Activator and Stroke: Review of the Literature for the Clinician.” *The Journal of Emergency Medicine* 43 (6): 1149–54.

Demongeot, Jacques, and Andrés Moreira. 2007. “A Possible Circular RNA at the Origin of Life.” *Journal of Theoretical Biology* 249 (2): 314–24.

Dénes, Ádám, Szilamér Ferenczi, József Halász, Zsuzsanna Környei, and Krisztina J. Kovács. 2008. “Role of CX3CR1 (fractalkine Receptor) in Brain Damage and Inflammation Induced by Focal Cerebral Ischemia in Mouse.” *Journal of Cerebral Blood Flow & Metabolism* 28 (10): 1707–21.

Dharap, A., and K. Bowen. 2009. “Transient Focal Ischemia Induces Extensive Temporal Changes in Rat Cerebral microRNAome.” *Journal of Cerebral Blood Flow & Metabolism* 29 (4): 675–87.

Dharap, Ashutosh, and Raghu Vemuganti. 2010. “Ischemic Pre-conditioning Alters Cerebral microRNAs That Are Upstream to Neuroprotective Signaling Pathways.” *Journal of Neurochemistry* 113 (6): 1685–91.

Diener, Hans-Christoph, Kennedy R. Lees, Patrick Lyden, Jim Grotta, Antoni Davalos, Stephen M. Davis, Ashfaq Shuaib, Tim Ashwood, Warren Wasiewski, and Vivian Alderfer. 2008a. “NXY-059 for the Treatment of Acute Stroke Pooled Analysis of the SAINT I and II Trials.” *Stroke* 39 (6): 1751–58.

———. 2008b. “NXY-059 for the Treatment of Acute Stroke Pooled Analysis of the SAINT I and II Trials.” *Stroke* 39 (6): 1751–58.

Dirnagl, Ulrich, Costantino Iadecola, and Michael A. Moskowitz. 1999. “Pathobiology of Ischaemic Stroke: An Integrated View.” *Trends in Neurosciences* 22 (9): 391–97.

Dmitriev, Petr, Luiza Stankevicins, Eugenie Ansseau, Andrei Petrov, Ana Barat, Philippe Dessen, Thomas Robert, Ahmed Turki, Vladimir Lazar, and Emmanuel Labourer. 2013. “Defective Regulation of microRNA Target Genes in Myoblasts from Facioscapulohumeral Dystrophy Patients.” *Journal of Biological Chemistry* 288 (49): 34989–2.

Dong, Wei-Qiang, Avital Schurr, Kenneth H. Reid, Christopher B. Shields, and Catherine A. West. 1988. “The Rat Hippocampal Slice Preparation as an in Vitro Model of Ischemia.” *Stroke* 19 (4): 498–502.

Donnan, Geoffrey A. 2015. “The Impact of MR CLEAN.” *International Journal of Stroke* 10 (2): 139–139.

Donnan, Geoffrey A., Jean-Claude Baron, Henry Ma, and Stephen M. Davis. 2009. “Penumbral Selection of Patients for Trials of Acute Stroke Therapy.” *The Lancet Neurology* 8 (3): 261–69.

Doolittle, W. Ford. 2013. “Is Junk DNA Bunk? A Critique of ENCODE.” *Proceedings of the National Academy of Sciences* 110 (14): 5294–5300.

Dostie, Josée, Zissimos Mourelatos, Michael Yang, Anup Sharma, and Gideon Dreyfuss. 2003. “Numerous microRNPs in Neuronal Cells Containing Novel microRNAs.” *Rna* 9 (2): 180–86.

Douglas, Joanne T., Buck E. Rogers, Maryland E. Rosenfeld, Sharon I. Michael, Meizhen Feng, and David T. Curiel. 1996. “Targeted Gene Delivery by Tropism-Modified Adenoviral Vectors.” *Nature Biotechnology* 14 (11): 1574–78.

Ehrenreich, Hannelore, Anne Kästner, Karin Weissenborn, Jackson Streeter, Swetlana Sperling, Kevin K. Wang, Hans Worthmann, Ronald L. Hayes, Nico von Ahsen, and Andreas Kastrup. 2011. “Circulating Damage Marker Profiles Support a Neuroprotective Effect of Erythropoietin in Ischemic Stroke Patients.” *Molecular Medicine* 17 (11-12): 1306.

Ehrenreich, Hannelore, Karin Weissenborn, Hilmar Prange, Dietmar Schneider, Christian Weimar, Katja Wartenberg, Peter D. Schellinger, Matthias Bohn, Harald Becker, and Martin

Wegrzyn. 2009. “Recombinant Human Erythropoietin in the Treatment of Acute Ischemic Stroke.” *Stroke* 40 (12): e647–56.
El-Hefnawy, Talal, Siva Raja, Lori Kelly, William L. Bigbee, John M. Kirkwood, James D. Luketich, and Tony E. Godfrey. 2004. “Characterization of Amplifiable, Circulating RNA in Plasma and Its Potential as a Tool for Cancer Diagnostics.” *Clinical Chemistry* 50 (3): 564–73.
Engerson, Todd D., T. G. McKelvey, Darryl B. Rhyne, Elizabeth B. Boggio, Stephanie J. Snyder, and Harold P. Jones. 1987. “Conversion of Xanthine Dehydrogenase to Oxidase in Ischemic Rat Tissues.” *Journal of Clinical Investigation* 79 (6): 1564.
Everett, R. S., B. L. Hodges, E. Y. Ding, F. Xu, D. Serra, and A. Amalfitano. 2003. “Liver Toxicities Typically Induced by First-Generation Adenoviral Vectors Can Be Reduced by Use of E1, E2b-Deleted Adenoviral Vectors.” *Human Gene Therapy* 14 (18): 1715–26.
Exiqon. 2011. “miRCURY LNA microRNA ISH Optimization Kit Manual.” http://www.exiqon.com/ls/documents/scientific/mircury-lna-microrna-ish-optimization-kit-manual.pdf.
Fasanaro, Pasquale, Yuri D’Alessandra, Valeria Di Stefano, Roberta Melchionna, Sveva Romani, Giulio Pompilio, Maurizio C. Capogrossi, and Fabio Martelli. 2008. “MicroRNA-210 Modulates Endothelial Cell Response to Hypoxia and Inhibits the Receptor Tyrosine Kinase Ligand Ephrin-A3.” *Journal of Biological Chemistry* 283 (23): 15878–83.
Fisher, C. M. 1962. “Concerning Recurrent Transient Cerebral Ischemic Attacks.” *Canadian Medical Association Journal* 86 (24): 1091.
———. 1969. “The Arterial Lesions Underlying Lacunes.” *Acta Neuropathologica* 12 (1): 1–15.
Fisher, C. M., E. H. Picard, A. Polak, P. Dalal, and R. G. Ojemann. 1965. “Acute Hypertensive Cerebellar Hemorrhage: Diagnosis and Surgical Treatment.” *The Journal of Nervous and Mental Disease* 140 (1): 38–57.
Fisher, Marc, and Birgul Bastan. 2012. “Identifying and Utilizing the Ischemic Penumbra.” *Neurology* 79 (13 Supplement 1): S79–85.
Fisher, Marc, Giora Feuerstein, David W. Howells, Patricia D. Hurn, Thomas A. Kent, Sean I. Savitz, and Eng H. Lo. 2009. “Update of the Stroke Therapy Academic Industry Roundtable Preclinical Recommendations.” *Stroke* 40 (6): 2244–50.
Fisher, Marc, Daniel F. Hanley, George Howard, Edward C. Jauch, and Steven Warach. 2007. “Recommendations from the STAIR V Meeting on Acute Stroke Trials, Technology and Outcomes.” *Stroke* 38 (2): 245–48.
Follert, Philipp, Harold Cremer, and Christophe Béclin. 2014. “MicroRNAs in Brain Development and Function: A Matter of Flexibility and Stability.” *Frontiers in Molecular Neuroscience* 7.
Fransen, Puck SS, Debbie Beumer, Olvert A. Berkhemer, Lucie A. van den Berg, Hester Lingsma, Aad van der Lugt, Wim H. van Zwam, Robert J. van Oostenbrugge, Yvo BWEM Roos, and Charles B. Majoie. 2014. “MR CLEAN, a Multicenter Randomized Clinical Trial of Endovascular Treatment for Acute Ischemic Stroke in the Netherlands: Study Protocol for a Randomized Controlled Trial.” *Trials* 15 (1): 343.
Freemon, Frank R. 1971. “Akinetic Mutism and Bilateral Anterior Cerebral Artery Occlusion.” *Journal of Neurology, Neurosurgery & Psychiatry* 34 (6): 693–98.
Fujita, Shuji, Taiji Ito, Taketoshi Mizutani, Shigeru Minoguchi, Nobutake Yamamichi, Kouhei Sakurai, and Hideo Iba. 2008. “< I> miR-21</i> Gene Expression Triggered by AP-1 Is Sustained through a Double-Negative Feedback Mechanism.” *Journal of Molecular Biology* 378 (3): 492–504.
Fulda, S., and K. M. Debatin. 2006. “Extrinsic versus Intrinsic Apoptosis Pathways in Anticancer Chemotherapy.” *Oncogene* 25 (34): 4798–4811.
Gao, Shan, Y. J. Wang, A. D. Xu, Y. S. Li, and D. Z. Wang. 2011. “Chinese Ischemic Stroke Subclassification.” *Frontiers in Neurology* 2.
Garcia, Julio H., Simone Wagner, Kai-Feng Liu, and Xiao-jiang Hu. 1995. “Neurological Deficit and Extent of Neuronal Necrosis Attributable to Middle Cerebral Artery Occlusion in Rats Statistical Validation.” *Stroke* 26 (4): 627–35.

Garzon, Ramiro, Catherine EA Heaphy, Violaine Havelange, Muller Fabbri, Stefano Volinia, Twee Tsao, Nicola Zanesi, Steven M. Kornblau, Guido Marcucci, and George A. Calin. 2009. "MicroRNA 29b Functions in Acute Myeloid Leukemia." *Blood* 114 (26): 5331–41.

Gebert, Luca FR, Mario AE Rebhan, Silvia EM Crivelli, Rémy Denzler, Markus Stoffel, and Jonathan Hall. 2014. "Miravirsen (SPC3649) Can Inhibit the Biogenesis of miR-122." *Nucleic Acids Research* 42 (1): 609–21.

Gilad, Shlomit, Eti Meiri, Yariv Yogev, Sima Benjamin, Danit Lebanony, Noga Yerushalmi, Hila Benjamin, Michal Kushnir, Hila Cholakh, and Nir Melamed. 2008. "Serum microRNAs Are Promising Novel Biomarkers." *PloS One* 3 (9): e3148.

Ginsberg, Myron D. 2008. "Neuroprotection for Ischemic Stroke: Past, Present and Future." *Neuropharmacology* 55 (3): 363–89.

Giraldez, Antonio J., Ryan M. Cinalli, Margaret E. Glasner, Anton J. Enright, J. Michael Thomson, Scott Baskerville, Scott M. Hammond, David P. Bartel, and Alexander F. Schier. 2005. "MicroRNAs Regulate Brain Morphogenesis in Zebrafish." *Science* 308 (5723): 833–38.

Gluck, J. P., T. DiPasquale, and F. B. Orlans. 2002. *Applied Ethics in Animal Research: Philosophy, Regulation, and Laboratory Applications*. Purdue University Press. https://books.google.co.uk/books?id=ZJXrkrX2uIAC.

Go, Alan S., Dariush Mozaffarian, Véronique L. Roger, Emelia J. Benjamin, Jarett D. Berry, Michael J. Blaha, Shifan Dai, Earl S. Ford, Caroline S. Fox, and Sheila Franco. 2014. "Executive Summary: Heart Disease and Stroke Statistics—2014 Update A Report From the American Heart Association." *Circulation* 129 (3): 399–410.

Goldberg, Mark P., and Dennis W. Choi. 1993. "Combined Oxygen and Glucose Deprivation in Cortical Cell Culture: Calcium-Dependent and Calcium-Independent Mechanisms of Neuronal Injury." *The Journal of Neuroscience* 13 (8): 3510–24.

Granger, D. NEIL, Gianfranco Rutili, and J. M. McCord. 1981. "Superoxide Radicals in Feline Intestinal Ischemia." *Gastroenterology* 81 (1): 22–29.

Grasso, Margherita, Paola Piscopo, Annamaria Confaloni, and Michela A. Denti. 2014. "Circulating miRNAs as Biomarkers for Neurodegenerative Disorders." *Molecules* 19 (5): 6891–6910.

Greer, D. M. 2008. *Acute Ischemic Stroke: An Evidence-Based Approach*. Wiley. http://books.google.co.uk/books?id=DQdgzBoOsYUC.

Gregory, Richard I., Kai-ping Yan, Govindasamy Amuthan, Thimmaiah Chendrimada, Behzad Doratotaj, Neil Cooch, and Ramin Shiekhattar. 2004. "The Microprocessor Complex Mediates the Genesis of microRNAs." *Nature* 432 (7014): 235–40.

Grishok, Alla, Amy E. Pasquinelli, Darryl Conte, Na Li, Susan Parrish, Ilho Ha, David L. Baillie, Andrew Fire, Gary Ruvkun, and Craig C. Mello. 2001. "Genes and Mechanisms Related to RNA Interference Regulate Expression of the Small Temporal RNAs That Control< I> C. Elegans</i> Developmental Timing." *Cell* 106 (1): 23–34.

Guduric-Fuchs, Jasenka, Anna O'Connor, Angela Cullen, Laura Harwood, Reinhold J. Medina, Christina L. O'Neill, Alan W. Stitt, Tim M. Curtis, and David A. Simpson. 2012. "Deep Sequencing Reveals Predominant Expression of miR-21 amongst the Small Non-coding RNAs in Retinal Microvascular Endothelial Cells." *Journal of Cellular Biochemistry* 113 (6): 2098–2111.

Guo, Dong, Junni Liu, Weihua Wang, Fang Hao, Xuedong Sun, Xiao Wu, Peili Bu, Yun Zhang, Yu Liu, and Fengqing Liu. 2013. "Alteration in Abundance and Compartmentalization of Inflammation-Related miRNAs in Plasma after Intracerebral Hemorrhage." *Stroke* 44 (6): 1739–42.

Haas, Sebastian, Norbert Weidner, and Jürgen Winkler. 2005. "Adult Stem Cell Therapy in Stroke." *Current Opinion in Neurology* 18 (1): 59–64.

Hacke, Werner, Greg Albers, Yasir Al-Rawi, Julien Bogousslavsky, Antonio Davalos, Michael Eliasziw, Michael Fischer, Anthony Furlan, Markku Kaste, and Kennedy R. Lees. 2005. "The Desmoteplase in Acute Ischemic Stroke Trial (DIAS) a Phase II MRI-Based 9-Hour Window Acute Stroke Thrombolysis Trial with Intravenous Desmoteplase." *Stroke* 36 (1): 66–73.

Hacke, Werner, Anthony J. Furlan, Yasir Al-Rawi, Antoni Davalos, Jochen B. Fiebach, Franz Gruber, Markku Kaste, Leslie J. Lipka, Salvador Pedraza, and Peter A. Ringleb. 2009. "Intravenous

Desmoteplase in Patients with Acute Ischaemic Stroke Selected by MRI Perfusion–diffusion Weighted Imaging or Perfusion CT (DIAS-2): A Prospective, Randomised, Double-Blind, Placebo-Controlled Study." *The Lancet Neurology* 8 (2): 141–50.

Hacke, Werner, Markku Kaste, Erich Bluhmki, Miroslav Brozman, Antoni Dávalos, Donata Guidetti, Vincent Larrue, Kennedy R. Lees, Zakaria Medeghri, and Thomas Machnig. 2008. "Thrombolysis with Alteplase 3 to 4.5 Hours after Acute Ischemic Stroke." *New England Journal of Medicine* 359 (13): 1317–29.

Hacke, Werner, Markku Kaste, Cesare Fieschi, Danilo Toni, Emmanuel Lesaffre, Rüdiger von Kummer, Gudrun Boysen, Erich Bluhmki, Godehard Höxter, and Marie-Helene Mahagne. 1995. "Intravenous Thrombolysis with Recombinant Tissue Plasminogen Activator for Acute Hemispheric Stroke: The European Cooperative Acute Stroke Study (ECASS)." *Jama* 274 (13): 1017–25.

Hacke, W., M. Kaste, E. Bluhmki, M. Brozman, A. Dávalos, D. Guidetti, V. Larrue, K. R Lees, Z. Medeghri, and T. Machnig. 2008. "Thrombolysis with Alteplase 3 to 4.5 Hours after Acute Ischemic Stroke." *New England Journal of Medicine* 359 (13): 1317–29.

Hall, Elin, Petr Volkov, Tasnim Dayeh, Jonathan Lou S. Esguerra, Sofia Salö, Lena Eliasson, Tina Rönn, Karl Bacos, and Charlotte Ling. 2014. "Sex Differences in the Genome-Wide DNA Methylation Pattern and Impact on Gene Expression, microRNA Levels and Insulin Secretion in Human Pancreatic Islets." *Genome Biology* 15 (12): 522.

Han, Mingyue, Jessica Toli, and Maha Abdellatif. 2011. "MicroRNAs in the Cardiovascular System." *Current Opinion in Cardiology* 26 (3): 181–89.

Harari, Olivier A., and James K. Liao. 2010. "NF-κB and Innate Immunity in Ischemic Stroke." *Annals of the New York Academy of Sciences* 1207 (1): 32–40.

Hashimoto, Terumasa, Keita Shibata, Koji Nobe, Keiji Hasumi, and Kazuo Honda. 2010a. "A Novel Embolic Model of Cerebral Infarction and Evaluation of Stachybotrys Microspora Triprenyl Phenol-7 (SMTP-7), a Novel Fungal Triprenyl Phenol Metabolite." *Journal of Pharmacological Sciences* 114 (1): 41.

———. 2010b. "A Novel Embolic Model of Cerebral Infarction and Evaluation of Stachybotrys Microspora Triprenyl Phenol-7 (SMTP-7), a Novel Fungal Triprenyl Phenol Metabolite." *Journal of Pharmacological Sciences* 114 (1): 41.

Hayashi, K., R. Morishita, H. Nakagami, S. Yoshimura, A. Hara, K. Matsumoto, T. Nakamura, T. Ogihara, Y. Kaneda, and N. Sakai. 2001. "Gene Therapy for Preventing Neuronal Death Using Hepatocyte Growth Factor: In Vivo Gene Transfer of HGF to Subarachnoid Space Prevents Delayed Neuronal Death in Gerbil Hippocampal CA1 Neurons." *Gene Therapy* 8 (15): 1167–73.

He, Fucheng, Pin Lv, Xue Zhao, Xi Wang, Xuehan Ma, Weiwei Meng, Xianchun Meng, and Shuling Dong. 2014. "Predictive Value of Circulating miR-328 and miR-134 for Acute Myocardial Infarction." *Molecular and Cellular Biochemistry*, 1–8.

"Hemorrhagic Strokes (Bleeds)." 2014. Accessed May 28. http://www.strokeassociation.org/STROKEORG/AboutStroke/TypesofStroke/HemorrhagicBleeds/Hemorrhagic-Strokes-Bleeds_UCM_310940_Article.jsp.

He, Yong, Cheng Huang, Xiang Lin, and Jun Li. 2013. "MicroRNA-29 Family, a Crucial Therapeutic Target for Fibrosis Diseases." *Biochimie* 95 (7): 1355–59.

Hillen, Thomas, Ruth Dundas, Enas Lawrence, Judith A. Stewart, Anthony G. Rudd, and Charles DA Wolfe. 2000. "Antithrombotic and Antihypertensive Management 3 Months After Ischemic Stroke A Prospective Study in an Inner City Population." *Stroke* 31 (2): 469–75.

Hill, Michael D., Renee H. Martin, David Mikulis, John H. Wong, Frank L. Silver, Karel G. terBrugge, Geneviève Milot, Wayne M. Clark, R. Loch MacDonald, and Michael E. Kelly. 2012. "Safety and Efficacy of NA-1 in Patients with Iatrogenic Stroke after Endovascular Aneurysm Repair (ENACT): A Phase 2, Randomised, Double-Blind, Placebo-Controlled Trial." *The Lancet Neurology* 11 (11): 942–50.

Hitoshi, Seiji, Tania Alexson, Vincent Tropepe, Dorit Donoviel, Andrew J. Elia, Jeffrey S. Nye, Ronald A. Conlon, Tak W. Mak, Alan Bernstein, and Derek van der Kooy. 2002. "Notch

Pathway Molecules Are Essential for the Maintenance, but Not the Generation, of Mammalian Neural Stem Cells." *Genes & Development* 16 (7): 846–58.
Hossmann, Konstantin-Alexander. 2012. "The Two Pathophysiologies of Focal Brain Ischemia: Implications for Translational Stroke Research." *Journal of Cerebral Blood Flow & Metabolism* 32 (7): 1310–16.
Hsu, An, Shu-Jen Chen, Yu-Sun Chang, Hua-Chien Chen, and Pao-Hsien Chu. 2014. "Systemic Approach to Identify Serum microRNAs as Potential Biomarkers for Acute Myocardial Infarction." *BioMed Research International* 2014.
Hunter, A. Jackie, A. Richard Green, and Alan J. Cross. 1995. "Animal Models of Acute Ischaemic Stroke: Can They Predict Clinically Successful Neuroprotective Drugs?" *Trends in Pharmacological Sciences* 16 (4): 123–28.
Huntzinger, Eric, and Elisa Izaurralde. 2011. "Gene Silencing by microRNAs: Contributions of Translational Repression and mRNA Decay." *Nature Reviews Genetics* 12 (2): 99–110.
Hutvágner, György, Juanita McLachlan, Amy E. Pasquinelli, Éva Bálint, Thomas Tuschl, and Phillip D. Zamore. 2001. "A Cellular Function for the RNA-Interference Enzyme Dicer in the Maturation of the Let-7 Small Temporal RNA." *Science* 293 (5531): 834–38.
Ikonomidou, Chrysanthy, Friederike Bosch, Michael Miksa, Petra Bittigau, Jessica Vöckler, Krikor Dikranian, Tanya I. Tenkova, Vanya Stefovska, Lechoslaw Turski, and John W. Olney. 1999. "Blockade of NMDA Receptors and Apoptotic Neurodegeneration in the Developing Brain." *Science* 283 (5398): 70–74.
Ikonomidou, Chrysanthy, and Lechoslaw Turski. 2002. "Why Did NMDA Receptor Antagonists Fail Clinical Trials for Stroke and Traumatic Brain Injury?" *The Lancet Neurology* 1 (6): 383–86.
Iorio, Marilena V., Manuela Ferracin, Chang-Gong Liu, Angelo Veronese, Riccardo Spizzo, Silvia Sabbioni, Eros Magri, Massimo Pedriali, Muller Fabbri, and Manuela Campiglio. 2005. "MicroRNA Gene Expression Deregulation in Human Breast Cancer." *Cancer Research* 65 (16): 7065–70.
"Ischemic Strokes (Clots)." 2014. Accessed May 28. http://www.strokeassociation.org/STROKEORG/AboutStroke/TypesofStroke/IschemicClots/Ischemic-Strokes-Clots_UCM_310939_Article.jsp.
Ivey, Kathryn N., Alecia Muth, Joshua Arnold, Frank W. King, Ru-Fang Yeh, Jason E. Fish, Edward C. Hsiao, Robert J. Schwartz, Bruce R. Conklin, and Harold S. Bernstein. 2008. "MicroRNA Regulation of Cell Lineages in Mouse and Human Embryonic Stem Cells." *Cell Stem Cell* 2 (3): 219–29.
Jagannath, Aarti, and Matthew JA Wood. 2009. "Localization of Double-Stranded Small Interfering RNA to Cytoplasmic Processing Bodies Is Ago2 Dependent and Results in up-Regulation of GW182 and Argonaute-2." *Molecular Biology of the Cell* 20 (1): 521–29.
Jamaluddin, Md Saha, Sarah M. Weakley, Lidong Zhang, Panagiotis Kougias, Peter H. Lin, Qizhi Yao, and Changyi Chen. 2011. "miRNAs: Roles and Clinical Applications in Vascular Disease."
Janeway, Charles A., Paul Travers, M. J. Walport, and Mark J. Shlomchik. 2001. *Immunobiology: The Immune System in Health and Disease*. Vol. 2. Churchill Livingstone.
Janssen, Harry LA, Hendrik W. Reesink, Eric J. Lawitz, Stefan Zeuzem, Maribel Rodriguez-Torres, Keyur Patel, Adriaan J. van der Meer, Amy K. Patick, Alice Chen, and Yi Zhou. 2013. "Treatment of HCV Infection by Targeting microRNA." *New England Journal of Medicine* 368 (18): 1685–94.
Janssen, H. L., Sakari Kauppinen, and Michael R. Hodges. 2013. "HCV Infection and Miravirsen." *The New England Journal of Medicine* 369 (9): 878–878.
Jarry, J., D. Schadendorf, C. Greenwood, A. Spatz, and L. C. van Kempen. 2014. "The Validity of Circulating microRNAs in Oncology: Five Years of Challenges and Contradictions." *Molecular Oncology*.
Jeyaseelan, K., K. Y. Lim, and A. Armugam. 2008a. "MicroRNA Expression in the Blood and Brain of Rats Subjected to Transient Focal Ischemia by Middle Cerebral Artery Occlusion." *Stroke* 39 (3): 959–66.
———. 2008b. "MicroRNA Expression in the Blood and Brain of Rats Subjected to Transient Focal Ischemia by Middle Cerebral Artery Occlusion." *Stroke* 39 (3): 959–66.

Kahles, Timo, and Ralf P. Brandes. 2012. “NADPH Oxidases as Therapeutic Targets in Ischemic Stroke.” *Cellular and Molecular Life Sciences* 69 (14): 2345–63.

Kahles, Timo, Andreas Kohnen, Sabine Heumueller, Angelika Rappert, Ingo Bechmann, Stefan Liebner, Ina M. Wittko, Tobias Neumann-Haefelin, Helmuth Steinmetz, and Katrin Schroeder. 2010. “NADPH Oxidase Nox1 Contributes to Ischemic Injury in Experimental Stroke in Mice.” *Neurobiology of Disease* 40 (1): 185–92.

Kalladka, Dheeraj, and Keith W. Muir. 2011. “Stem Cell Therapy in Stroke: Designing Clinical Trials.” *Neurochemistry International* 59 (3): 367–70.

Kanaan, Ziad, Henry Roberts, M. Robert Eichenberger, Adrian Billeter, Gairy Ocheretner, Jianmin Pan, Shesh N. Rai, Jeffery Jorden, Anna Williford, and Susan Galandiuk. 2013. “A Plasma microRNA Panel for Detection of Colorectal Adenomas: A Step toward More Precise Screening for Colorectal Cancer.” *Annals of Surgery* 258 (3): 400–408.

Kannel, William B., Thomas R. Dawber, Abraham Kagan, Nicholas Revotskie, and Joseph Stokes. 1961. “Factors of Risk in the Development of Coronary Heart Disease—Six-Year Follow-up ExperienceThe Framingham Study.” *Annals of Internal Medicine* 55 (1): 33–50.

Kay, Mark A., Joseph C. Glorioso, and Luigi Naldini. 2001. “Viral Vectors for Gene Therapy: The Art of Turning Infectious Agents into Vehicles of Therapeutics.” *Nature Medicine* 7 (1): 33–40.

Khanna, Savita, Cameron Rink, Reza Ghoorkhanian, Surya Gnyawali, Mallory Heigel, Dayanjan S. Wijesinghe, Charles E. Chalfant, Yuk Cheung Chan, Jaideep Banerjee, and Yue Huang. 2013. “Loss of miR-29b Following Acute Ischemic Stroke Contributes to Neural Cell Death and Infarct Size.” *Journal of Cerebral Blood Flow & Metabolism*.

Kirino, Takaaki. 2000. “Delayed Neuronal Death.” *Neuropathology* 20 (s1): 95–97.

Kirschenbaum, Barry, Fiona Doetsch, Carlos Lois, and Arturo Alvarez-Buylla. 1999. “Adult Subventricular Zone Neuronal Precursors Continue to Proliferate and Migrate in the Absence of the Olfactory Bulb.” *The Journal of Neuroscience* 19 (6): 2171–80.

Kirschner, Michaela B., J. James B. Edelman, Steven CH Kao, Michael P. Vallely, Nico Van Zandwijk, and Glen Reid. 2013. “The Impact of Hemolysis on Cell-Free microRNA Biomarkers.” *Frontiers in Genetics* 4.

Kirschner, Michaela B., Steven C. Kao, J. James Edelman, Nicola J. Armstrong, Michael P. Vallely, Nico van Zandwijk, and Glen Reid. 2011. “Haemolysis during Sample Preparation Alters microRNA Content of Plasma.” *PLoS One* 6 (9): e24145.

Kleinschnitz, Christoph, Henrike Grund, Kirstin Wingler, Melanie E. Armitage, Emma Jones, Manish Mittal, David Barit, Tobias Schwarz, Christian Geis, and Peter Kraft. 2010. “Post-Stroke Inhibition of Induced NADPH Oxidase Type 4 Prevents Oxidative Stress and Neurodegeneration.” *PLoS Biology* 8 (9): e1000479.

Klonjkowski, Bernard, Pascale Gilardi-Hebenstreit, Juliette Hadchouel, Voahangy Randrianarison, Sylvie Boutin, Patrice Yeh, Michel Perricaudet, and Eric J. Kremer. 1997. “A Recombinant E1-Deleted Canine Adenoviral Vector Capable of Transduction and Expression of a Transgene in Human-Derived Cells and in Vivo.” *Human Gene Therapy* 8 (17): 2103–15.

Kochanek, Patrick M., and John M. Hallenbeck. 1992. “Polymorphonuclear Leukocytes and Monocytes/macrophages in the Pathogenesis of Cerebral Ischemia and Stroke.” *Stroke* 23 (9): 1367–79.

Koizumi, Naoya, Tomoko Yamaguchi, Kenji Kawabata, Fuminori Sakurai, Tomomi Sasaki, Yoshiteru Watanabe, Takao Hayakawa, and Hiroyuki Mizuguchi. 2007. “Fiber-Modified Adenovirus Vectors Decrease Liver Toxicity through Reduced IL-6 Production.” *The Journal of Immunology* 178 (3): 1767–73.

Korcok, Jasminka, Lin N. Raimundo, Hua Z. Ke, Stephen M. Sims, and S. Jeffrey Dixon. 2004. “Extracellular Nucleotides Act Through P2X7 Receptors to Activate NF-κB in Osteoclasts.” *Journal of Bone and Mineral Research* 19 (4): 642–51.

Krichevsky, Anna M., and Galina Gabriely. 2009. “miR-21: A Small Multi-faceted RNA.” *Journal of Cellular and Molecular Medicine* 13 (1): 39–53.

Kriegel, Alison J., Yong Liu, Yi Fang, Xiaoqiang Ding, and Mingyu Liang. 2012. “The miR-29 Family: Genomics, Cell Biology, and Relevance to Renal and Cardiovascular Injury.” *Physiological Genomics* 44 (4): 237–44.

Krol, Jacek, Inga Loedige, and Witold Filipowicz. 2010. “The Widespread Regulation of microRNA Biogenesis, Function and Decay.” *Nature Reviews Genetics* 11 (9): 597–610.

Krupinski, Jerzy, Pat Kumar, Shant Kumar, and Jozef Kaluza. 1996. “Increased Expression of TGF-β1 in Brain Tissue after Ischemic Stroke in Humans.” *Stroke* 27 (5): 852–57.

Kuehbacher, Angelika, Carmen Urbich, Andreas M. Zeiher, and Stefanie Dimmeler. 2007. “Role of Dicer and Drosha for Endothelial microRNA Expression and Angiogenesis.” *Circulation Research* 101 (1): 59–68.

Kulshreshtha, Ritu, Manuela Ferracin, Sylwia E. Wojcik, Ramiro Garzon, Hansjuerg Alder, Francisco J. Agosto-Perez, Ramana Davuluri, Chang-Gong Liu, Carlo M. Croce, and Massimo Negrini. 2007. “A microRNA Signature of Hypoxia.” *Molecular and Cellular Biology* 27 (5): 1859–67.

Kwiecinski, M., A. Noetel, N. Elfimova, J. Trebicka, S. Schievenbusch, I. Strack, L. Molnar, M. von Brandenstein, U. Töx, and R. Nischt. 2011. “Hepatocyte Growth Factor (HGF) Inhibits Collagen I and IV Synthesis in Hepatic Stellate Cells by miRNA-29 Induction.” *PloS One* 6 (9): e24568.

Lagos-Quintana, Mariana, Reinhard Rauhut, Winfried Lendeckel, and Thomas Tuschl. 2001. “Identification of Novel Genes Coding for Small Expressed RNAs.” *Science* 294 (5543): 853–58.

Lagos-Quintana, Mariana, Reinhard Rauhut, Abdullah Yalcin, Jutta Meyer, Winfried Lendeckel, and Thomas Tuschl. 2002. “Identification of Tissue-Specific microRNAs from Mouse.” *Current Biology* 12 (9): 735–39.

Lai, Chi-Yu, Sung-Liang Yu, Ming H. Hsieh, Chun-Houh Chen, Hsuan-Yu Chen, Chun-Chiang Wen, Yung-Hsiang Huang, Po-Chang Hsiao, Chuhsing Kate Hsiao, and Chih-Min Liu. 2011. “MicroRNA Expression Aberration as Potential Peripheral Blood Biomarkers for Schizophrenia.” *PloS One* 6 (6): e21635.

Lai, Karen, Brian K. Kaspar, Fred H. Gage, and David V. Schaffer. 2003. “Sonic Hedgehog Regulates Adult Neural Progenitor Proliferation in Vitro and in Vivo.” *Nature Neuroscience* 6 (1): 21–27.

Lai, Ted Weita, Shu Zhang, and Yu Tian Wang. 2013. “Excitotoxicity and Stroke: Identifying Novel Targets for Neuroprotection.” *Progress in Neurobiology*.

Lander, Eric S., Lauren M. Linton, Bruce Birren, Chad Nusbaum, Michael C. Zody, Jennifer Baldwin, Keri Devon, Ken Dewar, Michael Doyle, and William FitzHugh. 2001. “Initial Sequencing and Analysis of the Human Genome.” *Nature* 409 (6822): 860–921.

Lanfranconi, Silvia, and Hugh S. Markus. 2013. “Stroke Subtyping for Genetic Association Studies? A Comparison of the CCS and TOAST Classifications.” *International Journal of Stroke* 8 (8): 626–31.

Laurent, J. P., G. F. Molinari, and J. I. Moseley. 1975. “Clinicopathological Validation of a Primate Stroke Model.” *Surgical Neurology* 4 (5): 449–55.

Lawrie, Charles H., Shira Gal, Heather M. Dunlop, Beena Pushkaran, Amanda P. Liggins, Karen Pulford, Alison H. Banham, Francesco Pezzella, Jacqueline Boultwood, and James S. Wainscoat. 2008. “Detection of Elevated Levels of Tumour-associated microRNAs in Serum of Patients with Diffuse Large B-cell Lymphoma.” *British Journal of Haematology* 141 (5): 672–75.

Lee, Rosalind C., Rhonda L. Feinbaum, and Victor Ambros. 1993. “The C. Elegans Heterochronic Gene< I> Lin-4</i> Encodes Small RNAs with Antisense Complementarity To< I> Lin-14</i>.” *Cell* 75 (5): 843–54.

Lees, Kennedy R. 1997. “Cerestat and Other NMDA Antagonists in Ischemic Stroke.” *Neurology* 49 (5 Suppl 4): S66–69.

Lee, Soon-Tae, Kon Chu, Keun-Hwa Jung, Hye-Jin Yoon, Daejong Jeon, Kyoung-Mook Kang, Ki-Ho Park, Eun-Kee Bae, Manho Kim, and Sang Kun Lee. 2010. “MicroRNAs Induced during Ischemic Preconditioning.” *Stroke* 41 (8): 1646–51.

Lee, Yi, Samir EL Andaloussi, and Matthew JA Wood. 2012. “Exosomes and Microvesicles: Extracellular Vesicles for Genetic Information Transfer and Gene Therapy.” *Human Molecular Genetics* 21 (R1): R125–34.

Lee, Yoontae, Chiyoung Ahn, Jinju Han, Hyounjeong Choi, Jaekwang Kim, Jeongbin Yim, Junho Lee, Patrick Provost, Olof Rådmark, and Sunyoung Kim. 2003. “The Nuclear RNase III Drosha Initiates microRNA Processing.” *Nature* 425 (6956): 415–19.

Lee, Yoontae, Kipyoung Jeon, Jun-Tae Lee, Sunyoung Kim, and V. Narry Kim. 2002. “MicroRNA Maturation: Stepwise Processing and Subcellular Localization.” *The EMBO Journal* 21 (17): 4663–70.

Lennox, K. A., and M. A. Behlke. 2011. “Chemical Modification and Design of Anti-miRNA Oligonucleotides.” *Gene Therapy* 18 (12): 1111–20.

Lindley, Richard I., Joanna M. Wardlaw, and Peter AG Sandercock. 2005. “Alteplase and Ischaemic Stroke: Have New Reviews of Old Data Helped?” *The Lancet Neurology* 4 (4): 249–53.

Li, Qiaoling, Jun Xie, Ruotian Li, Jian Shi, Jiayin Sun, Rong Gu, Liang Ding, Lian Wang, and Biao Xu. 2014. “Overexpression of microRNA-99a Attenuates Heart Remodelling and Improves Cardiac Performance after Myocardial Infarction.” *Journal of Cellular and Molecular Medicine* 18 (5): 919–28.

Li, Sung-Chou, Yu-Lun Liao, Meng-Ru Ho, Kuo-Wang Tsai, Chun-Hung Lai, and Wen-chang Lin. 2012. “miRNA Arm Selection and isomiR Distribution in Gastric Cancer.” *BMC Genomics* 13 (Suppl 1): S13.

Liu, Amon Y., Joseph A. Maldjian, Linda J. Bagley, Grant P. Sinson, and Robert I. Grossman. 1999. “Traumatic Brain Injury: Diffusion-Weighted MR Imaging Findings.” *American Journal of Neuroradiology* 20 (9): 1636–41.

Liu, Shimin, Honglian Shi, Wenlan Liu, Takamitsu Furuichi, Graham S. Timmins, and Ke Jian Liu. 2004. “Interstitial pO2 in Ischemic Penumbra and Core Are Differentially Affected Following Transient Focal Cerebral Ischemia in Rats.” *Journal of Cerebral Blood Flow & Metabolism* 24 (3): 343–49.

Li, Yang, Fenfen Wang, Junfen Xu, Feng Ye, Yuanming Shen, Jiansong Zhou, Weiguo Lu, Xiaoyun Wan, Ding Ma, and Xing Xie. 2011. “Progressive miRNA Expression Profiles in Cervical Carcinogenesis and Identification of HPV-related Target Genes for miR-29.” *The Journal of Pathology* 224 (4): 484–95.

Li, Yanmei, Laura M. Vecchiarelli-Federico, You-Jun Li, Sean E. Egan, David Spaner, Margaret R. Hough, and Yaacov Ben-David. 2012. “The miR-17-92 Cluster Expands Multipotent Hematopoietic Progenitors Whereas Imbalanced Expression of Its Individual Oncogenic miRNAs Promotes Leukemia in Mice.” *Blood* 119 (19): 4486–98.

Lo, Eng H., Turgay Dalkara, and Michael A. Moskowitz. 2003. “Mechanisms, Challenges and Opportunities in Stroke.” *Nature Reviews Neuroscience* 4 (5): 399–414.

Longa, E. Zea, Philip R. Weinstein, Sara Carlson, and Robert Cummins. 1989. “Reversible Middle Cerebral Artery Occlusion without Craniectomy in Rats.” *Stroke* 20 (1): 84–91.

Long, Guangwen, Feng Wang, Huaping Li, Zhongwei Yin, Chaugai Sandip, Yan Lou, Yan Wang, Chen Chen, and Dao Wen Wang. 2013. “Circulating miR-30a, miR-126 and Let-7b as Biomarker for Ischemic Stroke in Humans.” *BMC Neurology* 13 (1): 178.

Lu, David P., Rebecca L. Read, David T. Humphreys, Fiona M. Battah, David IK Martin, and John EJ Rasko. 2005. “PCR-Based Expression Analysis and Identification of microRNAs.” *Journal of RNAi and Gene Silencing: An International Journal of RNA and Gene Targeting Research* 1 (1): 44.

Lyons, Anthony, Eric J. Downer, Suzanne Crotty, Yvonne M. Nolan, Kingston HG Mills, and Marina A. Lynch. 2007. “CD200 Ligand–receptor Interaction Modulates Microglial Activation in Vivo and in Vitro: A Role for IL-4.” *The Journal of Neuroscience* 27 (31): 8309–13.

Machi, Paolo, Vincent Costalat, Kyriakos Lobotesis, Igor Lima Maldonado, Jean Francois Vendrell, Carlos Riquelme, and Alain Bonafé. 2012. “Solitaire FR Thrombectomy System: Immediate Results in 56 Consecutive Acute Ischemic Stroke Patients.” *Journal of Neurointerventional Surgery* 4 (1): 62–66.

Macrae, I. M. 2011. “Preclinical Stroke Research–advantages and Disadvantages of the Most Common Rodent Models of Focal Ischaemia.” *British Journal of Pharmacology* 164 (4): 1062–78.

Maegdefessel, Lars, Junya Azuma, and Philip S. Tsao. 2014. “MicroRNA-29b Regulation of Abdominal Aortic Aneurysm Development.” *Trends in Cardiovascular Medicine* 24 (1): 1–6.
Majid, Arshad. 2014. “Neuroprotection in Stroke: Past, Present, and Future.” *ISRN Neurology* 2014.
Ma, Liuqing, Liangming Wei, Fei Wu, Zhenhua Hu, Zhenguo Liu, and Weien Yuan. 2013. “Advances with microRNAs in Parkinson’s Disease Research.” *Drug Design, Development and Therapy* 7: 1103.
Marcus, Michelle E., and Joshua N. Leonard. 2013. “FedExosomes: Engineering Therapeutic Biological Nanoparticles That Truly Deliver.” *Pharmaceuticals* 6 (5): 659–80.
Marsh, Brenda J., Rebecca L. Williams-Karnesky, and Mary P. Stenzel-Poore. 2009. “Toll-like Receptor Signaling in Endogenous Neuroprotection and Stroke.” *Neuroscience* 158 (3): 1007–20.
Martins, Madalena, Alexandra Rosa, Leonor C. Guedes, Benedita V. Fonseca, Kristina Gotovac, Sara Violante, Tiago Mestre, Miguel Coelho, Mário M. Rosa, and Eden R. Martin. 2011. “Convergence of miRNA Expression Profiling, A-Synuclein Interacton and GWAS in Parkinson’s Disease.” *PloS One* 6 (10): e25443.
Matranga, Christian, Yukihide Tomari, Chanseok Shin, David P. Bartel, and Phillip D. Zamore. 2005. “Passenger-Strand Cleavage Facilitates Assembly of siRNA into Ago2-Containing RNAi Enzyme Complexes.” *Cell* 123 (4): 607–20.
Mattson, M. P., P. Dou, and S. B. Kater. 1988. “Outgrowth-Regulating Actions of Glutamate in Isolated Hippocampal Pyramidal Neurons.” *The Journal of Neuroscience* 8 (6): 2087–2100.
McDonald, Robert A., Katie M. White, Junxi Wu, Brian C. Cooley, Keith E. Robertson, Crawford A. Halliday, John D. McClure, Sheila Francis, Ruifaug Lu, and Simon Kennedy. 2013. “miRNA-21 Is Dysregulated in Response to Vein Grafting in Multiple Models and Genetic Ablation in Mice Attenuates Neointima Formation.” *European Heart Journal*, eht105.
Mehra, Manik, Nils Henninger, Joshua A. Hirsch, Juyu Chueh, Ajay K. Wakhloo, and Matthew J. Gounis. 2012. “Preclinical Acute Ischemic Stroke Modeling.” *Journal of Neurointerventional Surgery* 4 (4): 307–13.
Melani, Alessia, Daria Turchi, Maria Giuliana Vannucchi, Sara Cipriani, Marco Gianfriddo, and Felicita Pedata. 2005. “ATP Extracellular Concentrations Are Increased in the Rat Striatum during in Vivo Ischemia.” *Neurochemistry International* 47 (6): 442–48.
Mendell, Joshua T. 2008. “miRiad Roles for the miR-17-92 Cluster in Development and Disease.” *Cell* 133 (2): 217–22.
Mergenthaler, Philipp, Ulrich Dirnagl, and Andreas Meisel. 2004. “Pathophysiology of Stroke: Lessons from Animal Models.” *Metabolic Brain Disease* 19 (3-4): 151–67.
Metz, Gerlinde A., and Ian Q. Whishaw. 2009a. “The Ladder Rung Walking Task: A Scoring System and Its Practical Application.” *Journal of Visualized Experiments: JoVE*, no. 28.
———. 2009b. “The Ladder Rung Walking Task: A Scoring System and Its Practical Application.” *Journal of Visualized Experiments: JoVE*, no. 28.
Meyer, F., and M. Finer. 2001. “Gene Therapy: Progress and Challenges.” *Cellular and Molecular Biology* 47 (8): 1277–94.
Ming, Guo-li, and Hongjun Song. 2011. “Adult Neurogenesis in the Mammalian Brain: Significant Answers and Significant Questions.” *Neuron* 70 (4): 687–702.
Mishmar, Dan, Ayelet Rahat, Stephen W. Scherer, Gerald Nyakatura, Bernd Hinzmann, Yoshinori Kohwi, Yael Mandel-Gutfroind, Jeffrey R. Lee, Bernd Drescher, and Dean E. Sas. 1998. “Molecular Characterization of a Common Fragile Site (FRA7H) on Human Chromosome 7 by the Cloning of a Simian Virus 40 Integration Site.” *Proceedings of the National Academy of Sciences* 95 (14): 8141–46.
Mohr, J. P., Louis R. Caplan, John W. Melski, Robert J. Goldstein, Gary W. Duncan, J. P. Kistler, Michael S. Pessin, and Howard L. Bleich. 1978. “The Harvard Cooperative Stroke Registry a Prospective Registry.” *Neurology* 28 (8): 754–754.
Mojsilovic-Petrovic, Jelena, Debbie Callaghan, Hong Cui, Clare Dean, Danica B. Stanimirovic, and Wandong Zhang. 2007. “Hypoxia-Inducible Factor-1 (HIF-1) Is Involved in the Regulation of Hypoxia-Stimulated Expression of Monocyte Chemoattractant Protein-1 (MCP-1/CCL2) and MCP-5 (Ccl12) in Astrocytes.” *J Neuroinflammation* 4: 12.

Morris, K. V. 2012. *Non-Coding RNAs and Epigenetic Regulation of Gene Expression: Drivers of Natural Selection*. Caister Academic Press. https://books.google.co.uk/books?id=HvWuosBg2tcC.

Moseley, John I., John P. Laurent, and Gaetano F. Molinari. 1975. "Barbiturate Attenuation of the Clinical Course and Pathologic Lesions in a Primate Stroke Model." *Neurology* 25 (9): 870–870.

Mowa, Mohube Betty, Carol Crowther, and Patrick Arbuthnot. 2010. "Therapeutic Potential of Adenoviral Vectors for Delivery of Expressed RNAi Activators." *Expert Opinion on Drug Delivery* 7 (12): 1373–85.

Murlidharan, Giridhar, Richard J. Samulski, and Aravind Asokan. 2014. "Biology of Adeno-Associated Viral Vectors in the Central Nervous System." *Frontiers in Molecular Neuroscience* 7.

Nana-Sinkam, S. P., and C. M. Croce. 2012. "Clinical Applications for microRNAs in Cancer." *Clinical Pharmacology & Therapeutics* 93 (1): 98–104.

Nitatori, T., N. Sato, S. Waguri, Y. Karasawa, H. Araki, K. Shibanai, E. Kominami, and Y. Uchiyama. 1995. "Delayed Neuronal Death in the CA1 Pyramidal Cell Layer of the Gerbil Hippocampus Following Transient Ischemia Is Apoptosis." *The Journal of Neuroscience* 15 (2): 1001–11.

Noetel, Andrea, Monika Kwiecinski, Natalia Elfimova, Jia Huang, and Margarete Odenthal. 2012. "microRNA Are Central Players in Anti-and Profibrotic Gene Regulation during Liver Fibrosis." *Frontiers in Physiology* 3.

Nour, May, Fabien Scalzo, and David S. Liebeskind. 2013. "Ischemia-Reperfusion Injury in Stroke." *Interventional Neurology* 1 (3-4): 185.

O'Donnell, Kathryn A., Erik A. Wentzel, Karen I. Zeller, Chi V. Dang, and Joshua T. Mendell. 2005. "C-Myc-Regulated microRNAs Modulate E2F1 Expression." *Nature* 435 (7043): 839–43.

Okamura, Katsutomo, Joshua W. Hagen, Hong Duan, David M. Tyler, and Eric C. Lai. 2007. "The Mirtron Pathway Generates microRNA-Class Regulatory RNAs In< I> Drosophila</i>." *Cell* 130 (1): 89–100.

Oki, Koichi, Jemal Tatarishvili, James Wood, Philipp Koch, Somsak Wattananit, Yutaka Mine, Emanuela Monni, Daniel Tornero, Henrik Ahlenius, and Julia Ladewig. 2012. "Human-Induced Pluripotent Stem Cells Form Functional Neurons and Improve Recovery After Grafting in Stroke-Damaged Brain." *Stem Cells* 30 (6): 1120–33.

Okita, Keisuke, Tomoko Ichisaka, and Shinya Yamanaka. 2007. "Generation of Germline-Competent Induced Pluripotent Stem Cells." *Nature* 448 (7151): 313–17.

Olive, Virginie, Iris Jiang, and Lin He. 2010. "Mir-17-92, a Cluster of miRNAs in the Midst of the Cancer Network." *The International Journal of Biochemistry & Cell Biology* 42 (8): 1348–54.

Olney, John W. 1969. "Brain Lesions, Obesity, and Other Disturbances in Mice Treated with Monosodium Glutamate." *Science* 164 (3880): 719–21.

Ord, Emily NJ, Rachel Shirley, John D. McClure, Christopher McCabe, Eric J. Kremer, I. Mhairi Macrae, and Lorraine M. Work. 2013. "Combined Antiapoptotic and Antioxidant Approach to Acute Neuroprotection for Stroke in Hypertensive Rats." *Journal of Cerebral Blood Flow & Metabolism* 33 (8): 1215–24.

Ord, Emily NJ, Rachel Shirley, Josie C. van Kralingen, Alice Graves, John D. McClure, Michael Wilkinson, Christopher McCabe, I. Mhairi Macrae, and Lorraine M. Work. 2012. "Positive Impact of Pre-Stroke Surgery on Survival Following Transient Focal Ischemia in Hypertensive Rats." *Journal of Neuroscience Methods*.

Ouyang, Yi-Bing, Yu Lu, Sibiao Yue, and Rona G. Giffard. 2012. "miR-181 Targets Multiple Bcl-2 Family Members and Influences Apoptosis and Mitochondrial Function in Astrocytes." *Mitochondrion* 12 (2): 213–19.

Ouyang, Yi-Bing, Yu Lu, Sibiao Yue, Li-Jun Xu, Xiao-Xing Xiong, Robin E. White, Xiaoyun Sun, and Rona G. Giffard. 2012. "miR-181 Regulates GRP78 and Influences Outcome from Cerebral Ischemia< I> in Vitro</i> And< I> in Vivo</i>." *Neurobiology of Disease* 45 (1): 555–63.

Papagiannakopoulos, Thales, Alice Shapiro, and Kenneth S. Kosik. 2008. “MicroRNA-21 Targets a Network of Key Tumor-Suppressive Pathways in Glioblastoma Cells.” *Cancer Research* 68 (19): 8164–72.

Parks, D. A., and D. N. Granger. 1986. “Xanthine Oxidase: Biochemistry, Distribution and Physiology.” *Acta Physiologica Scandinavica. Supplementum* 548: 87.

Parmet, Sharon, Tiffany J. Glass, and Richard M. Glass. 2004. “Hemorrhagic Stroke.” *JAMA: The Journal of the American Medical Association* 292 (15): 1916–1916.

Patrick, David M., Rusty L. Montgomery, Xiaoxia Qi, Susanna Obad, Sakari Kauppinen, Joseph A. Hill, Eva van Rooij, and Eric N. Olson. 2010. “Stress-Dependent Cardiac Remodeling Occurs in the Absence of microRNA-21 in Mice.” *The Journal of Clinical Investigation* 120 (120 (11)): 3912–16.

Peerschke, Ellinor I., Wei Yin, and Berhane Ghebrehiwet. 2010. “Complement Activation on Platelets: Implications for Vascular Inflammation and Thrombosis.” *Molecular Immunology* 47 (13): 2170–75.

Peltékian, Elise, Luis Garcia, and Olivier Danos. 2002. “Neurotropism and Retrograde Axonal Transport of a Canine Adenoviral Vector: A Tool for Targeting Key Structures Undergoing Neurodegenerative Processes.” *Molecular Therapy* 5 (1): 25–32.

Peng, Liu, Qiu Chun-guang, Bei-fang Li, Ding Xue-zhi, Wang Zi-hao, Li Yun-fu, Dang Yan-ping, Liu Yang-gui, Li Wei-guo, and Hu Tian-yong. 2014. “Clinical Impact of Circulating miR-133, miR-1291 and miR-663b in Plasma of Patients with Acute Myocardial Infarction.” *Diagnostic Pathology* 9 (1): 89.

Peters, Oliver, Tobias Back, Ute Lindauer, Christina Busch, Dirk Megow, Jens Dreier, and Ulrich Dirnagl. 1998. “Increased Formation of Reactive Oxygen Species after Permanent and Reversible Middle Cerebral Artery Occlusion in the Rat.” *Journal of Cerebral Blood Flow & Metabolism* 18 (2): 196–205.

Pinsky, David J., Yoshifumi Naka, Hui Liao, Mehmet C. Oz, Denisa D. Wagner, Tanya N. Mayadas, Robert C. Johnson, Richard O. Hynes, Mark Heath, and Charles A. Lawson. 1996. “Hypoxia-Induced Exocytosis of Endothelial Cell Weibel-Palade Bodies. A Mechanism for Rapid Neutrophil Recruitment after Cardiac Preservation.” *Journal of Clinical Investigation* 97 (2): 493.

Poliseno, Laura, Andrea Tuccoli, Laura Mariani, Monica Evangelista, Lorenzo Citti, Keith Woods, Alberto Mercatanti, Scott Hammond, and Giuseppe Rainaldi. 2006. “MicroRNAs Modulate the Angiogenic Properties of HUVECs.” *Blood* 108 (9): 3068–71.

Proctor, Peter H., and Lynsey P. Tamborello. 2007. “SAINT-I Worked, but the Neuroprotectant Is Not NXY-059.” *Stroke* 38 (10): e109–e109.

Purdy, Phillip D., M. D. Devous, H. Hunt Batjer, C. L. White, Yves Meyer, and Duke S. Samson. 1989. “Microfibrillar Collagen Model of Canine Cerebral Infarction.” *Stroke* 20 (10): 1361–67.

Qing, S., S. Yuan, C. Yun, H. Hui, P. Mao, F. Wen, Y. Ding, and Q. Liu. 2014. “Serum MiRNA Biomarkers Serve as a Fingerprint for Proliferative Diabetic Retinopathy.” *Cellular Physiology and Biochemistry* 34 (5): 1733–40.

Qin, Jizheng, Hongwei Liang, Dongquan Shi, Jin Dai, Zhihong Xu, Dongyang Chen, Xi Chen, and Qing Jiang. 2014. “A Panel of microRNAs as a New Biomarkers for the Detection of Deep Vein Thrombosis.” *Journal of Thrombosis and Thrombolysis*, 1–7.

Radak, Djordje, Ivana Resanovic, and Esma R. Isenovic. 2013. “Link between Oxidative Stress and Acute Brain Ischemia.” *Angiology*, 0003319713506516.

Raitoharju, Emma, Leo-Pekka Lyytikäinen, Mari Levula, Niku Oksala, Ari Mennander, Matti Tarkka, Norman Klopp, Thomas Illig, Mika Kähönen, and Pekka J. Karhunen. 2011. “miR-21, miR-210, miR-34a, and miR-146a/b Are up-Regulated in Human Atherosclerotic Plaques in the Tampere Vascular Study.” *Atherosclerosis* 219 (1): 211–17.

Ramdas, Vasudev, Martin McBride, Laura Denby, and Andrew H. Baker. 2013. “Canonical Transforming Growth Factor-B Signaling Regulates Disintegrin Metalloprotease Expression in Experimental Renal Fibrosis via miR-29.” *The American Journal of Pathology* 183 (6): 1885–96.

Rao, Pooja, Eva Benito, and André Fischer. 2013. “MicroRNAs as Biomarkers for CNS Disease.” *Frontiers in Molecular Neuroscience* 6.
Raposo, Graça, and Willem Stoorvogel. 2013. “Extracellular Vesicles: Exosomes, Microvesicles, and Friends.” *The Journal of Cell Biology* 200 (4): 373–83.
Raynaud, F., and A. Marcilhac. 2006. “Implication of Calpain in Neuronal Apoptosis.” *Febs Journal* 273 (15): 3437–43.
Rayner, Katey J., and Elizabeth J. Hennessy. 2013. “Extracellular Communication via microRNA: Lipid Particles Have a New Message.” *Journal of Lipid Research* 54 (5): 1174–81.
Redell, John B., Jing Zhao, and Pramod K. Dash. 2011. “Altered Expression of miRNA-21 and Its Targets in the Hippocampus after Traumatic Brain Injury.” *Journal of Neuroscience Research* 89 (2): 212–21.
Reynolds, I. J., and T. G. Hastings. 1995. “Glutamate Induces the Production of Reactive Oxygen Species in Cultured Forebrain Neurons Following NMDA Receptor Activation.” *The Journal of Neuroscience* 15 (5): 3318–27.
Rha, Joung-Ho, and Jeffrey L. Saver. 2007. “The Impact of Recanalization on Ischemic Stroke Outcome a Meta-Analysis.” *Stroke* 38 (3): 967–73.
Rice, Margaret E. 2011. “H2O2: A Dynamic Neuromodulator.” *The Neuroscientist*, 1073858411404531.
Rogers, Susan D., Linda B. Riemersma, and Stephen D. Clements. 1987. “Tissue Plasminogen Activator: An Evaluation of Clinical Efficacy in Acute Myocardial Infarction.” *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy* 7 (4): 111–20.
Rosenberg, G. A., E. Y. Estrada, and J. E. Dencoff. 1998. “Matrix Metalloproteinases and TIMPs Are Associated with Blood-Brain Barrier Opening after Reperfusion in Rat Brain.” *Stroke* 29 (10): 2189–95.
Russell, William Moy Stratton, Rex Leonard Burch, and Charles Westley Hume. 1959. “The Principles of Humane Experimental Technique.”
Sanderson, Thomas H., Christian A. Reynolds, Rita Kumar, Karin Przyklenk, and Maik Hüttemann. 2013. “Molecular Mechanisms of Ischemia–reperfusion Injury in Brain: Pivotal Role of the Mitochondrial Membrane Potential in Reactive Oxygen Species Generation.” *Molecular Neurobiology* 47 (1): 9–23.
Sasaki, Yukio, Christina Gross, Lei Xing, Yoshio Goshima, and Gary J. Bassell. 2013. “Identification of Axon-enriched MicroRNAs Localized to Growth Cones of Cortical Neurons.” *Developmental Neurobiology*.
Saver, Jeffrey L. 2007. “Clinical Impact of NXY-059 Demonstrated in the SAINT I Trial: Derivation of Number Needed to Treat for Benefit Over Entire Range of Functional Disability.” *Stroke* 38 (5): 1515–18.
Saver, Jeffrey L., Gregory W. Albers, Billy Dunn, Karen C. Johnston, and Marc Fisher. 2009a. “Stroke Therapy Academic Industry Roundtable (STAIR) Recommendations for Extended Window Acute Stroke Therapy Trials.” *Stroke* 40 (7): 2594–2600.
———. 2009b. “Stroke Therapy Academic Industry Roundtable (STAIR) Recommendations for Extended Window Acute Stroke Therapy Trials.” *Stroke* 40 (7): 2594–2600.
Savitz, Sean I., and Wolf-Rüdiger Schäbitz. 2008. “A Critique of SAINT II Wishful Thinking, Dashed Hopes, and the Future of Neuroprotection for Acute Stroke.” *Stroke* 39 (4): 1389–91.
Sayed, Danish, Minzhen He, Chull Hong, Shumin Gao, Shweta Rane, Zhi Yang, and Maha Abdellatif. 2010. “MicroRNA-21 Is a Downstream Effector of AKT That Mediates Its Antiapoptotic Effects via Suppression of Fas Ligand.” *Journal of Biological Chemistry* 285 (26): 20281–90.
Sayed, Danish, Chull Hong, Ieng-Yi Chen, Jacqueline Lypowy, and Maha Abdellatif. 2007. “MicroRNAs Play an Essential Role in the Development of Cardiac Hypertrophy.” *Circulation Research* 100 (3): 416–24.
Schäfer, Matthias, and Sabine Werner. 2008. “Cancer as an Overhealing Wound: An Old Hypothesis Revisited.” *Nature Reviews Molecular Cell Biology* 9 (8): 628–38.
Schiller, Francis. 1970. “Concepts of Stroke before and after Virchow.” *Medical History* 14 (2): 115.

Schipper, Hyman M., Olivier C. Maes, Howard M. Chertkow, and Eugenia Wang. 2007. “MicroRNA Expression in Alzheimer Blood Mononuclear Cells.” *Gene Regulation and Systems Biology* 1: 263.

Schneider, B., S. Nagel, M. Kaufmann, S. Winkelmann, J. Bode, H. G. Drexler, and R. A. F. MacLeod. 2007. “T (3; 7)(q27; q32) Fuses BCL6 to a Non-Coding Region at FRA7H near miR-29.” *Leukemia* 22 (6): 1262–66.

Shabalina, Svetlana A., and Eugene V. Koonin. 2008. “Origins and Evolution of Eukaryotic RNA Interference.” *Trends in Ecology & Evolution* 23 (10): 578–87.

Shayakhmetov, Dmitry M., Anuj Gaggar, Shaoheng Ni, Zong-Yi Li, and André Lieber. 2005. “Adenovirus Binding to Blood Factors Results in Liver Cell Infection and Hepatotoxicity.” *Journal of Virology* 79 (12): 7478–91.

Shi, G., Y. Liu, T. Liu, W. Yan, X. Liu, Y. Wang, J. Shi, and L. Jia. 2012. “Upregulated miR-29b Promotes Neuronal Cell Death by Inhibiting Bcl2L2 after Ischemic Brain Injury.” *Experimental Brain Research* 216 (2): 225–30.

Shingo, Tetsuro, S. Todd Sorokan, Takuya Shimazaki, and Samuel Weiss. 2001. “Erythropoietin Regulates the in Vitro and in Vivo Production of Neuronal Progenitors by Mammalian Forebrain Neural Stem Cells.” *The Journal of Neuroscience* 21 (24): 9733–43.

Shirley, Rachel, Emily NJ Ord, and Lorraine M. Work. 2014. “Oxidative Stress and the Use of Antioxidants in Stroke.” *Antioxidants* 3 (3): 472–501.

Silver, Jerry, and Jared H. Miller. 2004. “Regeneration beyond the Glial Scar.” *Nature Reviews Neuroscience* 5 (2): 146–56.

Simon, Jessica E., David L. Sandler, JH Warwick Pexman, Michael D. Hill, and Alastair M. Buchan. 2004. “Is Intravenous Recombinant Tissue Plasminogen Activator (rt-PA) Safe for Use in Patients over 80 Years Old with Acute Ischaemic stroke?–The Calgary Experience.” *Age and Ageing* 33 (2): 143–49.

Simons, Mikael, and Graça Raposo. 2009. “Exosomes–vesicular Carriers for Intercellular Communication.” *Current Opinion in Cell Biology* 21 (4): 575–81.

Siu, Kwong Lok, Dong-Geun Lee, Jae Ho Shim, Dae Chul Suh, and Deok Hee Lee. 2014. “Mechanical Thrombectomy Using the Solitaire FR System for Occlusion of the Top of the Basilar Artery: Intentional Detachment of the Device after Partial Retrieval.” *Neurointervention* 9 (1): 26–31.

Small, Daniel L., and Alastair M. Buchan. 2000. “Animal Models.” *British Medical Bulletin* 56 (2): 307–17.

Smith, Helen K., and Felicity NE Gavins. 2012. “The Potential of Stem Cell Therapy for Stroke: Is PISCES the Sign?” *The FASEB Journal* 26 (6): 2239–52.

Soundarapandian, Mangala M., Wei Hong Tu, Peter L. Peng, Antonis S. Zervos, and YouMing Lu. 2005. “AMPA Receptor Subunit GluR2 Gates Injurious Signals in Ischemic Stroke.” *Molecular Neurobiology* 32 (2): 145–55.

Stanika, Ruslan I., Idalis Villanueva, Galina Kazanina, S. Brian Andrews, and Natalia B. Pivovarova. 2012. “Comparative Impact of Voltage-Gated Calcium Channels and NMDA Receptors on Mitochondria-Mediated Neuronal Injury.” *The Journal of Neuroscience* 32 (19): 6642–50.

Stolberg, Sheryl Gay. 1999. “The Biotech Death of Jesse Gelsinger.” *New York Times Magazine* 28: 136–40.

Stroemer, Paul, Andrew Hope, Sara Patel, Kenny Pollock, and John Sinden. 2007. “Development of a Human Neural Stem Cell Line for Use in Recovery from Disability after Stroke.” *Frontiers in Bioscience: A Journal and Virtual Library* 13: 2290–92.

Stroke Association. 2015. “State of the Nation: Stroke Statistics.”

Suárez, Yajaira, Carlos Fernández-Hernando, Jordan S. Pober, and William C. Sessa. 2007. “Dicer Dependent microRNAs Regulate Gene Expression and Functions in Human Endothelial Cells.” *Circulation Research* 100 (8): 1164–73.

Sun, GuoQiang, Peng Ye, Kiyohito Murai, Ming-Fei Lang, Shengxiu Li, Heying Zhang, Wendong Li, et al. 2011. “miR-137 Forms a Regulatory Loop with Nuclear Receptor TLX and LSD1 in Neural Stem Cells.” *Nat Commun* 2 (November): 529. doi:10.1038/ncomms1532.

Sun, Hong-shuo. 2013. “Restoring Neuroprotection through a New Preclinical Paradigm: Translational Success for NA-1 in Stroke Therapy.” *Acta Pharmacologica Sinica* 34 (1): 3–5.
Sun, Tao, Xiangyu Kong, Yiqi Du, and Zhaoshen Li. 2014. “Aberrant MicroRNAs in Pancreatic Cancer: Researches and Clinical Implications.” *Gastroenterology Research and Practice* 2014.
Suzuki, Shigeaki, Kortaro Tanaka, Shigeru Nogawa, Tomohisa Dembo, Arifumi Kosakai, and Yasuo Fukuuchi. 2001. “Phosphorylation of Signal Transducer and Activator of Transcription-3 (Stat3) after Focal Cerebral Ischemia in Rats.” *Experimental Neurology* 170 (1): 63–71.
Symonds, Charles. 1955. “The Circle of Willis.” *British Medical Journal* 1 (4906): 119.
Tamura, A_, D. I. Graham, J. McCulloch, and G. M. Teasdale. 1981. “Focal Cerebral Ischaemia in the Rat: 1. Description of Technique and Early Neuropathological Consequences Following Middle Cerebral Artery Occlusion.” *Journal of Cerebral Blood Flow & Metabolism* 1 (1): 53–60.
Tan, Jun Rong, Kay Sin Tan, Yu Xuan Koo, Fung Lin Yong, Chee Woon Wang, Arunmozhiarasi Armugam, and Kandiah Jeyaseelan. 2013. “Blood microRNAs in Low or No Risk Ischemic Stroke Patients.” *International Journal of Molecular Sciences* 14 (1): 2072–84.
Tan, K. S., A. Armugam, S. Sepramaniam, K. Y. Lim, K. D. Setyowati, C. W. Wang, and K. Jeyaseelan. 2009. “Expression Profile of MicroRNAs in Young Stroke Patients.” *PLoS One* 4 (11): e7689.
Tatsuguchi, Mariko, Hee Young Seok, Thomas E. Callis, J. Michael Thomson, Jian-Fu Chen, Martin Newman, Mauricio Rojas, Scott M. Hammond, and Da-Zhi Wang. 2007. “Expression of microRNAs Is Dynamically Regulated during Cardiomyocyte Hypertrophy.” *Journal of Molecular and Cellular Cardiology* 42 (6): 1137–41.
Temple, Sally. 2001. “The Development of Neural Stem Cells.” *Nature* 414 (6859): 112–17.
“The Facts Behind ‘1 in 6’ - World Stroke Campaign.” 2015. Accessed February 12. http://www.worldstrokecampaign.org/learn/the-facts-behind-1-in-6.html.
Thum, Thomas. 2012. “MicroRNA Therapeutics in Cardiovascular Medicine.” *EMBO Molecular Medicine* 4 (1): 3–14.
Thum, Thomas, Carina Gross, Jan Fiedler, Thomas Fischer, Stephan Kissler, Markus Bussen, Paolo Galuppo, Steffen Just, Wolfgang Rottbauer, and Stefan Frantz. 2008. “MicroRNA-21 Contributes to Myocardial Disease by Stimulating MAP Kinase Signalling in Fibroblasts.” *Nature* 456 (7224): 980–84.
“Tissue Plasminogen Activator for Acute Ischemic Stroke.” 1995. *New England Journal of Medicine* 333 (24): 1581–88.
Tranmer, Bruce I., Ted S. Keller, Glenn W. Kindt, and David Archer. 1992. “Loss of Cerebral Regulation during Cardiac Output Variations in Focal Cerebral Ischemia.” *Journal of Neurosurgery* 77 (2): 253–59.
Trialists’Collaboration, Stroke Unit. 1997. “How Do Stroke Units Improve Patient Outcomes? A Collaborative Systematic Review of the Randomized Trials.” *Stroke* 28 (11): 2139–44.
———. 2001. “Organised Inpatient (stroke Unit) Care for Stroke.” *Cochrane Database of Systematic Reviews* 3.
Tsai, P.-C., Y.-C. Liao, Y.-S. Wang, H.-F. Lin, R.-T. Lin, and S.-HH Juo. 2013. “Serum microRNA-21 and microRNA-221 as Potential Biomarkers for Cerebrovascular Disease.” *Journal of Vascular Research* 50 (4): 346–54.
Tsivgoulis, Georgios, Aristeidis H. Katsanos, and Andrei V. Alexandrov. 2014. “Reperfusion Therapies of Acute Ischemic Stroke: Potentials and Failures.” *Frontiers in Neurology* 5.
Urbich, Carmen, Angelika Kuehbacher, and Stefanie Dimmeler. 2008. “Role of microRNAs in Vascular Diseases, Inflammation, and Angiogenesis.” *Cardiovascular Research* 79 (4): 581–88.
Vaca, Luis. 2014. “Point-of-Care Diagnostic Tools to Detect Circulating MicroRNAS as Biomarkers of Disease.” *Sensors* 14 (5): 9117–31.
Valadi, Hadi, Karin Ekström, Apostolos Bossios, Margareta Sjöstrand, James J. Lee, and Jan O. Lötvall. 2007. “Exosome-Mediated Transfer of mRNAs and microRNAs Is a Novel Mechanism of Genetic Exchange between Cells.” *Nature Cell Biology* 9 (6): 654–59.

Vallet, P., Yves Charnay, K. Steger, E. Ogier-Denis, E. Kovari, François Herrmann, J.-P. Michel, and I. Szanto. 2005. “Neuronal Expression of the NADPH Oxidase NOX4, and Its Regulation in Mouse Experimental Brain Ischemia.” *Neuroscience* 132 (2): 233–38.

Van Huyen, Jean-Paul Duong, Marion Tible, Arnaud Gay, Romain Guillemain, Olivier Aubert, Shaida Varnous, Franck Iserin, Philippe Rouvier, Arnaud François, and Dewi Vernerey. 2014. “MicroRNAs as Non-Invasive Biomarkers of Heart Transplant Rejection.” *European Heart Journal*, ehu346.

Van Niel, Guillaume, Isabel Porto-Carreiro, Sabrina Simoes, and Graça Raposo. 2006. “Exosomes: A Common Pathway for a Specialized Function.” *Journal of Biochemistry* 140 (1): 13–21.

Van Rooij, Eva, and Sakari Kauppinen. 2014. “Development of microRNA Therapeutics Is Coming of Age.” *EMBO Molecular Medicine*.

Van Rooij, Eva, Lillian B. Sutherland, Ning Liu, Andrew H. Williams, John McAnally, Robert D. Gerard, James A. Richardson, and Eric N. Olson. 2006. “A Signature Pattern of Stress-Responsive microRNAs That Can Evoke Cardiac Hypertrophy and Heart Failure.” *Proceedings of the National Academy of Sciences* 103 (48): 18255–60.

Van Rooij, Eva, Lillian B. Sutherland, Jeffrey E. Thatcher, J. Michael DiMaio, R. Haris Naseem, William S. Marshall, Joseph A. Hill, and Eric N. Olson. 2008. “Dysregulation of microRNAs after Myocardial Infarction Reveals a Role of miR-29 in Cardiac Fibrosis.” *Proceedings of the National Academy of Sciences* 105 (35): 13027–32.

Venter, J. Craig, Mark D. Adams, Eugene W. Myers, Peter W. Li, Richard J. Mural, Granger G. Sutton, Hamilton O. Smith, Mark Yandell, Cheryl A. Evans, and Robert A. Holt. 2001. “The Sequence of the Human Genome.” *Science* 291 (5507): 1304–51.

Vergeade, Aurélia, Paul Mulder, Cathy Vendeville-Dehaudt, François Estour, Dominique Fortin, Renée Ventura-Clapier, Christian Thuillez, and Christelle Monteil. 2010. “Mitochondrial Impairment Contributes to Cocaine-Induced Cardiac Dysfunction: Prevention by the Targeted Antioxidant MitoQ.” *Free Radical Biology and Medicine* 49 (5): 748–56.

Visvanathan, Jaya, Seunghee Lee, Bora Lee, Jae W. Lee, and Soo-Kyung Lee. 2007. “The microRNA miR-124 Antagonizes the Anti-Neural REST/SCP1 Pathway during Embryonic CNS Development.” *Genes & Development* 21 (7): 744–49.

Vornov, James J., Robert C. Tasker, and Joseph T. Coyle. 1994. “Delayed Protection by MK-801 and Tetrodotoxin in a Rat Organotypic Hippocampal Culture Model of Ischemia.” *Stroke* 25 (2): 457–64.

Wahlgren, Nils, Niaz Ahmed, Antoni Dávalos, Werner Hacke, Mónica Millán, Keith Muir, Risto O. Roine, Danilo Toni, and Kennedy R. Lees. 2008. “Thrombolysis with Alteplase 3–4· 5 H after Acute Ischaemic Stroke (SITS-ISTR): An Observational Study.” *The Lancet* 372 (9646): 1303–9.

Wang, Jiaji, Zhuqing Jia, Chenguang Zhang, Min Sun, Weiping Wang, Ping Chen, Kangtao Ma, Youyi Zhang, Xianhui Li, and Chunyan Zhou. 2014. “miR-499 Protects Cardiomyocytes from H2O2-Induced Apoptosis via Its Effects on Pdcd4 and Pacs2.” *RNA Biology* 11 (4): 0–1.

Wang, Mian, Wen Li, Guang-Qi Chang, Cai-Sheng Ye, Jing-Song Ou, Xiao-Xi Li, Yong Liu, Tuck-Yun Cheang, Xue-Ling Huang, and Shen-Ming Wang. 2011. “MicroRNA-21 Regulates Vascular Smooth Muscle Cell Function via Targeting Tropomyosin 1 in Arteriosclerosis Obliterans of Lower Extremities.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 31 (9): 2044–53.

Wang, Shusheng, and Eric N. Olson. 2009. “AngiomiRs—key Regulators of Angiogenesis.” *Current Opinion in Genetics & Development* 19 (3): 205–11.

Wang, Yang, Xinwei Zhang, Hui Li, Jinpu Yu, and Xiubao Ren. 2013. “The Role of miRNA-29 Family in Cancer.” *European Journal of Cell Biology* 92 (3): 123–28.

Weber, Martina, Meredith B. Baker, Jeffrey P. Moore, and Charles D. Searles. 2010. “MiR-21 Is Induced in Endothelial Cells by Shear Stress and Modulates Apoptosis and eNOS Activity.” *Biochemical and Biophysical Research Communications* 393 (4): 643–48.

“Welcome to Multi-PART.” 2015. Accessed February 12. http://www.dcn.ed.ac.uk/multipart/.

White, Kevin, Yvonne Dempsie, Paola Caruso, Emma Wallace, Robert A. McDonald, Hannah Stevens, Mark E. Hatley, Eva Van Rooij, Nicholas W. Morrell, and Margaret R. MacLean. 2014. “Endothelial Apoptosis in Pulmonary Hypertension Is Controlled by a

microRNA/Programmed Cell Death 4/Caspase-3 Axis." *Hypertension*, HYPERTENSIONAHA. 113.03037.

Widera, Darius, Ilja Mikenberg, Margitta Elvers, Christian Kaltschmidt, and Barbara Kaltschmidt. 2006. "Tumor Necrosis Factor A Triggers Proliferation of Adult Neural Stem Cells via IKK/NF-κB Signaling." *BMC Neuroscience* 7 (1): 1–18.

Wightman, Bruce, Ilho Ha, and Gary Ruvkun. 1993. "Posttranscriptional Regulation of the Heterochronic Gene< I> Lin-14</i> By< I> Lin-4</i> Mediates Temporal Pattern Formation in C. Elegans." *Cell* 75 (5): 855–62.

Wolffe, Alan P., and Marjori A. Matzke. 1999. "Epigenetics: Regulation through Repression." *Science* 286 (5439): 481–86.

Wolf, Philip A., Ralph B. D'Agostino, Albert J. Belanger, and William B. Kannel. 1991. "Probability of Stroke: A Risk Profile from the Framingham Study." *Stroke* 22 (3): 312–18.

Yamato, Mayumi, Toru Egashira, and Hideo Utsumi. 2003. "Application of in Vivo ESR Spectroscopy to Measurement of Cerebrovascular ROS Generation in Stroke." *Free Radical Biology and Medicine* 35 (12): 1619–31.

Yang, Wei J., Derek D. Yang, Songqing Na, George E. Sandusky, Qing Zhang, and Genshi Zhao. 2005. "Dicer Is Required for Embryonic Angiogenesis during Mouse Development." *Journal of Biological Chemistry* 280 (10): 9330–35.

Yao, X.-L., S. Nakagawa, and J.-Q. Gao. 2011. "Current Targeting Strategies for Adenovirus Vectors in Cancer Gene Therapy." *Current Cancer Drug Targets* 11 (7): 810–25.

Yilmaz, Gokhan, and D. Neil Granger. 2010. "Leukocyte Recruitment and Ischemic Brain Injury." *Neuromolecular Medicine* 12 (2): 193–204.

Ying, Weihai, Shan-Kuo Han, Johann W. Miller, and Raymond A. Swanson. 1999. "Acidosis Potentiates Oxidative Neuronal Death by Multiple Mechanisms." *Journal of Neurochemistry* 73 (4): 1549–56.

Yin, Ke-Jie, Zhen Deng, Milton Hamblin, Yi Xiang, Huarong Huang, Jifeng Zhang, Xiaodan Jiang, Yanzhuang Wang, and Y. Eugene Chen. 2010. "Peroxisome Proliferator-Activated Receptor Δ Regulation of miR-15a in Ischemia-Induced Cerebral Vascular Endothelial Injury." *The Journal of Neuroscience* 30 (18): 6398–6408.

Yin, Ke-Jie, Zhen Deng, Huarong Huang, Milton Hamblin, Changqing Xie, Jifeng Zhang, and Y. Eugene Chen. 2010. "miR-497 Regulates Neuronal Death in Mouse Brain after Transient Focal Cerebral Ischemia." *Neurobiology of Disease* 38 (1): 17–26.

Yi, Youngsuk, Sung H. Hahm, and Kwan Hee Lee. 2005. "Retroviral Gene Therapy: Safety Issues and Possible Solutions." *Current Gene Therapy* 5 (1): 25–35.

Yoda, Mayuko, Tomoko Kawamata, Zain Paroo, Xuecheng Ye, Shintaro Iwasaki, Qinghua Liu, and Yukihide Tomari. 2010. "ATP-Dependent Human RISC Assembly Pathways." *Nature Structural & Molecular Biology* 17 (1): 17–23.

Young, D. B., J. G. Devane, and J. Butler. 2013. *In Vitro-In Vivo Correlations*. Springer US. https://books.google.co.uk/books?id=u6jhBwAAQBAJ.

Yu, S. P., C.-H. Yeh, U. Strasser, M. Tian, and D. W. Choi. 1999. "NMDA Receptor-Mediated K+ Efflux and Neuronal Apoptosis." *Science* 284 (5412): 336–39.

Zhao, Chunnian, GuoQiang Sun, Shengxiu Li, and Yanhong Shi. 2009. "A Feedback Regulatory Loop Involving microRNA-9 and Nuclear Receptor TLX in Neural Stem Cell Fate Determination." *Nat Struct Mol Biol* 16 (4): 365–71. doi:10.1038/nsmb.1576.

Zhao, Dong-Sheng, Yan Chen, Hui Jiang, Jing-Ping Lu, Gang Zhang, Jie Geng, Qing Zhang, Jian-Hua Shen, Xin Zhou, and Wei Zhu. 2013. "Serum miR-210 and miR-30a Expressions Tend to Revert to Fetal Levels in Chinese Adult Patients with Chronic Heart Failure." *Cardiovascular Pathology* 22 (6): 444–50.

Zhao, Jian-Jun, Jianhong Lin, Tint Lwin, Hua Yang, Jianping Guo, William Kong, Sophie Dessureault, Lynn C. Moscinski, Dorna Rezania, and William S. Dalton. 2010. "microRNA Expression Profile and Identification of miR-29 as a Prognostic Marker and Pathogenetic Factor by Targeting CDK6 in Mantle Cell Lymphoma." *Blood* 115 (13): 2630–39.

Zhou, Jiansuo, and Jie Zhang. 2014. "Identification of miRNA-21 and miRNA-24 in Plasma as Potential Early Stage Markers of Acute Cerebral Infarction." *Molecular Medicine Reports*.

Zhou, Liang, Lijun Wang, Leina Lu, Peiyong Jiang, Hao Sun, and Huating Wang. 2012. “Inhibition of miR-29 by TGF-Beta-Smad3 Signaling through Dual Mechanisms Promotes Transdifferentiation of Mouse Myoblasts into Myofibroblasts.” *PLoS One* 7 (3): e33766.

Ziu, Mateo, Lauren Fletcher, Shushan Rana, David F. Jimenez, and Murat Digicaylioglu. 2011. “Temporal Differences in microRNA Expression Patterns in Astrocytes and Neurons after Ischemic Injury.” *PloS One* 6 (2): e14724.

Zufferey, Romain, Dea Nagy, Ron J. Mandel, Luigi Naldini, and Didier Trono. 1997. “Multiply Attenuated Lentiviral Vector Achieves Efficient Gene Delivery in Vivo.” *Nature Biotechnology* 15 (9): 871–75.