Molecular Biomarkers and Regulators of Susceptibility to Drug-Induced Kidney Injury

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

By

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Declaration

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented wholly, or in part, for any other degree or qualification.

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This research was undertaken at the Department of Molecular & Clinical Pharmacology, at the Centre for Drug Safety Science, University of Liverpool.

Contents

Abstract

Adverse drug reactions (ADRs) are undesirable effects of any therapeutic compound beyond its desired pharmacological effect. They are not only a serious issue for the sufferer but also bear a large societal cost and are one of the main reasons for the withdrawal of drugs from the market. Nephrotoxicity is the toxic affect of any exogenous compound on the kidney. The kidney is particularly susceptible to adverse effects of drugs due to its adaptations which allow it to carry out its physiological role efficiently. The kidney receives approximately 25 % of the cardiac output so is exposed to any blood borne toxin at high levels. The kidney also concentrates the filtrate as it passes through the nephron which exposes the epithelial cells of the nephron to much greater concentrations of any toxin present. The fact that the cells of the nephron are metabolically active and have active transport mechanisms also contribute towards the susceptibility of the kidney to ADRs. Adverse events which affect the kidney are often initially very subtle but can rapidly progress into more serious events if not detected early. A biomarker is any quantifiable change in an endogenous protein or molecule which can be indicative of a disease process or state. Current gold standard biomarkers of kidney injury include Serum Creatinine and blood urea nitrogen. Both of these are suboptimal biomarkers of kidney injury in terms of sensitivity and specificity so there is a real need for the development of more specific, translational biomarkers of kidney injury. Ideally these next generation biomarkers would also provide information on the location of the injury or the extent of the injury. MicroRNAs (miRNAs) are short ribonucleic acid sequences which are the smallest functional non-coding RNA units in plants and animals. Recent research has implicated miRNAs in many disease states, particularly cancers where many miRNA species have been shown to be aberrantly expressed. MiRNAs have also been shown to have potential as biomarkers of drug induced liver injury (DILI). This thesis focuses on the potential of miRNAs to serve as translational biomarkers of kidney injury.

Techniques used to isolate, purify and quantify miRNA species were validated to determine the suitability of them for routine quantification in any laboratory. The qPCR technique was shown to be highly precise and sensitive for miRNA quantification. Intra-assay and inter-assay variation was low (<12 %), recovery was variable (54 – 89 %). Variable recovery was controlled for by the development of an internal synthetic c.elegans standard. Significant urinary elevations in four kidney enriched miRNAs were observed at all time points (3hr) preceding SCr elevations (9hr IR; 0.2±0.01 mg/dL. 9hr Control; 0.1±0.01 mg/dL) following 10 minute ischemic reperfusion (IR). MiRNA elevations (3hr IR; miR-26b 7.01. 3hr control; 0.38 (ΔΔCt normalised to U6snRNA)) were also comparable to Kim-1 (3hr IR; 443±71pg/mg.UCr. 3hr Control; 96±3171pg/mg.UCr) and histological changes (3hr). No significant elevations in miR-26b, 30b or 30c were observed in the serum of a murine model of paracetamol induced liver injury whereas miR-192 was found to be significantly elevated. NAG (Control 122 mU/ml mg UCr, Cisplatin 313 mU/ml mg UCr) and Kim-1 (Control 1003 pg/mg UCr, Cisplatin 10617pg/mg UCr) were significantly elevated in the urine of a Cisplatin murine model of

kidney injury 72 hours post-administration whereas a total of 18 miRNA species were significantly elevated by greater than twofold 48 hours post-administration. Five miRNAs were found to be significantly elevated and two decreased in the serum and four were elevated in the kidney tissue of Cisplatin administered mice. In the urine of an Adriamycin induced model of kidney injury one miRNA was significantly elevated and three reduced. 28 were significantly elevated in the serum and three were reduced while 14 were significantly elevated in the kidney tissue. MiR-34a was found to be significantly elevated in the kidney tissue of both the Adriamycin and Cisplatin models. Transfection of HEK293 cells with a miR-34a mimic was confirmed through the quantification of the level of Bcl-2 and CDK6 protein (mean 0.56 and 0.83 respectively). Cisplatin (50 μM) caused a significant reduction in cell viability in transfected cells (MTS fold change 0.12) whereas it did not in control cells. Cisplatin (50 μM) caused significantly greater expression of cleaved caspase 3 in miR-34a mimic transfected cells than in control cells.

This work shows that the assays and techniques used to isolate, purify and quantify miRNA species are precise and sensitive. It shows that kidney enriched miRNA species can be quantified in the urine of animals with IR induced kidney injury and are significantly elevated when compared to control animals with at least the same sensitivity as urinary Kim-1 and greater sensitivity than SCr. It also identifies a number of miRNA species which are significantly altered in a Cisplatin and Adriamycin murine model of drug induced kidney injury (DIKI) which may have utility as biomarkers. They may also present locational biomarkers of kidney injury and warrant further investigation. Finally it shows that the modulation of miR-34a can alter susceptibility to toxicity in vitro which suggests that miRNAs may represent therapeutic interventions in the future.

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Publications

Research Articles

Sharkey JW, Antoine DJ, Park BK (2012). Validation of the isolation and quantification of kidney enriched miRNAs for use as biomarkers. *Biomarkers* 17(3): 231-239.

Antoine DJ, **Sharkey JW**, Sabbisetti V, Bonventre JV, Park BK. Novel Urinary Markers of Kidney Injury to Facilitate Early Detection and Treatment of Acute Kidney Injury. *Manuscript in preparation.*

Sharkey JW, Antoine DJ, Rak-Raszewska A, Park BK. MicroRNA profiles in the kidney tissue, serum and urine of in vivo models of DIKI. *Manuscript in preparation.*

Antoine DJ, **Sharkey JW,** Park BK. Identification and Potential Utility of Urinary miRNAs as Non-Invasive, Translational Biomarkers of Drug induced Liver Injury. *Manuscript in preparation.*

Abstracts

Sharkey JW, Antoine DJ, Park BK (2011). Validation of methods involving miRNA isolation, purification and quantification and the potential of miRNAs to be used as biomarkers of nephrotoxicity. *Toxicology*, *Volume 290, Issues 2–3*, *18, Pages 136-137*

Abbreviations

α-GST	Alpha Glutathione-S-transferase
A	Amperes
AAP	Alanine aminopeptidase
ACE	Angiotensin Converting Enzyme
ADH	Anti Diuretic Hormone
ADR	Adverse Drug Reaction
ADQI	Acute Dialysis Quality Initiative
ALT	Alanine Aminotransferase
AKI	Acute Kidney Injury
AKIN	Acute Kidney Injury Network
AP	Alkaline Phoshatase
APAP	Acetaminophen
AUC	Area Under Curve
Bcl-2	B Cell Lymphoma-2
BUN	Blood Urea Nitrogen
bp	Base Pairs
ΔΔCt	Delta Delta Cycle Threshold
%CV	Coefficient of Variation
°C	Degrees Centigrade
CDK4/6	Cyclin Dependent Kinase 4/6
cDNA	Complimentary DNA
CKD	Chronic Kidney Disease
cm^2	Square Centimetre
CO_2	Carbon Dioxide

Ct	Cycle Threshold
Ctr1	Copper Transporter 1
CYP	Cytochrome P450
CVD	Cardiovascular Disease
DIKI	Drug Induced Kidney Injury
DILI	Drug Induced Liver Injury
DISC	Death Induced Silencing Complex
dl	Decilitre
DMEM	Dulbecco Minimal Essential Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide
ECACC	European Collection of Cell Cultures
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EMEA	European Medicines Agency
ESRD	End Stage Renal Disease
FACS	Fluorescence Activated Cell Sorting
FDA	Food and Drug Administration
fM	Femto Molar
FSGS	Focal Segmental Glomerulosclerosis
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
g	Gram
GFR	Glomerular Filtration Rate

GSH	Glutathione
GTP	Guanosine Triphosphate
GGT	γ-glutamyltranspeptidase
H^+	Hydrogen ion
HEK293	Human Embronic Kidney Cells 293
HIV	Human Immunodefficiency Virus
HMGB1	High Mobility Group Box 1
Hr	Hour
IgA	Immunoglobulin A
IL-18	Interleukin 18
IP	Intraperitoneally
IR	Ischemic Reperfusion
JGA	Juxtaglomerular Apparatus
K^+	Potassium ion
KDa	Kilo Dalton
KDOQI	Kidney Disease Outcomes Quality Initiative
Kg	Kilo Gram
KI	Kidney Injury
Kim-1	Kidney Injury Molecule 1
L	Litre
LDH	Lactate Dehydrogenase
M	Molar

MDRD	Modification of Diet in Renal Disease
mg	milligram
MHRA	Medicines and Healthcare products Regulatory Agency
min	Minute
miRNA	Micro RNA
miRNA*	Micro RNA (Complementary Strand)
mRNA	Messenger RNA
ml	millilitre
MMP-9	Metalloproteinase 9
mosm	Milliosmole
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
mU	MilliUnit
NAPQI	N-Acetyl-p-Quinoneimine
NaCl	Sodium Chloride
NAG	N-Acetyl-β-D-Glucosaminidase
NGAL	Neutrophil Gelatinase –associated Lipocalin
NHS	National Health Service
NKF	National Kidney Foundation
nM	Nano Molar
nm	Nanometre
NSAID	Non-Steroidal Anti Inflammatory Drug
P53	(Tumour) Protein 53
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PIWI	P-element Wimpy Testis
PLP	Pyridoxal 5'-phosphate

Pre-miRNA	Precursor MicroRNA
Pri-miRNA	Primary MicroRNA
qPCR	Quantitative Polymerase Chain Reaction
RAAS	Renin-Aldosterone-Angiotensin-System
RBP	Retinol Binding Protein
RISC	RNA Induced Silencing Complex
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPA-1	Renal Papillary Antigen 1
rpm	Revolutions Per Minute
RT	Room Temperature
SCID	Severe Combined Immunodeficiency
SEM	Standard Error of the Mean
snRNA	Small Nuclear RNA
SNP	Single Nucleotide Polymorphism
SiRNA	Silencing RNA
StRNA	Small Temporal RNA
TLDA	Taqman Low Density Array
TNF-α	Tumour Necrosis Factor-α
TNFR1	Tumour Necrosis Factor Receptor 1
UCr	Urinary Creatinine
µg	Micro Gram
µl	Micro Litre

µM	Micro Molar
UK	United Kingdom
USA	United States of America
UTR	Untranslated Region
V	Volts
VEGF	Vascular Epithelial Growth Factor
v/v	Volume/Volume
WHO	World Health Organisation
w/v	Weight per Volume
ZVAD-FMK	(carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone)

Foreword

In chapter two, parts of the text and figures are taken directly from a published article, namely:

Sharkey JW, Antoine DJ, Park BK (2012). Validation of the isolation and quantification of kidney enriched miRNAs for use as biomarkers. *Biomarkers* 17(3): 231-239.

In chapter three, the surgery and urine collection from the ischemic reperfusion mouse model of kidney injury was carried out in the Harvard institute of medicine, Boston, USA in the laboratory of J Bonventre.

In chapter four, the Adriamycin administered mice samples were generated by A Rak-Raszewska at the University of liverpool.

All work presented herein is my own. I am the sole author of the contents of this thesis.

Jack William Sharkey

Chapter 1

General Introduction

Contents

1 - **Adverse Drug Reactions**

An adverse drug reaction (ADR) is any undesirable effect of a drug beyond its desired therapeutic effects during normal therapeutic use. The World Health Organisation (WHO) defines an ADR as "a response to a drug which is noxious, and unintended, and which occurs at doses normally used in man for prophylaxis, diagnosis or therapy of disease, or for the modification of physiological function". An ADR is a serious issue for the sufferer as well as a large financial burden to society (Smyth *et al.*, 2012) and are a leading reason for the withdrawal of drugs from the market (Jaeschke *et al.*, 2002; Lasser *et al.*, 2002). A recent study estimated that the financial cost of ADRs to the NHS was up to £466 million annually in the UK and that ADRs were responsible for the death of approximately 5700 hospital patients in 2002 (Park *et al.*, 1998; Pirmohamed *et al.*, 2004). ADRs are thought to be one of the leading causes of mortality in the western world and were thought to be responsible for approximately five % of hospital admissions in the United Kingdom and can occur in 10 to 20 % of inpatients (Lazarou *et al.*, 1998; Park *et al.*, 2005; Pirmohamed *et al.*, 2004). There is also evidence to suggest that ADRs are responsible for increasing the length of time spent in hospital so as such they increase the financial burden of the patient to society and decrease the patient's quality of life (Pirmohamed *et al.*, 1998). ADRs are also capable of mimicking most disease states resulting in unnecessary clinical investigations and increased time before accurate diagnosis and treatment. Research also shows that up to 80 % of ADRs in hospitals could be preventable with better informed clinicians and more sensitive biomarkers able to predict the onset of ADRs (Pirmohamed *et al.*, 2004).

There are two main types of ADR; those which are related to the pharmacological action of the drug, and so therefore can be predicted, and those which are unrelated to the main

pharmacological action of the drug (idiosyncratic). These are often referred to as type A or type B ADRs respectively. Type A reactions are often reversible as they are a result of the pharmacological action of the drug, they are dose dependent and therefore they can often be rectified by reducing the dose or cessation of the drug in question. The factors aside from dose which can affect susceptibility to type ADRs include variation in drug formulations, pharmacokinetic and dynamic abnormalities and most importantly interactions which other drugs. Certain disease states can also increase susceptibility to type A ADRs such as renal disease which can reduce the clearance of drugs and therefore increase serum concentrations of the compound leading to ADRs. β-blockers are an example of drugs which cause type A ADRs: they can cause bradycardia and heart block through exaggerated primary pharmacological action but can also cause bronchospasm through a secondary pharmacological site of action. Nowadays the potential secondary pharmacological effects of a compound are evaluated thoroughly in preclinical trials to attempt to predict possible adverse events which may arise when administered to humans. Type A reactions are much more common than type B reactions and account for over 80 % of all ADRs (Pirmohamed *et al.*, 1998; Pirmohamed *et al.*, 2004).

Type B reactions cannot be predicted from the pharmacology of the drug and may be a result of a metabolite of the parent drug rather than the parent drug itself. The adverse effect can be direct but is often immunological in nature and can be serious. Type B reactions often only occur in a small fraction of the general population so are often missed in the development of a drug due to the compound being exposed to a relatively small sample of patients. Genetic variations in the population are often responsible for predisposing an individual to a type B reaction. Single nucleotide polymorphisms (SNPs) in

drug metabolising enzymes can play a large role in type B reactions. Perhexiline is a drug which is metabolised by the hugely polymorphic CYP2D6 cytochrome P450 enzyme. Type B reactions occur in individuals who are deficient in this enzyme due to accumulation of the parent compound leading to hepatotoxicity and peripheral neuropathy. In this instance the lack of the enzyme leads to the ADR whereas the converse is also possible where the over expression of an enzyme can metabolise a compound to a more chemically reactive metabolite which then causes the ADR (Park *et al.*, 2005). Immunological responses in certain individuals are also a cause of many type B ADRs. These responses occur in individuals to compounds which act as haptens; they bind covalently to proteins or other endogenous macromolecules. The most studied of these reactions is the response to penicillins which cause severe anaphylactic reactions in one in 2000 patients when the penicillin conjugates to a protein (Park *et al.*, 1998; Pirmohamed *et al.*, 1998).

2 - **Kidney Physiology and Function**

The kidney is an extremely important organ in the body which is adapted for the filtration of the blood and reabsorption of electrolytes and water. One of its most important functions is to adjust the volume and osmolarity of urine in response to water and salt levels in the body. This is managed via nervous and hormonal signals. Antidiuretic hormone (ADH) is produced in the hypothalamus and stored in the pituitary gland. It is released when the osmolarity of blood increases past a certain point and acts on the distal tubules and collecting ducts to increase the permeability to water. Another important system in the regulation of kidney function is the renin-angiotensin-aldosterone system (RAAS) which involves a tissue called the juxtaglomerular apparatus (JGA). The JGA is located near the afferent arteriole; its function is to detect a reduction in blood pressure leading to the

conversion of angiotensin to angiotensin II by renin. Angiotensin II raises blood pressure and volume by causing constriction of arterioles and causing the proximal tubules to reabsorb more salt and water. Angiotensin II also stimulates the adrenal glands to release aldosterone which causes distal tubules to reabsorb greater amounts of Na+ and water.

The kidneys account for less than one % of total body mass in humans yet receive approximately 20 % of the cardiac output at rest through the renal artery and the renal vein. Between 1,100 and 2,000 L of blood flows through the kidneys on a daily basis and they in turn process approximately 180 L of filtrate. The kidney is capable of excreting urine which is as much as four times more concentrated than the blood (1,200 mosm/L and 300 mosm/L respectively). The kidney has two distinct areas known as the renal cortex and the medulla which are situated on the outside and the inside of the kidney respectively. The functional unit of the kidney is the nephron which consists of a ball of capillaries called the glomerulus from which liquid is forced into the lumen of the Bowman's capsule which surrounds the glomerulus. The Bowman's capsule contains specialised cells known as podocytes and is non-selectively permeable to water and small molecules but not blood cells or larger plasma proteins. The Bowman's capsule receives the filtrate from the blood in the glomerulus which then flows to the proximal tubule, the loop of Henle, the distal tubule and finally the collecting duct before passing into the ureter. The glomerulus, the Bowman's capsule, the proximal and distal tubules are situated in the renal cortex while the loop of Henle and the collecting ducts are situated in the renal medulla. Each nephron in the kidney is supplied by an afferent arteriole which flows away from the nephron as the efferent arteriole. The afferent arterioles divide into the capillaries of the glomerulus and also the peritubular capillaries which supply the proximal and distal tubules.

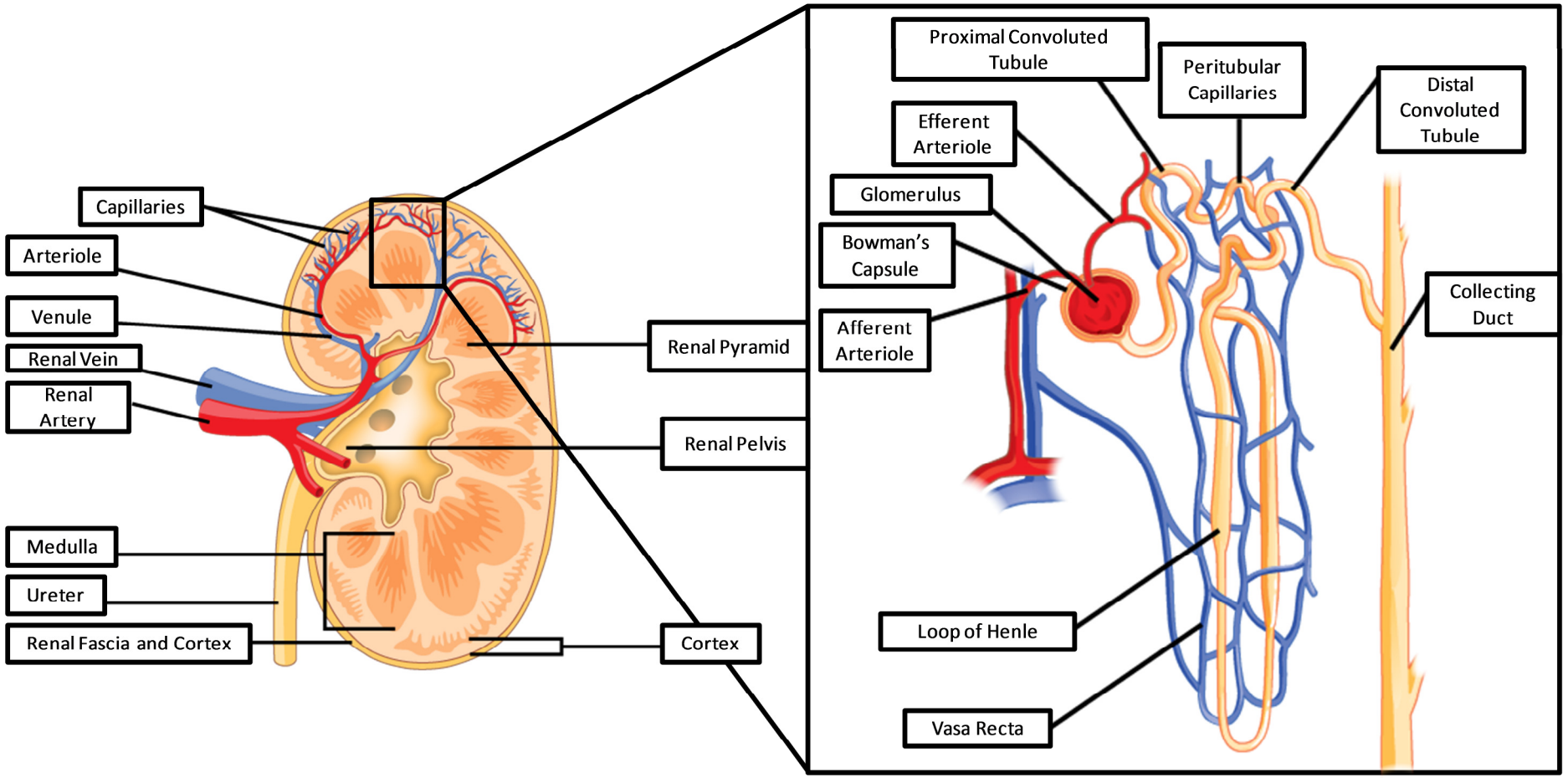

Figure 1: Functional units of the kidney and structure and functional units of a single nephron. Adapted from OpenStax college 2013. OpenStax College. 2013. Biology. Connexions, May 30, 2013. http://cnx.org/content/col11448/1.9/.

The proximal tubule is adapted for secretion and reabsorption and serves to alter the volume and content of the filtrate. The epithelial cells are adapted to maintain a constant pH throughout the tubule through the secretion of hydrogen ions and also synthesise and secrete ammonia which neutralises acids. The proximal tubules also serve to reabsorb approximately 90 % of bicarbonate which is essential in maintaining acid base balance in the body. Nutrients such as glucose and amino acids are either actively transported or passively diffuse from the lumen into the endothelial cells where they will ultimately be reabsorbed into the peritubular capillaries. Drugs and other exogenous compounds can also diffuse or be actively transported into proximal tubular epithelial cells and can also pass from the peritubular capillaries into the interstitial fluid and then into the lumen of the proximal tubules. Arguably the most important function of the proximal tubule from a physiological viewpoint is the reabsorption of the majority of NaCl and water from the filtrate; this diffuses into the epithelial cells of the proximal tubule where the Na^+ is actively transported into the interstitial fluid, drawing water into the interstitial fluid by osmosis. The active transport of positively charged Na^+ out of the proximal tubular epithelial cells is balanced by the passive transfer of negatively charged Cl^- ions from the tubule.

The descending limb of the loop of Henle is adapted for the reabsorption of water from the filtrate. Water moves from the lumen of the nephron across the epithelium which is permeable to water but not to other soluble molecules. Water moves across the epithelium via osmosis which requires the filtrate to be hypo-osmotic compared to the interstitial fluid which surrounds it. This gradient in osmolarity is maintained throughout the descending loop of Henle which allows water to be continually drawn from the lumen of the loop of Henle to the ever increasing osmolarity of the interstitial fluid which is generated through

the concentration of the solute increasing as water leaves. After the filtrate reaches the tip of the loop of Henle which is situated deep in the medulla the descending limb becomes the ascending limb and begins to return towards the cortex of the kidney. The ascending limb of the loop of Henle is permeable to salt but impermeable to water unlike the descending limb. The ascending limb also differs from the descending limb in that it has two distinct regions; the thin segment which is close to the tip of the loop of Henle and the thick segment which is closer to the distal tubule. In the thin segment salt diffuses into the interstitial fluid, this is facilitated by the fact that the filtrate is highly concentrated due to the removal of water in the descending limb. Salt is also removed in the thick segment of the loop of Henle however here the process is active. The loss of salt from the filtrate in the ascending limb without the loss of more water dilutes the filtrate before it enters the distal tubule.

The distal tubules are also crucial in the reabsorption of salts and play a large role in the regulation of NaCl and K^+ levels through the variation of the amount of K^+ which is secreted into and the amount of NaCl which is reabsorbed from the filtrate. Another role of the distal tubules is to maintain pH like the proximal tubules by the secretion of H^+ and reabsorption of bicarbonate. From the distal tubule the filtrate moves into the collecting duct which carries it down through the medulla and out of the kidney. The collecting duct actively reabsorbs NaCl, and water can leave the filtrate by passive diffusion along a gradient of osmolarity much like in the descending limb of the loop of Henle, this makes the filtrate more concentrated as it descends into the medulla. Deep in the medulla the collecting duct becomes permeable to urea which is highly concentrated in the filtrate by the time it reaches this point of the nephron. Urea now diffuses into the interstitial fluid where it is

crucial in maintaining the high osmolarity which allows the kidney to conserve water by excreting hyper osmotic urine.

The aforementioned physiology of the kidney and the precise arrangement of the nephrons enable the most efficient reabsorption of water. This illustrates the delicate balance necessary for its physiological functions to work efficiently. This efficient process is energy dependent due to the active transport of various solutes against concentration gradients. These important physiological functions and the many delicately balanced processes within the kidney makes it an essential organ in the body but also contribute to its susceptibility to toxicity. As previously stated the kidney receives up to 25 % of the cardiac output, this large volume of blood exposes the cells of the nephron to higher concentrations of any toxins which are present in the blood than other organs or tissues (Ferguson *et al.*, 2008). Tubular epithelial cells are particularly susceptible to toxicity because they are adapted to concentrate the filtrate as described previously; this serves to concentrate any toxin which is present in the filtrate. The tubular epithelium also has active transport processes which transport toxins into epithelial cells leading to accumulations of toxins and reactive metabolites intracellularly (Ferguson *et al.*, 2008). Finally the epithelial cells have metabolic capabilities which allow metabolism of compounds into chemically reactive products and also, along with active transport mechanisms, require energy leading to susceptibility to disturbances in the REDOX state of the cells.

The characteristics outlined here illustrate the kidneys susceptibility to various forms of injury. This could be direct damage to cells of the kidney such as tubular epithelial cells, obstruction of the tubules of the kidney caused by precipitates of drugs or their metabolites, inflammation or be secondary to altered haemodynamics in the body. Due to the many

different sections of the nephron with varying functions and exposure to concentrated filtrate there are a range of different compounds and mechanisms which can adversely affect the organ as a whole. The effects of these adverse events are often initially very subtle such as small alterations in the ability of the kidney to concentrate urine or handle the transport of various ions and would be difficult to spot clinically without very scrutinous monitoring. For this reason it can be very difficult to detect kidney injury without significant damage to the organ and therefore significant reduction in kidney function (Ferguson *et al.*, 2008).

3 - **Acute Kidney Injury**

Acute kidney injury (AKI) is a major worldwide health issue, and particularly so in the developed western world. It is a complex disorder which can manifest itself in the form of a subtle elevation in serum creatinine (SCr) ranging to complete renal failure (Ricci *et al.*, 2008). Due to its rapid onset it is often very difficult to diagnose clinically before it has progressed substantially and some evidence suggests that an AKI event can increase susceptibility to the future development of chronic kidney disease (CKD) (Ricci *et al.*, 2008). AKI is also a common adverse event associated with cardiac bypass surgery, an increasingly common problem in the developed world (Liangos *et al.*, 2009). A recently formed group known as The Acute Kidney Injury Network (AKIN) defines AKI as “an abrupt (within 48 hours) reduction in kidney function currently defined as an absolute increase in serum creatinine of either over or equal to 0.3 mg/dl or a percentage increase of over 50 % or a documented reduction in urine output (Mehta *et al.*, 2007). Patients who suffer from CKD are more likely to experience AKI (Coca *et al.*, 2008). AKIN was formed to help improve the care for patients who suffer from or who are susceptible to AKI. It has been estimated to

affect and complicate up to as many as seven % of all hospital admissions, up to 25 % of all intensive care unit admissions and is responsible for increasing the length of stay in hospital (Chertow *et al.*, 2005a). It is extremely difficult to accurately state the mortality rate for AKI due to large variations in studies carried out by research groups and also due to the accepted definition of AKI in various studies. However it is thought that over the 50 years the mortality rates have not changed significantly and remain between 50 to 70 % in the adult population and approximately 30 % in an infant population. AKIN was founded to try and place widely accepted definitions on AKI. Although there are challenges in accurately determining mortality rates from AKI, it is widely accepted that drug induced AKI plays a large role in AKI acquired in hospitals, in fact it has been shown to contribute to eight to 60 % of cases of drug induced AKI. A study by Uchino et al in 2005 (Uchino *et al.*, 2005) suggested that the number of cases of AKI which were thought to be contributed to by drugs was 19 %. Older studies tended to predict this number to be lower suggesting that drug induced AKI is increasing, although as previously stated this may be due to variations in the definition of AKI or the methods of detecting it (Ferguson *et al.*, 2008; Ho *et al.*, 2008).

Another major effort to properly define AKI was made by the Acute Dialysis Quality Initiative (ADQI) who developed the RIFLE criteria. This initiative was set up to define AKI and produce a widely accepted clinical consensus for the definition of the term. Without this definition the term AKI remained a concept which was difficult to diagnose clinically (Ricci *et al.*, 2008). A widely accepted definition was also essential to allow the selection of randomised groups in clinical trials to study the disease itself, the development of animal models to help study the disease pre-clinically, to study the epidemiology of the disease and to develop biomarkers of the AKI (Ricci *et al.*, 2008). The acronym itself relates firstly to the increasing

severity classes of AKI followed by the two outcome classes which are risk, injury, failure, loss and end stage renal disease (Kellum, 2008; Kellum *et al.*, 2012). The severity of the AKI is assessed by changes in glomerular filtration rate (GFR), serum creatinine (SCr) and urine output whereas the outcome criteria are defined by the duration of loss of function. The RIFLE criteria was developed with a number of key features in mind which included the ease of use, applicability by different centres with the same outcome, a similar sensitivity and specificity for the disease in different populations and genders and ability to take into account changes in SCr (Ricci *et al.*, 2008). It was also developed to allow the distinction between early and late cases and mild and severe cases of AKI.

AKI is generally diagnosed through the quantification of SCr or the detection of oliguria however it is widely accepted that SCr is a sub optimal marker of AKI which will be discussed in a later section. In AKI SCr is possibly a poorer marker than in CKD due to the fact that the sufferers do not reach steady state due to the rapid onset of injury. This means that significant elevations in SCr are often not observed until up to 72 hours post any AKI (Coca *et al.*, 2008). Furthermore the kidney has a renal reserve of creatinine, it can also secrete more creatinine in the tubules, that muscle wasting increases SCr and an altered volume of distribution can affect creatinine. These facts reveal its poor sensitivity as a biomarker of AKI (Coca *et al.*, 2008; Star, 1998). For example, patients who have acute renal failure are often oedematous which increases the volume in which creatinine is distributed, therefore reducing its concentration thus preventing recognition of the disease (Star, 1998). The major factor and rate limiting step impairing the progress in the treatment and diagnosis of AKI patients and pre-clinical studies is the lack of more sensitive and specific biomarkers for the condition. This will be discussed in more detail in a later section. However it is thought to be

unlikely that any single biomarker will be sufficient to diagnose the various stages of AKI and it is more likely that a panel of biomarkers with be the best option in the future (Han *et al.*, 2009; Han *et al.*, 2008; Koyner *et al.*, 2010; Liangos *et al.*, 2009).

4 - Chronic Kidney Disease

Chronic kidney disease (CKD) is a renal disease which develops over a much longer period of time than AKI. It is a worldwide health issue and the incidence of it is increasing, especially as the population ages (Collins *et al.*, 2012; Coresh *et al.*, 2001; Levey *et al.*, 2003). Historically glomerulonephritis was one of the leading causes of CKD but in the western world it has been overtaken by hypertension and diabetes (Zhang *et al.*, 2008). The prognosis of CKD is often extremely poor and the costs of treating it are high and of a long duration. In the USA alone the amount of patients who were suffering with CKD who were treated with either dialysis or transplantation was estimated to almost double from approximately 340,000 in 1999 to 650,000 in 2010. Approximately 11 % of the population of the USA are thought to be affected. CKD can develop into kidney failure and cardiovascular disease (CVD) as well as other conditions that are a result of a decreased kidney function. CKD can also develop into end stage renal disease (ESRD) if left untreated (although recent data from the USA suggests that only two % of CKD patients progress to ESRD (Zhang *et al.*, 2008)). ESRD has a very high rate of mortality and is associated with accelerating cardiovascular disease and often requires dialysis or transplantation (Zhang *et al.*, 2008). Maintaining patients with ESRD is extremely costly and in 2001 the worldwide annual burden was estimated to be $75 billion (Zhang *et al.*, 2008). CKD also has a complex relationship with other diseases such as CVD amongst other adverse events (Aqeilan *et al.*, 2010; Huang *et al.*, 2012; McClellan *et al.*, 1997; Obrador *et al.*, 1999; Remuzzi *et al.*, 2002;

Sanchez *et al.*, 1997; Tonelli *et al.*, 2006).There is growing evidence which suggests that with earlier detection of CKD through the development of improved biomarkers of chronic kidney injury the associated adverse events could be delayed or prevented completely. CKD is often not diagnosed nor treated in its early stages due to the guidelines which are used to define CKD. Much like with AKI, historically there has been a lack of a uniform definition to classify the disease. In CKD this includes a lack of agreement into what defines the various stages of CKD and lack of simple tests to detect and evaluate the disease (Lund, 2010; Ruggenenti *et al.*, 2001; Zhang *et al.*, 2008). To address this issue in the USA the Kidney Disease Outcomes Quality Initiative (KDOQI) of the National Kidney Foundation (NKF) published a number of guidelines to define the disease and to recommend best practices to treat and detect the early stages:

The first guideline was to define the stages of chronic kidney disease which was defined as "either kidney damage or decreased kidney function (decreased GFR) for three or more months". The guidelines suggest diagnosing the disease without necessarily having knowledge of its route cause through markers such as persistent proteinuria, albumin-creatinine ratio, urine sediment and GFR measurements. GFR is considered the best measurement of overall kidney function but if a patient has a normal GFR but other markers are elevated then they are considered to have an increased risk of adverse CKD outcomes. Considerations in GFR must be taken for sex, age and weight. This first guideline also states that the level of kidney function determines the stage of the CKD no matter what the diagnosis is. Stage one is the presence of kidney damage but with a normal or increased GFR (90 ml/min), stage two is kidney damage with a mildly decreased GFR (60-89 ml/min), stage three is a moderately decreased GFR (30-59 ml/min), stage four is a severely decreased GFR

(15-29) and stage five is kidney failure (<15 ml/min)(Davies *et al.*, 1950; Lindeman *et al.*, 1985; Rowe *et al.*, 1976).

The second guideline regards the evaluation and the treatment of the patient and states that each patient should have a treatment plan based on the stage of their disease as classified by the first guideline. The disease is diagnosed by examining the patient's history, laboratory results of various markers previously described and a physical examination. After this the treatment type depends on the cause of the CKD and the symptoms which are presented. Patients who are in stage four of CKD are candidates for dialysis and kidney transplant and if a patient's GFR is below 30 ml/min a nephrologist should also be involved in their care (Davies *et al.*, 1950; Levey *et al.*, 2003; Lindeman *et al.*, 1985).

The third guideline is designed to identify patients who are at an increased risk of CKD who have a normal or elevated GFR. This guideline suggests that as part of a routine health assessment GFR should be assessed and the patient notified if it is elevated and sent for further assessment of markers of kidney function. There are a number of conditions which increase the risk factor for development of CKD such as diabetes and hypertension. A low protein diet, stopping smoking and lipid lowering drugs have all been shown to slow the progression of CKD (Davies *et al.*, 1950; Levey *et al.*, 2003; Lindeman *et al.*, 1985; Zhang *et al.*, 2008).

The final two guidelines involve the laboratory measurements used to assess kidney disease in patients. The fourth guideline involves the estimation of GFR using the Cockcroft-Gault equation and/or the modification of diet in renal disease (MDRD) equation which takes into account age, sex, race, body size and serum creatinine concentration. Females have been shown to be slightly more susceptible to CKD than males and Caucasians and Asians more

susceptible than African Americans although the most important factor has been repeatedly shown to be age (Zhang *et al.*, 2008). In healthy individuals GFR stays constant after infancy until the age of 30 where it begins to start to decrease by approximately one ml/min per year (Zhang *et al.*, 2008). The MDRD equation has greater precision and accuracy than the Cockroft-Gault equation in patients with a GFR of below 90 ml/min and takes into account serum creatinine. This guideline also states that serum creatinine measurements should not be used as the sole means to assess kidney function as it depends on muscle mass, creatinine secretion and extra renal excretion. Also in elderly patients serum creatinine is often reduced due to reduced muscle mass which can make an effective diagnosis difficult. Finally, clinical laboratories should all use the same calibration of equipment when analysing serum creatinine so as to minimise inter-laboratory variation. The fifth and final guideline regards the use of the measurement of proteinuria as a marker of CKD. It states that untimed urine samples are usually adequate in detecting and monitoring proteinuria and the ratio of protein or albumin to creatinine can replace a 24 hour assessment of protein excretion. If a patient has two or more positive results for proteinuria in the space of three months then they are deemed to have persistent proteinuria and should have further evaluation for CKD (Davies *et al.*, 1950; Levey *et al.*, 2003; Lindeman *et al.*, 1985; Zhang *et al.*, 2008).

5 - **Ischemic Reperfusion**

Another common and potentially devastating form of kidney injury which can cause acute kidney failure or loss of function is ischemic reperfusion (IR) injury. It has a persistently high rate of mortality in severe cases, which may require dialysis, even though there have been considerable advances in supportive care for the condition (Rabb *et al.*, 1995).It has been

suggested to be responsible for up to 50 % of acute renal failure cases (Mishra *et al.*, 2003; Star, 1998). IR injury is caused by a period of lack of blood to the kidney tissue so therefore lack of oxygen leading to ischemia followed by a subsequent reperfusion of blood and oxygen (Pulsinelli *et al.*, 1979). The degree of organ damage or degree of loss of function is dependent on the duration of the period of ischemia and mild cases usually resolve themselves (Nogae *et al.*, 1998). This resupply of oxygen to the kidney tissue leads to widespread oxidative stress and inflammation rather than restoration of the natural function of the tissue due to the initial period of ischemia. Reactive oxygen species are generated in part by the infiltration of leukocytes which also bring with them proteolytic enzymes (Rabb *et al.*, 1995). The oxidative stress and inflammation lead to tubular atrophy, endothelial monolayer disruption and apoptosis. Although the damage to the renal tissue itself is largely due to apoptotic cell death, necrotic cell death has also been observed (Renolleau *et al.*, 1998). Vasoconstriction, tubular obstruction, metabolic alterations and inflammation are all thought to play a role in the development of IR although the development of the condition in humans is thought to be complex and multifactoral involving the response to nephrotoxins as well as the ischemia (Mishra *et al.*, 2003). Studies have confirmed the involvement of a number of genes which have been significantly upregulated in the ischemic murine kidney, including cysteine-rich protein 61 and neutrophil gelatinase-associated lipocalin (NGAL). Although IR occurs in humans and can cause renal failure it is also an excellent in vivo model of kidney damage and has been used in this thesis to study potential biomarkers of kidney injury (Mishra *et al.*, 2003).

6 - **Nephrotoxicity**

Nephrotoxicity is the toxic affect of any exogenous compound whether it is a medication or other drug on the kidney. It has been estimated that drugs cause approximately 20 % of acute renal failure events which present themselves in hospitals. Recently nephrotoxicity is most associated with the older population due to the larger amounts of medication patients are often on, the higher incidence of cardiovascular disease and diabetes and the increased amount of tests and procedures they may be exposed to compared with more historical cases. In the aging population the incidence of nephrotoxicity can be as high as 66 %. Other risk factors which have been reported to influence susceptibility to nephrotoxicity include sexual, racial and genetic differences (Naughton, 2008). Acute kidney injury induced by nephrotoxins (and other causalities) is more often than not reversible by removing the root cause of the injury. It still has a large societal cost which is incurred by the possible need for many interventions or hospitalisations (Gandhi *et al.*, 2000; He *et al.*, 2007; Hoste *et al.*, 2006; Kohli *et al.*, 2000; Kong *et al.*, 2012; Naughton, 2008; Vakkila *et al.*, 2004). The majority of compounds which are found to be nephrotoxic all cause kidney injury through common mechanisms which will be discussed in the following section:

Tubular cell toxicity is the most important mechanism of nephrotoxicity to this thesis, particularly in proximal tubular cells. The majority of drug induced nephrotoxicity affects the proximal tubular cells and even when drugs target the glomerulus they often cause damage to the tubules secondarily (Bonventre *et al.*, 2010). Tubular cells are very susceptible to nephrotoxicity due to their physiological role in concentrating the filtrate which serves to increase the concentration of the nephrotoxin exposed to the epithelial cells. This leads to cell toxicity through interference with mitochondrial activity and transport mechanisms

which in turns leads to oxidative stress and generation of reactive oxygen species (ROS). From initiation of tubular cell toxicity the epithelial cells of the kidney go through a number of events during the progression of the damage; these are a loss of polarity and cytoskeletal integrity of the cells, followed by necrosis and apoptosis which then progresses into dedifferentiation of the surviving cells which is followed by the covering of the newly differentiated cells over the basement membrane. Finally the surviving proximal tubular epithelial cells proliferate and differentiate to once again form a functioning proximal tubular epithelium (Ichimura *et al.*, 2004). Compounds which cause nephrotoxicity through this mechanism include Aminoglycosides and Cisplatin. The latter causes tubular epithelial cell toxicity through the induction of oxidative stress and DNA damage and features heavily in this thesis (Broker *et al.*, 2005; Elmore, 2007; Morley *et al.*, 2000; Shelton *et al.*, 2013; Yamakuchi *et al.*, 2008)

Inflammation is another cause of kidney injury as a result of nephrotoxic agents and can be induced in the glomerulus, renal tubular cells and also the surrounding interstitium. This can lead to fibrosis if not resolved quickly. Agents such as lithium, gold and non-steroidal anti-inflammatory agents have all been reported to induce glomerulonephritis which is an inflammatory condition which leads to proteinuria. Acute interstitial nephritis is an inflammatory condition which can be a result of a type B ADR (discussed previously). This condition is caused by the toxin in question forming haptens in the kidney which enter the interstitial fluid and induce an immune response which may or may not also induce the more classical symptoms of this type of reaction. Drugs which are associated with this type of reaction include beta lactam antibiotics, the antiviral acyclovir and proton pump inhibitors. Finally drugs can also induce chronic interstitial nephritis however this is much

more unlikely to be drug induced than the other forms. It also often presents itself without other typical signs of hypersensitivity. Drugs which have been reported to induce this condition include certain chemotherapeutics, certain NSAIDs when administered in high doses for a long period of time or in patients with a pre-existing kidney condition(Geevasinga *et al.*, 2006; Simpson *et al.*, 2006).

Drugs can also cause nephrotoxicity via modulation of intraglomerular haemodynamics which causes issues with glomerular filtration rate (GFR) and the kidneys ability to self-regulate its local blood pressure. Drugs which can cause this include those that interfere with prostaglandin activity, angiotensin-II activity or angiotensin receptor antagonists. These include NSAID's, angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers respectively. Calcineurin inhibitors like cyclosporine can also cause vasoconstriction of afferent arterioles which can impair renal function in already high risk patients (Naughton, 2008).

Crystal nephropathy is another condition which can be caused by various nephrotoxic compounds. Crystal nephropathy is a condition which results in renal impairment through the development of crystals which are insoluble in urine. The likelihood of these crystals precipitating is a result of the concentration of the drug and the pH of the urine. If a patient has an underlying condition which leads to a loss of renal function then they are at a greater risk. These crystals often precipitate in the distal tubular lumen and leading to obstruction of urine and causing a reaction in the interstitium. Drugs which can form these insoluble crystals include sulphonamide antibiotics, and certain antivirals such as acyclovir and methotrexate (Elmore, 2007; Morley *et al.*, 2000; Yamakuchi *et al.*, 2008).

Drugs which induce rhabdomyolysis can cause nephrotoxicity indirectly through the release of myoglobin into the circulation. This myoglobin can induce nephrotoxicity usually secondarily to other forms of kidney injury such as tubular obstruction, altered GFR or direct toxicity. The most common drugs involved in rhabdomyolysis are the statins although many more drugs have also been implicated in causing rhabdomyolysis including alcohol and many other drugs of abuse. Finally, drugs which can induce thrombi in the microvasculature around the nephron can also cause nephrotoxicity indirectly. Drugs which have been associated with this include cyclosporine and various antiplatelet agents (Greene *et al.*, 2010; Majid *et al.*, 2010).

Although the main risk factor for the development of nephrotoxicity is the nephrotoxic agent which has been taken by the patient there are also a number of patient based risk factors which are shared in all types of nephrotoxins. Factors such as the patients age (where an age of over 60 years is strongly associated with a lowered GFR), exposure to more than one nephrotoxin, diabetes and heart failure all increase the likelihood of the development of nephrotoxicity (Naughton, 2008).

Table 1:- Various nephrotoxicants, their location of injury and their mechanism of toxicity. Adapted from (Ferguson *et al.*, 2008; Naughton, 2008).

Mechanism of Injury	Location of Injury	Responsible Compounds
Tubular Cell Toxicity	Proximal Tubules	Cisplatin
		Gentamicin
		Tenofovir
		Lead
		Cadmium
	Distal Tubules	Amphotericin B
		Lithium
Crystal Nephropathy	Interstitium and Distal Tubules	Acyclovir
		Sulphonamides
		Methotrexate
Interstitial Nephritis	Glomerulus, Interstitium, Tubules	Acetaminophen
		Lithium
		Omeprazole
		Phenytoin
Altered Intraglomerular Haemodynamics	Glomerulus	Cyclosporine
		ACE inhibitors
		Angiotensin Receptor Blockers
		NSAID's
		Diuretics
Thrombic Microangiopathy	Glomerulus and Bowman’s Capsule	Quinine
		Clopidogrel
		Micromycin-C

7 - **Biomarkers**

A biomarker is defined as any quantifiable change in an endogenous protein or molecule which can be indicative of a disease process or state. As has been previously discussed, the currently used clinical "gold standard" biomarkers of kidney injury, whether it be drug induced or otherwise, have limitations regarding their use clinically and pre clinically. In the last decade there has been a great effort by the research community to develop biomarkers for the detection of kidney injury earlier than previously possible. It is widely accepted that the most efficient way to prevent or help treat nephrotoxicity is to have more sensitive and specific biomarkers of kidney injury which are translatable from pre-clinical animal models to clinical scenarios (Blowey, 2005; Bonventre *et al.*, 2010; Mehta *et al.*, 2004). Ideally these biomarkers would help the researcher or clinician make a decision on whether a drug makes it to market depending on its risk benefit ratio or whether a therapy is worth continuing for an individual patient. An ideal biomarker of kidney injury would be able to identify kidney injury early (high specificity), would reflect the degree of damage, would provide information on the site of damage, would be able to analyse the progression of the damage and recovery, would be quantifiable in non-invasive samples matter and would be well characterised in terms of its strengths and weaknesses (Bonventre *et al.*, 2010; Coca *et al.*, 2008). Obviously a new biomarker need not possess all of the aforementioned attributes to be considered but should possess at least some of them.

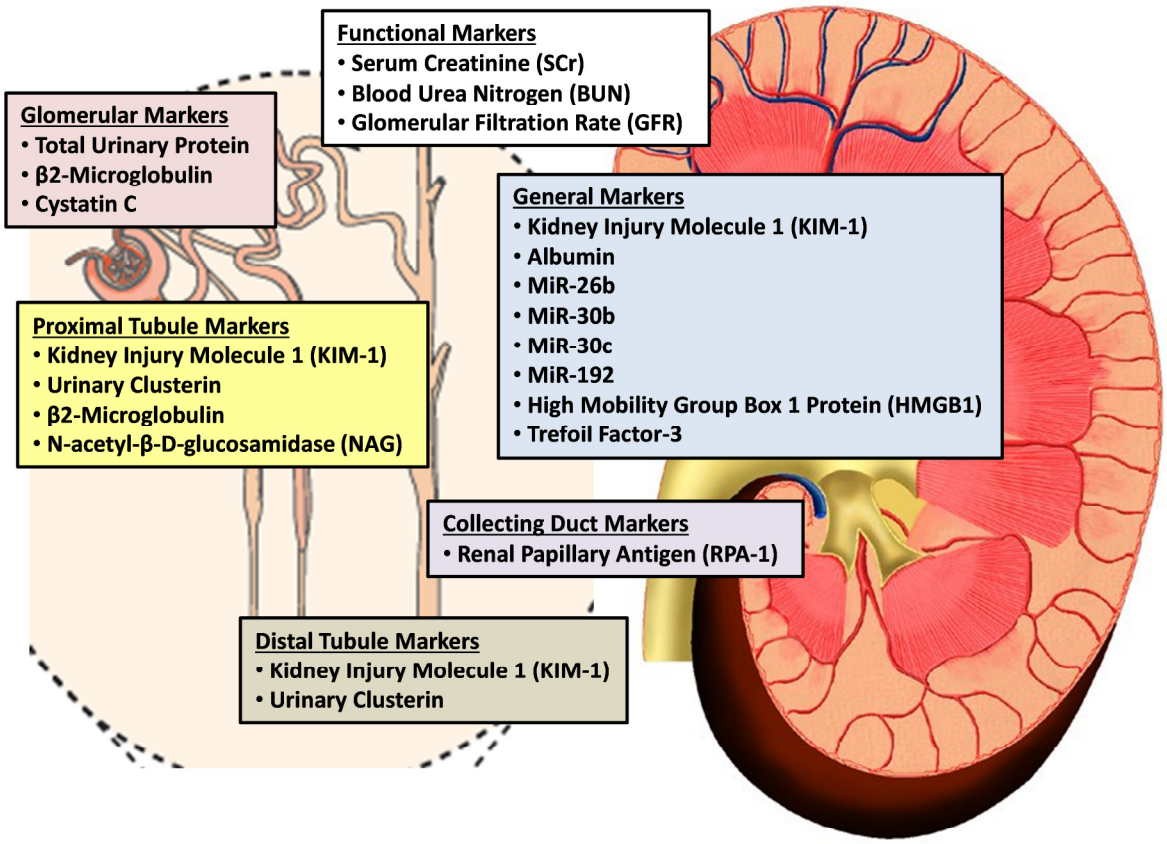

Figure 1: Molecules which have been investigated and have been proposed as biomarkers of kidney injury and the locations of injury which they may provide information on.

There are some blood based molecules but a larger number of urine based molecules which research has shown to have efficacy as biomarkers of kidney injury. The most commonly used blood based biomarkers of kidney injury are measurements of serum creatinine (SCr) and blood urea nitrogen (BUN). As previously described the diagnostic criteria for AKI and CKD both rely in some part on quantification of SCr and it is also widely used in preclinical animal studies. SCr and BUN both have a number of limitations as a biomarker of kidney injury which are summarised in table two. (Bonventre *et al.*, 2010; Chertow *et al.*, 2005b; Ferguson *et al.*, 2008; He *et al.*, 2009; Kellum *et al.*, 2002; Molitoris *et al.*, 2007; Vaidya *et al.*, 2010; Welch *et al.*, 2007; Wingard *et al.*, 1999). The lack of sensitivity of SCr and BUN causes major issues in the development of newer therapies and treatments as well as in the diagnosis of kidney injury itself. It also impacts clinical trials for new therapies as the disease cannot be detected with a great enough sensitivity (Ferguson *et al.*, 2008; Vakkila *et al.*, 2004; Welch *et al.*, 2007).

The urine based molecules which can be measured to indicate damage to the kidney can be broken into two groups; constitutive markers and inducible biomarkers. Constitutive urinary biomarkers are endogenous to renal tubular cells and are not found in urine under normal conditions but can be found in urine as a direct response to injury to the tubular cells. Inducible urinary biomarkers, on the other hand, are markers which are not normally found in tubular cells or urine but are present in the urine upon their expression being upregulated in response to injury (Koyner *et al.*, 2010). Urinary enzymes have been studied widely as biomarkers of AKI and CKD including responses to nephrotoxins, diabetic nephropathy, ischemia and glomerulopathy (Ferguson *et al.*, 2008; Vakkila *et al.*, 2004; Welch *et al.*, 2007). These enzymes can either be constitutive or inducible biomarkers which are involved in

Table 2: Summary of biomarkers of kidney injury, their location, the species they are validated in, the method of their detection and any issues associated with their use. Data collected from (Antoine *et al.*, 2010; Bernard *et al.*, 1987; Cannell *et al.*, 2010a; Cannell *et al.*, 2010b; Coca *et al.*, 2008; Ferguson *et al.*, 2008; Han *et al.*, 2009; Han *et al.*, 2008; Koyner *et al.*, 2010; Liangos *et al.*, 2007; Liangos *et al.*, 2009; Shlipak *et al.*, 2005; Yu *et al.*, 1983)

Biomarker	Matrix in which Quantified	Location of Marker	Type	Species	Specificity	Method of Detection	Nephron Specificity	Comments
Serum Creatinine	Serum	Filtered by Kidney	Protein	Human, mouse, rat	Diet, sex, race, muscle mass affect levels	Colourimetric		Cannot inform on time or severity of injury. Up to 50 % epithelial mass loss required before significant elevation
Blood Urea Nitrogen	Serum	Filtered by Kidney	Waste product of protein digestion	Human, mouse, rat	Dietary Supplements alter levels	Colourimetric		Increases can occur in absence of injury due to volume depletion or dietary supplements
NAG	Urine	Primarily Proximal Tubules	Enzyme	Human, mouse, rat	Endogenous urea and heavy metals can inhibit	Colourimetric	Proximal tubule	Elevations have also been observed in rheumatoid arthritis, impaired glucose tolerance and hyperthyroidism
NGAL	Plasma/ Urine	Filtered by Kidney	Protein	Human, rat, urine	Specificity for sepsis	ELISA	Proximal tubule	Utility varies greatly between adults and children
Albumin	Urine	Filtered by Kidney	Protein	Human, mouse	Elevated after exercise, dehydration, infections		Glomerulus and Proximal tubules	May reflect alterations in glomerular permeability or defects in proximal tubules
Cystatin C	Urine	Filtered by kidney, Reabsorbed by Proximal Tubules	Protein	Human, mouse, rat	Not specific enough to be considered a biomarker of AKI	ELISA, nephelometer	Tubules	Levels consistent over sex, age and weight

Biomarker	Matrix in which Quantified	Location of Marker	Type	Species	Specificity	Method of Detection	Nephron Specificity	Comments
IL-18	Urine	Filtered by Kidney	Cytokine	Human	Immunological role limits use	ELISA	Proximal tubule	Holds potential as marker of mortality in critically ill patients
Kim-1	Urine	Shedded from proximal tubule epithelial cells	Transmembrane protein	Human, mouse, rat, zebrafish, dog, monkey	Not detectable in healthy urine	ELISA	Proximal tubule	Large degree of proximal tubule specificity
Alanine aminopeptidase (AAP)	Urine	Primarily Proximal Tubules	Brush border enzyme	Rat, Dog, Human		Colourimetric	Proximal tubule	Unstable which limits utility
Alkaline phosphatase (AP)	Urine	Primarily Proximal Tubules	Brush border enzyme	Rat, human	Levels don't reflect degree of injury	Colourimetric	Proximal tubule	Unstable which limits utility
Microalbumin	Urine	Filtered by kidney	Protein	Human, mouse, rat, dog, monkey, human	Lack of specificity for AKI	ELISA	Proximal tubule	Established marker of diabetic kidney disease
α-GST	Urine	Primarily Proximal Tubules	Cytosolic Enzyme	Mouse, rat, human	Upregulated in renal cell carcinoma	Colourimetric	Proximal tubule	Stabilisation of samples required
RBP	Urine	Filtered by Kidney, reabsorbed by proximal tubules	Protein	Human, mouse, rat	Levels decreased in vitamin A deficiency	ELISA, nephelometer	Proximal tubule	Elevations precede elevations in NAG
α1-microglobulin	Urine	Filtered by Kidney, reabsorbed by proximal tubules	Protein	Human, mouse, rat	HIV associated with elevated levels	ELISA, nephelometer		Stable over a range of pHs. Clinical use limited by lack of standardisation of reference levels

repair and regeneration. Studies have shown that urinary enzymes show a dose dependent increase after kidney injury and research has increased the understanding of the source of these enzymes in the kidney so they can reflect localised damage (Ferguson *et al.*, 2008). The location of the enzyme can also be indicative of the severity of damage; for example enzymes which are found in lysosomes or cytosol would indicate more severe damage than those found in the brush border (Ferguson *et al.*, 2008). Although many enzymes are sensitive for tubular injury they have a low threshold for release; this means it is unclear whether the increase is a result of a lack of specificity or an increase in sensitivity (preceding changes in SCr). Many enzymes are also only stable for a number of hours post collection of urine and require gel filtration to remove impurities prior to processing.

8 - **MicroRNAs as Next Generation Biomarkers**

Micro ribonucleic acids (miRNAs) are short ribonucleic acid sequences which are between 18 and 25 nucleotides in length. They are the smallest functional non-coding RNA units in plants and animals (Schmittgen *et al.*, 2004). They are a relatively recent discovery and were first described in 1993 by *Lee et al* (Yang *et al.*, 2009a). The first miRNA species to be discovered were in the c.elegans nematode species and is known as Lin-4. This miRNA was discovered as the product of the Lin-4 gene which was noticed to code not for a protein but for two small RNA molecules. One of these RNA molecules was a 22 nucleotide sequence and the other a 61 nucleotide sequence. The smaller molecule was found to have a degree of complementarity for the 3' untranslated region of the Lin-4 gene which had previously been proposed to be the region that mediated repression of another gene (Lin-14) by Lin-4 (Bartel, 2004; Bartel, 2009; Lee *et al.*, 2001; Wightman *et al.*, 1993). It was later discovered that the longer of the two gene products was the precursor to the shorter product. The

same lab went on to note that the binding of the Lin-4 miRNA to the mRNA of Lin-14 caused a reduction in the transcription of the Lin-14 gene product without reduction in the amount of the Lin-14 messenger RNA (mRNA). Lin-4 is now recognised as the first miRNA to be discovered. A second miRNA was not discovered until seven years later when a second *c.elegans* miRNA was discovered, known as Let-7. Let-7 and Lin-4 are only expressed in certain stages of larval development which led to them being known as small temporal RNAs (stRNAS). Importantly Let-7 was also discovered in the human and fly genomes which lead to the discovery of thousands of other miRNA species which were not necessarily expressed at specific times of development but rather in specific cell types. This resulted in stRNAs being reclassified as the newly founded miRNA class of small RNAs, the functions of most of which were unknown. Since the discovery of miRNAs the research interest into the regulatory roles of miRNAs continues to grow and they have been shown to be involved in a number a physiological functions such as fatty acid metabolism, growth and repair and cell death to name but a few. They have also since been discovered in a huge number of species including plants, viruses and all animals and there are now over 1000 known human miRNA species (Bartel, 2004; Bartel, 2009; Waldman *et al.*, 2008). More recent research has implicated various miRNA species as biomarkers of various disease states. This thesis will focus on the use of miRNA as biomarkers of kidney injury and in the next section will further describe the physiological role, the synthesis and the utility of these molecules as biomarkers (Bartel, 2004; Bartel, 2009). The fact that many miRNA species are highly conserved throughout evolution between many species including vertebrates and invertebrates suggests an extremely important physiological role (Grishok *et al.*, 2001; Lagos-Quintana *et al.*, 2001; Lagos-Quintana *et al.*, 2002; Lee *et al.*, 2001; Lee *et al.*, 2003; Pasquinelli *et al.*, 2000; Yang *et al.*, 2009a).

As mentioned briefly many miRNAs have very distinct expression patterns. Lin-4 and Let-7 are expressed only at certain stages of larval development in nematodes. In humans there are many miRNAs which are expressed only in specific cell or tissue types, examples of this include miR-1 and miR-122 which are expressed primarily in the heart and liver respectively (Bartel, 2004). Although miR-1 has also been shown to be expressed in the mammalian liver and mid-brain but at much lower levels than in the heart where it accounts for 45 % of all miRNAs. MiR-122 however is not detectable in any other tissue except the liver where it makes up 72 % of all the miRNAs (Lagos-Quintana *et al.*, 2002). As well having tissue specific expression profiles some miRNA species can be present in amounts up to 50,000 copies per cell but can also be found at levels as low as 500 copies per cell. MiRNAs are similar to the endogenous silencing RNA molecules (siRNA) which also serve to silence mRNA transcripts. Both species serve to silence mRNA transcripts however miRNAs are transcribed from genes which often have no relation to their target mRNAs (hetero-silencing) whereas siRNAs originate from the same gene locus to which their target mRNAs originate (homo-silencing). MiRNAs are transcribed from genomic DNA often in areas of the genome previously thought not to code for any proteins in the nucleus with use of two polymerases, PolI and PolII which are responsible for transcribing the miRNA genes to pri-miRNAs which are an intermediate in the synthesis of miRNAs (Grosshans *et al.*, 2002). Pri-miRNAs are hairpin loop double stranded RNA sequences which are then cleaved in the nucleus by a specific endonuclease called Drosha to a 60 to 70 nucleotide hairpin loop sequence known as a pre-miRNA. Drosha cleaves the pri-miRNA at the base of the hairpin structure of both strands leaving a single double stranded hairpin loop. This pre-miRNA is then actively transported from the nucleus to the cytosol by Ran-GTP and the specific Exportin-5 transport protein.

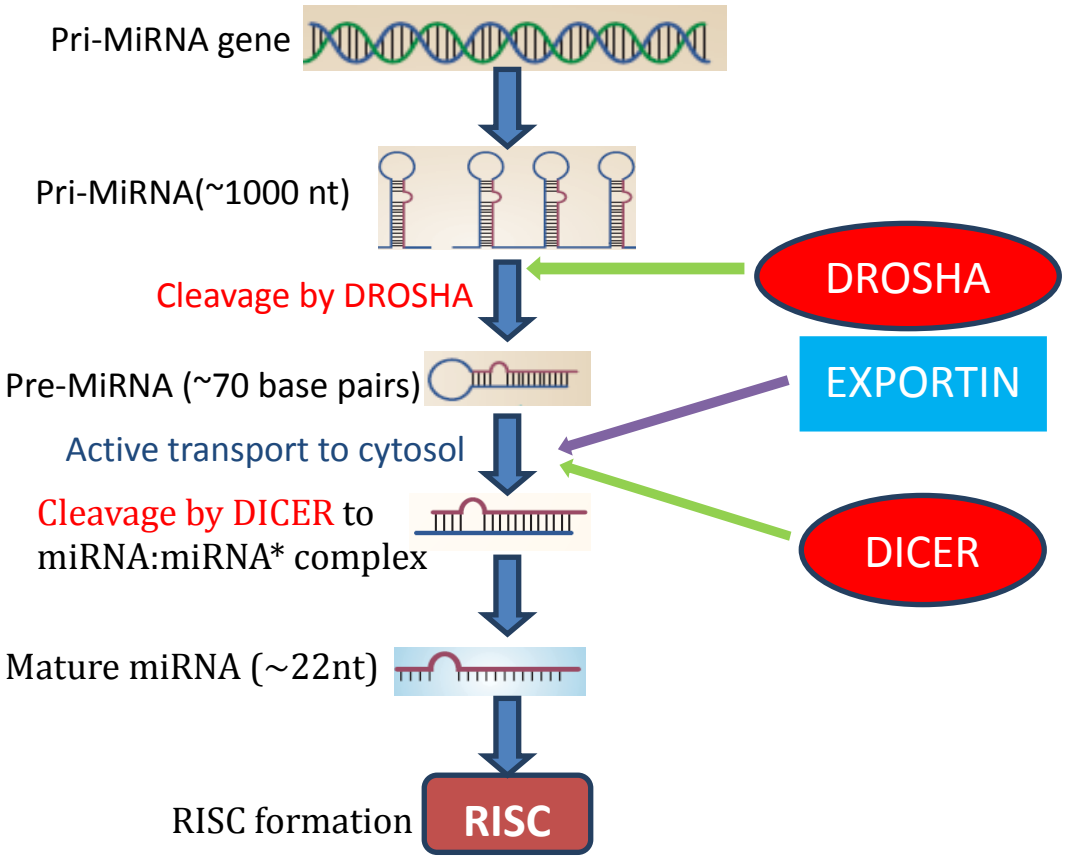

Figure 2:- Summary of the synthesis of miRNAs and the formation of the functional RNA induced silencing complex (RISC).

Once in the nucleus the opposite end that was cut by Drosha is cut by a second endonuclease called Dicer which leaves an 18 to 25 nucleotide double stranded RNA molecule which contains the mature miRNA and its complimentary sequence (Bartel, 2004; Bernstein *et al.*, 2001; Hutvágner *et al.*, 2001; Ketting *et al.*, 2001; Lee *et al.*, 2001; Lee *et al.*, 2003). The complimentary sequence to the mature miRNA is known as the miRNA* sequence and is known to have a much shorter lifespan than the mature sequence and is found at approximately 100 fold lower amounts in cells than the mature sequence. The mature miRNA is then processed into a ribonuclease complex called the RNA induced silencing complex (RISC). It is during this incorporation into the RISC that the miRNA* sequence is removed from the miRNA sequence and degraded. The RISC is thought to protect the mature miRNA strand from degradation while the mature miRNA (miRNA*) sequence is degraded (Billy *et al.*, 2001; Hammond *et al.*, 2001; Lagos-Quintana *et al.*, 2002; Majno *et al.*, 1995). The RISC contains a number of proteins from the argonaute family and then binds to mRNA transcripts specifically depending on the degree of complementarity between the miRNA and the mRNA transcript. Due to the fact that imperfect complementarity is required for miRNA binding to mRNA it means that a single miRNA species can have a number of mRNA transcripts, possible even hundreds of possible mRNA binding partners (Inui *et al.*, 2010). However it has been shown that even though there is a huge potential number of binding partners for each miRNA, in actual fact the number is fewer than has been predicted computationally or that many of these interactions do occur but do not elicit a noticeable phenotypic change. If however the activity of Dicer is abolished dramatic changes within cells can be elicited which alter a cells homeostasis (Inui *et al.*, 2010). It is thought that perfect or nearly perfect complementarity is required for binding of the RISC. The argonaute proteins are approximately 100 KDa and all contain a PAZ domain

which includes a PIWI (P-element wimpy testis) and a Zwillie domain which aid binding to RNAs.

MiRNAs negatively regulate gene expression in one of two ways; they either cause translational repression (or mRNA destabilisation) or cleave the mRNA transcript. It is thought that the mechanism which occurs is a result of the degree of complementarity which the miRNA has to the mRNA transcript. The RISC containing the miRNA binds to the 3' untranslated region (UTR) of the mRNA transcript with complementarity in the second to the eighth nucleotides of the 5' region of the miRNA(Inui *et al.*, 2010; Lagos-Quintana *et al.*, 2002). Evidence to support this is the fact that often the first eight nucleotides of miRNAs which provide the same function in various species but differ in large parts of the sequence are often conserved. The miRNA specifies mRNA cleavage if it has sufficient complementarity with the mRNA but will repress translation if it does not have sufficient complementarity but still a high enough degree to confer binding of the RISC. miRNA induced cleavage occurs between the part of the mRNA which is between the tenth and eleventh nucleotide of the miRNA (Bartel, 2004).

In the years after their discovery the most widely practiced method to quantify miRNAs was northern blotting to detect the presence of miRNAs. Northern blotting was useful as it provided information regarding the size of the miRNA as well as its abundance. However it was not quantitative, it was low throughput and had low sensitivity. This has now been replaced with the much more sensitive quantitative polymerase chain reaction (qPCR) (Schmittgen *et al.*, 2004).

8.1 - MiRNAs in disease

The expression profiles of various miRNA species differs between different tissues and cell types as discussed briefly previously. There has also been a great amount of research into the role miRNAs may play in various disease states and the changes in miRNA expression which occur during disease (Waldman *et al.*, 2008). The genes which code for miRNAs are often in areas of the genome which are associated with various cancers including regions which contain oncogenes, tumour suppressor genes or area of the genome which are prone to breaking (Waldman *et al.*, 2008). Tumours also commonly express altered miRNA profiles and these profiles can often provide some information on the pathogenesis of a tumour. This role of miRNAs in tumours provides a potential therapeutic target and also potential biomarkers of tumourogenesis. MiR-21 was shown to be expressed in 87 % of patients with colon cancer and furthermore was shown to be associated with poor outcome. This high expression of miR-21 could not only provide a biomarker of outcome but could also provide a specific therapeutic target as miR-21 may be involved in the progression of the tumour(Waldman *et al.*, 2008). Reduced expression of the Let-7 family has been reported in lung, colon, ovarian and gastric cancers and is associated with shorter survival in lung cancer sufferers (Brase *et al.*, 2010). MiRNAs are not only involved in cancers but also in the normal physiological functions of tissues. The liver has been studied widely and it has been shown that the liver abundantly expresses a number of miRNA species; miR-122 has already been mentioned and is the most abundant but miR-16, miR-27b, miR126, miR-133 and miR-143 are also abundantly expressed (He *et al.*, 2004). MiR-122 in particular has been shown to be involved in liver metabolism, specifically fatty acid metabolism (Esau *et al.*, 2006; Fabbri *et al.*, 2008; Girard *et al.*, 2008). This was shown through the knock-out of miR-122 in high fat fed mice which were found to have a much reduced cholesterol level than wild type mice.

MiR-122 also seems to play a role in the replication of the Hepatitis C virus and it has actually been shown that the virus cannot replicate in cells which do not express miR-122. It seems that miR-122 facilitates viral replication by targeting the viral 5' non coding region (Chen *et al.*, 2008; Chen, 2009; Girard *et al.*, 2008; Jopling *et al.*, 2005; Meijer *et al.*, 2013). A role for miR-122 in human hepatocellular cancer has also been proposed as it has been reported to be significantly down regulated in human hepatocellular cancer and rodent hepatocellular cancer.

8.2 - MiRNAs as biomarkers

MiRNAs are not only detectable in tissues providing information on the pathogenesis or development of cancer but can also be detected in other various bodily fluids; the majority of the research into their detection has focussed on their presence in serum (Chen *et al.*, 2008; Chen, 2009; Starkey Lewis *et al.*, 2011; Wang *et al.*, 2009; Wang *et al.*, 2010). MiRNAs have been shown to be functional in the cells in which they are synthesised but also functional in other cells after packaging into exosomes and transferred to other cells. This active process could provide a way in which cells communicate with each other and also a potential delivery mechanism for miRNA based therapeutics (Brase *et al.*, 2010; Chen *et al.*, 2008; Chen, 2009; Kosaka *et al.*, 2010a; Kosaka *et al.*, 2010b; Valadi *et al.*, 2007; Wang *et al.*, 2009; Wang *et al.*, 2010). The miRNAs are stable once in the serum and are resistant to RNase digestion which make the quantification of miRNAs in the serum promising biomarkers (Chen *et al.*, 2008). Studies by *Gilad et al* reported that miRNAs in fact are incredibly stable in serum and were stable over a number of freeze-thaw cycles and at extreme temperatures and pHs for up to four hours (Gilad *et al.*, 2008). The majority of the research into circulating blood based miRNAs as biomarkers is into their use as biomarkers

of cancer. Various studies have shown that the profile of circulating miRNAs is significantly changed in various cancers; miR-155 is elevated in B-cell lymphoma, miR-16 in breast cancer, miR-223 in lung cancer, miR-210 in pancreatic cancer and miR-141 in prostate cancer to name but a few (Chen *et al.*, 2008; Chen, 2009; Ho *et al.*, 2010; Ho *et al.*, 2008; Valadi *et al.*, 2007; Wang *et al.*, 2009; Wang *et al.*, 2010). However there is research to suggest that the circulating miRNA profile is altered in a wide range of inflammatory diseases, cardiovascular diseases and liver disease (Brase *et al.*, 2010). Another study has shown that a number of serum miRNA species were significantly elevated in pregnant women and that the quantification of specific miRNA species could help determine the stage of pregnancy (Gilad *et al.*, 2008). Perhaps the most important study involving miRNAs as biomarkers to this thesis was the study by *Wang et al* who published a study identifying a number of miRNAs which were significantly elevated in the serum of acetaminophen overdosed mice (Wang *et al.*, 2009). This study found that miR-122 and miR-192 were significantly elevated after acetaminophen overdose and importantly were significantly elevated earlier than the currently used clinical gold standard biomarker of liver injury alanine aminotransferase (ALT). This study showed that miRNAs may have utility as specific biomarkers of drug induced liver injury, with greater sensitivity than current gold standards (Wang *et al.*, 2009). This study formed the basis of much of the work in this thesis.

9 - **Aims and Hypothesis**

This thesis set out to improve the current state of the detection, diagnosis, and outcome of various types of kidney injury and also to try and improve the preclinical determination of kidney injury. Currently the subset of biomarkers used to identify kidney injury lack sensitivity and specificity making detection of the injury and its progression difficult until

significant damage to the kidney epithelium has occurred. Many of the biomarkers used currently are not fully translatable from preclinical to clinical situations and cannot provide accurate mechanistic or information on the location of the injury. The overall hypothesis of this thesis is that miRNAs will provide translatable biomarkers of kidney injury which could also provide information on the location or mechanism of the toxicity in the kidney. This will be tested by addressing the following hypotheses through the following aims:

- The techniques used to isolate, purify and quantify miRNAs from various biological matrices are precise, robust and repeatable. This will form the basis of the following work in this thesis and will achieve a number of objectives:
    - Determine the precision of the techniques used to quantify miRNAs
    - Provide information on the limitations of the techniques used to isolate, purify and quantify miRNAs
    - Provide information on the stability of isolated miRNA species over various conditions

- Kidney enriched miRNA species can be used as preclinical biomarkers of kidney injury.
    - Replicate a published method of detection of circulating miRNAs post DILI
    - Quantification of kidney enriched miRNA species in a IR model of kidney injury in vivo
    - Quantification of kidney enriched miRNA species in an APAP model of DILI

- Examining miRNA profiles of Cisplatin or Adriamycin dosed mice can provide potential locational biomarkers of DIKI and support the hypothesis that miRNAs can serve as biomarkers of kidney injury
    - Quantification of current biomarkers of kidney injury in a Cisplatin model of nephrotoxicity
    - Profiling of miRNAs in the kidney tissue, serum and urine of a Cisplatin and an Adriamycin induced models of nephrotoxicity.

- Modulation of miRNA activity can influence susceptibility to toxicity in vitro
    - Validate the toxic response to Cisplatin and Staurosporine in terms of apoptosis and necrosis
    - Determine if modulation of miRNA activity alters response to Cisplatin or Staurosporine

Chapter 2

Validation of Methods Involving Micro RNA Isolation, Purification and Quantification

Contents

1 - Introduction

Micro RNAs (miRNAs) are small non-coding RNA molecules which are between 18 and 25 nucleotides in length (Girard *et al.*, 2008). Physiologically they serve to negatively regulate gene expression through the binding of an RNA induced silencing complex to mRNA transcripts; this either targets the mRNA for degradation or represses mRNA translation (Bartel, 2004; Bartel, 2009; Grosshans *et al.*, 2002; Lagos-Quintana *et al.*, 2002; Lewis *et al.*, 2003). There are currently over 1000 known human miRNA species, many of which have sequences which are shared between many organisms, a factor that makes them attractive targets for translational toxicological research. Moreover, the fact that many miRNA species are expressed at higher levels in certain tissues and in different cell states makes miRNAs potential candidates for biomarkers of disease states (Lewis *et al.*, 2003).

Much research regarding miRNAs has focussed on the use of miRNAs as prognostic or diagnostic markers, particularly in the field of cancer. Many studies have investigated the expression profiles of miRNA species in various forms of cancer and certain miRNA species have been labelled either oncogenes or tumour suppressor genes as a result of these studies (Fabbri *et al.*, 2008; Mitchell *et al.*, 2008; Wong *et al.*, 2008). MiR-126 and miR-182 have been shown to be elevated in the urine of urothelial bladder cancer sufferers and miR-210 has shown promise as a marker of hypoxia in pancreatic cancer (Hanke *et al.*, 2010; Ho *et al.*, 2008). Recent research has focussed on the potential of the liver specific miRNA, miR-122, to be used as a biomarker of drug induced liver injury (DILI) and has been shown to have a huge dynamic range in the serum of patients with DILI (Wang *et al.*, 2009). MiR-122 is an extremely promising candidate biomarker of DILI due to its abundance in the liver (miR-

122 has been shown to represent 70 % of all liver miRNAs) and relatively low expression in other organs (Girard *et al.*, 2008; Jopling *et al.*, 2005).

Many miRNA species have tissue specific expression profiles, making them exciting candidate biomarkers for many clinical settings. As many miRNAs have shared sequences in many species, the assays used to quantify them are fully translatable between pre-clinical species and man. Due to the aforementioned concepts and the increasing interest and emerging importance of miRNAs in the fields of pharmacology, toxicology and medicine it is of uttermost importance that the assays and techniques used to isolate, purify and quantify these analytes are rugged, reproducible, sensitive and specific, more so if these molecules were to go on to be used in a clinical setting.

The aims of this current investigation are:

- To validate a commercially available approach to isolate, purify and quantify miRNA species from various biological matrices with focus on urine as a platform for quantification of miRNAs as potential biomarkers of kidney injury.
- To determine the precision of the qPCR techniques used to quantify miRNAs
- To determine the fractional recovery of miRNA from the isolation and purification procedure of miRNAs.
- To determine the stability of isolated miRNA species post isolation and purification from biological matrices.

2 - **Materials and Methods**

RNA oligonucleotides (synthetic miRNAs) were purchased from integrated DNA technologies (IDT). RNA isolation and purification kits were purchased from QIAGEN (miRNeasy mini kit, RNeasy minElute cleanup kit). Reverse transcription specific and qPCR specific miRNA primers (TaqMan® MicroRNA Assays) were obtained from Applied Biosystems. Reagents for reverse transcription and for qPCR were also obtained from Applied Biosystems (TaqMan® MicroRNA Reverse Transcription Kit and TaqMan® Universal PCR Master Mix). All other products were obtained from Sigma (Poole, UK). Human serum, plasma and urine were obtained from healthy volunteers. Protocols were undertaken in accordance with a licence granted under the Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Ethics Committee.

2.1 - miRNA isolation and purification

MiRNA isolation and purification was carried out according to manufacturer's instructions. Briefly, 50 µl miRNA containing sample (serum, plasma, urine, water) were mixed with Qiazol reagent (700 µl). 100 % chloroform was added (140 µl) and mixed vigorously. Samples were centrifuged for 15 minutes (12 000 g, 4°C) and the supernatant transferred to a separate tube. An equal volume of ethanol (70 %) was added to the supernatant and mixed well. Sample was transferred (700 µl) at a time to RNeasy mini spin columns and centrifuged for 15 seconds (8 000 g, room temperature). The flow through was transferred to a separate tube and 0.65 volumes of ethanol (100 %) was added and mixed well. Samples were transferred (700 µl at a time) to RNeasy MinElute spin columns and centrifuged for 15 seconds (8000 g, RT). The flow through was discarded and RWT (a stringent washing buffer) buffer (700 µl) was added to columns before centrifuging for 15 seconds (8 000 g, RT). Flow

through was again discarded before the same being repeated with RPE buffer (500 µl). Ethanol (80 %) was added and centrifuged for two minutes (8 000 g, RT) and flow through discarded. Columns were the centrifuged for five minutes with their caps open (8 000 g, RT) followed by the addition of RNase-free water (14 µl) to the column membrane and final centrifugation for one minute (8 000g, RT) to elute the purified miRNA in RNase-free water.

2.2 - Reverse transcription of miRNA to cDNA

Reverse transcription was carried out according to manufacturer's instructions in a total reaction volume of 15 µl. Briefly, a master mix was made up in RNase free water according to instructions from the manufacturer. MiRNA containing sample was added to non-skirted PCR plates (2 µl) followed by RNase free water (3 µl). RT master mix was added to each well (7 µl) followed by the miRNA RT specific primer (3 µl).

The following primer sequences were used:

miR-192: CUGACCUAUGAAUUGACAGCC

miR-194: UGUAACAGCAACUCCAUGUGGA

Lin-4: UCCCUGAGACCUCAAGUGUGA

U6snRNA:
GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATACAGAGAAGATTAGCATGGCCCCTGC
GCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTT

miR-26b: UUCAAGUAAUUCAGGAUAGGU

miR-30b: UGUAAACAUCCUACACUCAGCU

miR-30c: UGUAAACAUCCUACACUCUCAGC

The plates were then sealed using PCR plate caps and centrifuged briefly (1 500 g). The plate was then run on a GeneAmp PCR system 9700 using the following parameters: 30

minutes at 16 °C, 30 minutes at 42 °C, 5 minutes at 85 °C and then held at 4 °C. The cDNA product was then stored at -80°C until required.

2.3 - Quantification of cDNA by qPCR

Amplification and quantification of cDNA by qPCR was carried out according to manufacturer's instructions at a total reaction volume of 20 µl. The cDNA product from reverse transcription (1.33 µl) was added to separate wells in a skirted 96 well PCR plate in duplicate. Master mix (17.67 µl) and miRNA specific primer (1 µl) was then added to each well in triplicate. Plates were then sealed and centrifuged briefly prior to running on the ABI PRISM 7000 sequence detection system using the following parameters: 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds.

2.4 - Validation experiments

All the following validation experiments used RNA oligonucleotides which were designed to mimic endogenous miRNA species. RNA oligonucleotides were designed to the same sequence as miR-194 which is endogenous and enriched in the kidney. Stock solutions of each oligonucleotide were created and diluted accordingly for validation experiments.

2.5 - Dynamic range

Range finding experiments were carried out with each of the oligonucleotides to determine the dynamic range of the qPCR. Serial dilutions of the oligonucleotide stocks were created in RNase free water which spanned 10 orders of magnitude (10 fM – 10 µM). Reverse transcription and quantitative PCR was then carried out as described previously. The range of the serial dilutions was altered subsequently to generate linear standard curves for the subsequent validation experiments.

2.6 - Assay precision

To assess the precision of reverse transcribing miRNA to cDNA and quantifying cDNA using qPCR, inter and intra-assay variation were measured. Intra-assay variation was measured by generating serial dilutions from the oligonucleotide stocks, followed by reverse transcription and qPCR. This process was repeated in five independent experiments on the same day to generate an n of five. A similar process was carried out to determine inter-assay variation but with the process carried on five separate days. The coefficients of variation were determined at each oligonucleotide concentration as a measure of the precision of the qPCR.

2.7 - Recovery of the miRNA isolation and purification procedure

To determine how the miRNA extraction procedure performed in terms of analyte recovery, known amounts of the miR-194 miRNA oligonucleotide were “spiked” into RNase free water and RNA was quantified using nanodrop. MiRNA isolation was then carried out as described previously followed by nanodrop quantification of the isolated product to determine the fraction of the starting miRNA which was recovered.

2.8 - miRNA stability after freeze-thawing and in various biological matrices

The stability of the miRNA oligonucleotides were evaluated under a range of storage conditions. Serial dilutions of the oligonucleotides were stored at -80°C, thawed, reverse transcribed and quantified using qPCR before re-freezing. This process was repeated to judge the effect of numerous freeze-thaw cycles on miRNA stability. A similar process was repeated with serial dilutions being stored at 4°C to further assess miRNA stability.

The stability of three endogenous miRNAs was also assessed in RNase free water. MiRNA was isolated and purified from urine from a healthy Sprague-Dawley rat in triplicate as

previously described. The miRNA containing RNase free water product was then aliquoted into separate RNase free tubes and miRNA species quantified at zero, one, four and eight days post-extraction.

The stability of synthetic miRNA species was assessed in serum, urine and plasma. Synthetic miR-194 was spiked into each biofluid at a range of concentrations (100 fM – 1 nM). The miRNA isolation and purification procedure was carried out as previously described and the product reverse transcribed and qPCR carried out to generate standard curves.

2.9 - Normalisation of qPCR data

Quantified miRNA species were normalised in two separate ways to allow comparison of two routinely used normalisation techniques. The first method involved the measurement of the endogenous small nuclear ribonucleoprotein, U6snRNA, in all urine samples alongside miRNA measurements. Each individual miRNA species measured was then normalised by the ΔΔCt method to its corresponding U6snRNA measurement in the corresponding sample.

The second method of normalisation was implemented following investigation into the fractional miRNA recovery from the extraction and purification kits. A synthetic oligonucleotide was spiked into serum and/or urine samples prior to the isolation and purification of miRNA species to allow for correction to variations in miRNA recovery during the isolation and purification procedure. To prevent the degradation of the synthetic miRNA species by RNase activity in sample matrices the protocol to isolate and purify miRNAs was modified slightly. The procedure was carried out as described above except the Qiazol reagent was added to the sample and incubated for five minutes (RT) prior to the addition of the lin-4 oligonucleotide to ensure thorough denaturation. The lin-4 (5 µl) oligonucleotide was added to the solution at a final concentration of 50 fM.

2.10 - Statistical analysis

Each experiment involved triplicate qPCR repeats of the reverse transcription cDNA product. Each independent experiment was repeated to generate at least an n of three. Error bars represent standard error of the means (SEM) of the three independent experiments. Shapiro Wilk test was used to test all data for normality followed by the Kruskal Wallis test to assess statistical significance.

3 - **Results**

3.1 - Assay precision

To validate the precision of the reverse transcription and quantitative PCR assays used to quantify miRNAs, the intra-assay and inter-assay variation of the procedures was determined. Inter-assay and intra-assay variations were determined through the generation of a series of standard curves using the synthetic miR-194 oligonucleotide which was repeated, by the generation of new standards on five separate or five times on the same day respectively (figure 1, table 1). There was very little inter-assay variation between any of the five replicates. The coefficients of variation are all below 10 % except for the smallest concentration. There was, again, very little intra-assay variation between any of the five standard curves.

3.2 - miRNA Recovery

The ability of the QIAGEN miRNeasy kit to recover miRNA was determined to assess the effectiveness of the isolation procedure. Known concentrations of a synthetic miR-194 oligonucleotide were spiked into water prior to the isolation procedure. The miR-194 oligonucleotide was then re-quantified to determine the fraction of the oligonucleotide which was recovered during the isolation procedure. The miRNA recovery varied significantly from between 89 and 54 % (figure 2). Recovery not only varied between experiments but also varied significantly between different oligonucleotide concentrations. The results also indicate the recovery is most varied at lower concentrations of miRNA.

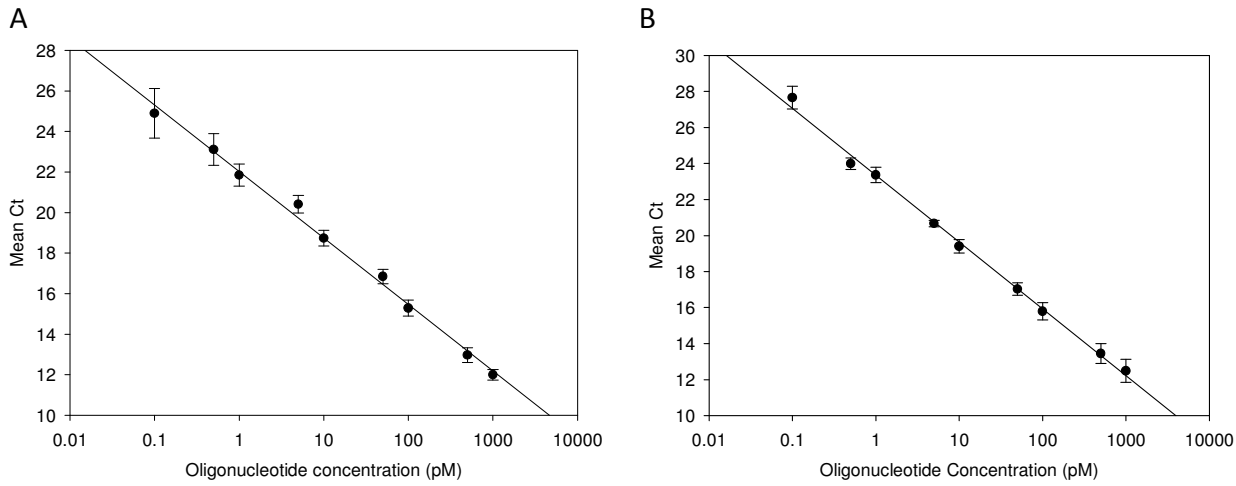

Figure 1: Inter-assay (A) and intra-assay (B) variation of reverse transcription and qPCR procedures for miR-194 oligonucleotide. The results show the mean of five independent experiments, each with and n=3. Error bars represent the standard error of mean of the three replicates.

Table 1: Intra-assay variation of miR-194 oligonucleotide Spiked into RNase free water. Ct values are the mean of five independent experiments. Precision is calculated as coefficients of variation.

miR-194 oligo conc. (pM)	Inter-assay standard deviation	Inter-assay coefficient of variation	Intra-assay standard deviation	Intra-assay coefficient of variation
0.1	2.75	11.04%	1.42	5.12%
0.5	1.75	7.58%	0.71	2.97%
1	1.22	5.56%	0.95	4.07%
5	0.98	4.79%	0.39	1.89%
10	0.86	4.60%	0.84	4.34%
50	0.79	4.70%	0.77	4.51%
100	0.87	5.70%	1.07	6.80%
500	0.80	6.18%	1.24	9.23%
1000	0.59	4.91%	1.43	11.44%

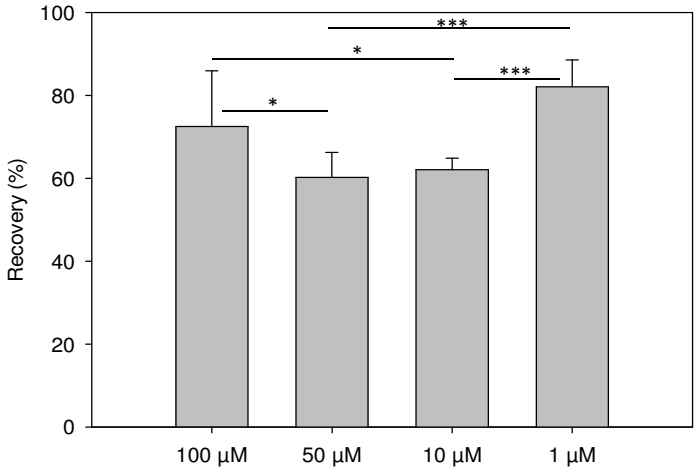

Figure 2: Mean recovery of miR-194 oligonucleotide at a range of concentrations from the QIAGEN miRNA extraction kits. Each bar represents the mean of three independent experiments and each independent experiment encompasses three technical replicates. Error bars represent the SEM of the three experiments. *** P<0.001, *P<0.05

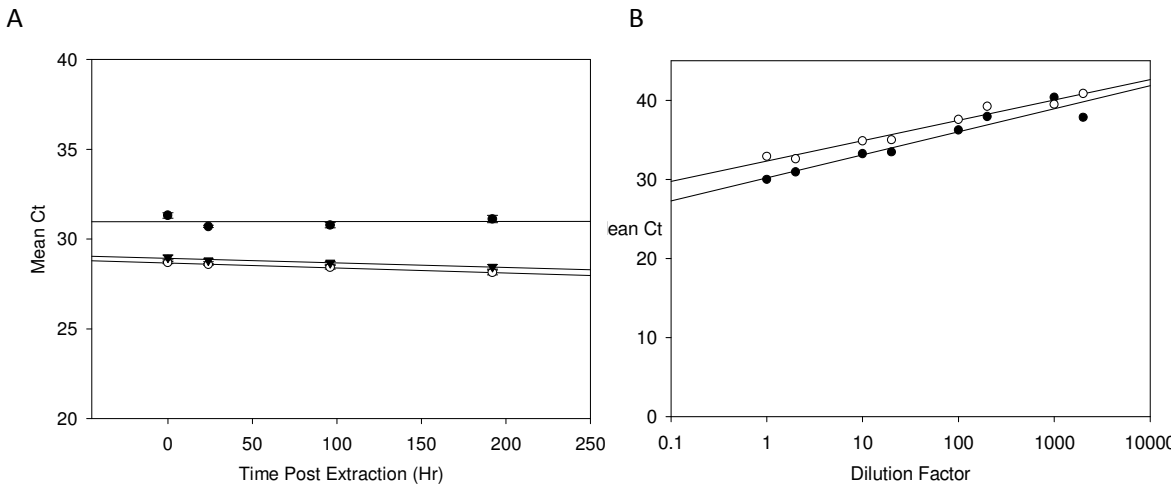

Figure 3: (A) Mean Ct values (n=3) of three endogenous miRNA species (miR-26b (circles), miR-30b (open circles), miR-30c (triangles) in RNase free water quantified at various time points post isolation and purification from healthy rat urine. **(B)** Mean Ct values of the endogenous miR-26b isolated from control urine. miR-26b was then quantified after one freeze thaw cycle (circles) and five freeze-thaw cycles (open circles).

3.3 - Endogenous miRNA stability

The majority of commercially available miRNA isolation and purification procedures yield the purified miRNA product in a small volume of RNase free water which may be then frozen and used at a later date. The stability of the endogenous kidney enriched miR-26b, miR-30b and miR-30c in RNase free water were assessed after their isolation and purification from urine from a healthy Sprague-Dawley rat. There were no significant differences in the quantifiable levels of any of the three endogenous kidney enriched miRNAs when quantified at varying time points up to eight days post miRNA isolation and purification (Figure 3A).

The endogenous miRNA stability was also assessed in control Sprague-Dawley rat urine over a number of freeze-thaw cycles. MiRNAs were isolated and purified from control rat urine after the urine had been subjected to a number of freeze-thaw cycles. Serial dilutions from the miRNA product were then made and quantified miRNAs using qPCR. This procedure was repeated after freeze-thawing the urine samples a number of times (figure 3B). A non-significant decrease in the quantified miRNA between the first and fifth freeze-thaw cycle was found.

3.4 - Stability of synthetic miRNA oligonucleotides

As it was found that the recovery of the miRNA isolation and purification procedure was inconsistent it was necessary to normalise qPCR results to an internal standard which could be spiked into the sample matter prior to the isolation and purification of miRNA species. The stability of synthetic miRNAs in RNase free water was assessed over a number of freeze-thaw cycles and over a number of temperatures. Synthetic miR-194 was added to RNase free water over a range of concentrations and a standard curve generated. The standards

were then subjected to five freeze-thawing cycles (-80 ˚C) and the standard curves repeated after each cycle. No significant difference between any of the standard curves generated after each freeze-thaw cycle were observed (figure 4A).

The stability of the miR-194 oligonucleotide at 4 ˚C was assessed on a weekly basis for a period of three weeks. There was no significant reduction in the quantified miRNA levels over the three weeks as shown by the coefficients of variation (table 2).

Finally the stability of synthetic miRNA species in human serum, plasma and rat urine was investigated through the generation of standard curves using the same oligonucleotide concentrations as described previously. There was no correlation between the miR-194 oligonucleotide concentration and the mean Ct values in serum while it was found that in urine, as the oligonucleotide concentration increased, so too did the Ct value. MiR-194 oligonucleotide was not detected in plasma at any of the concentrations used (figure 4B).

3.5 - Development of a method to allow the spiking in of an endogenous synthetic miRNA internal standard

To allow the Spiking of a synthetic exogenous miRNA species into serum or urine without degradation the protocol to denature the RNases in the biofluid prior to the addition of the exogenous miRNA was modified. To determine whether this approach would prevent the denaturation of the oligonucleotides the synthetic miRNA internal standard was spiked into both normal serum and urine and denatured serum and urine at the same concentrations.

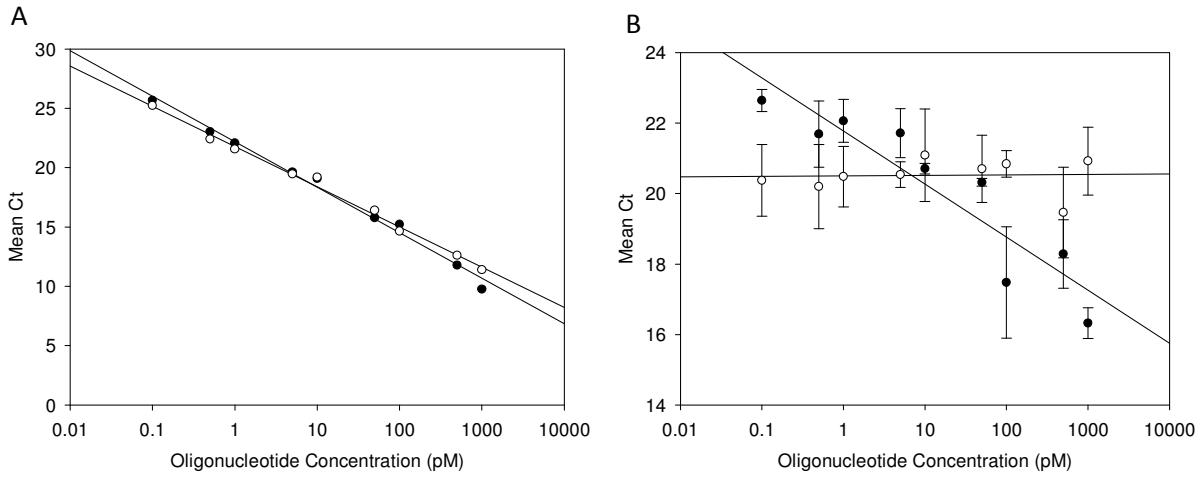

Figure 4:-(A) Mean Ct values (n=3) of synthetic miR-194 in RNase free water over a range of concentrations (circles) and the same standards after five freeze thaw cycles, shown as the mean of the five cycles (open circles). **(B)** Standard curves generated by the spiking of synthetic miR-194 oligonucleotide directly into serum (circles), plasma and urine (open circles). Each line represents the mean of three independent experiments and each independent experiment represents the mean of three technical replicates. Error bars represent the SEM of the three independent experiments.

Table 2:- Stability of synthetic miR-194 oligonucleotide measured on a weekly basis when stored at 4 ˚C. Each weekly reading is the mean of three technical replicates.

miR-194 oligo conc (pM)	Mean Ct weekly stability check run 1	Mean Ct weekly stability check run 2	Mean Ct weekly stability check run 3	Mean Ct	Standard dev	Coefficient of variation	SEM
0.1	26.405	24.385	25.435	25.408	0.850	3.3%	0.491
0.5	22.990	24.540	23.940	23.823	0.634	2.7%	0.366
1	22.180	22.015	22.035	22.077	0.070	0.3%	0.040
5	20.115	21.700	20.800	20.872	0.678	3.3%	0.392
10	18.975	17.935	18.380	18.430	0.428	2.3%	0.247
50	16.415	17.490	17.770	17.225	0.561	3.3%	0.324
100	15.030	16.745	14.860	15.545	1.004	6.5%	0.580
500	12.705	14.310	12.850	13.288	0.828	6.2%	0.478
1000	11.745	11.715	12.985	12.148	0.690	5.7%	0.399

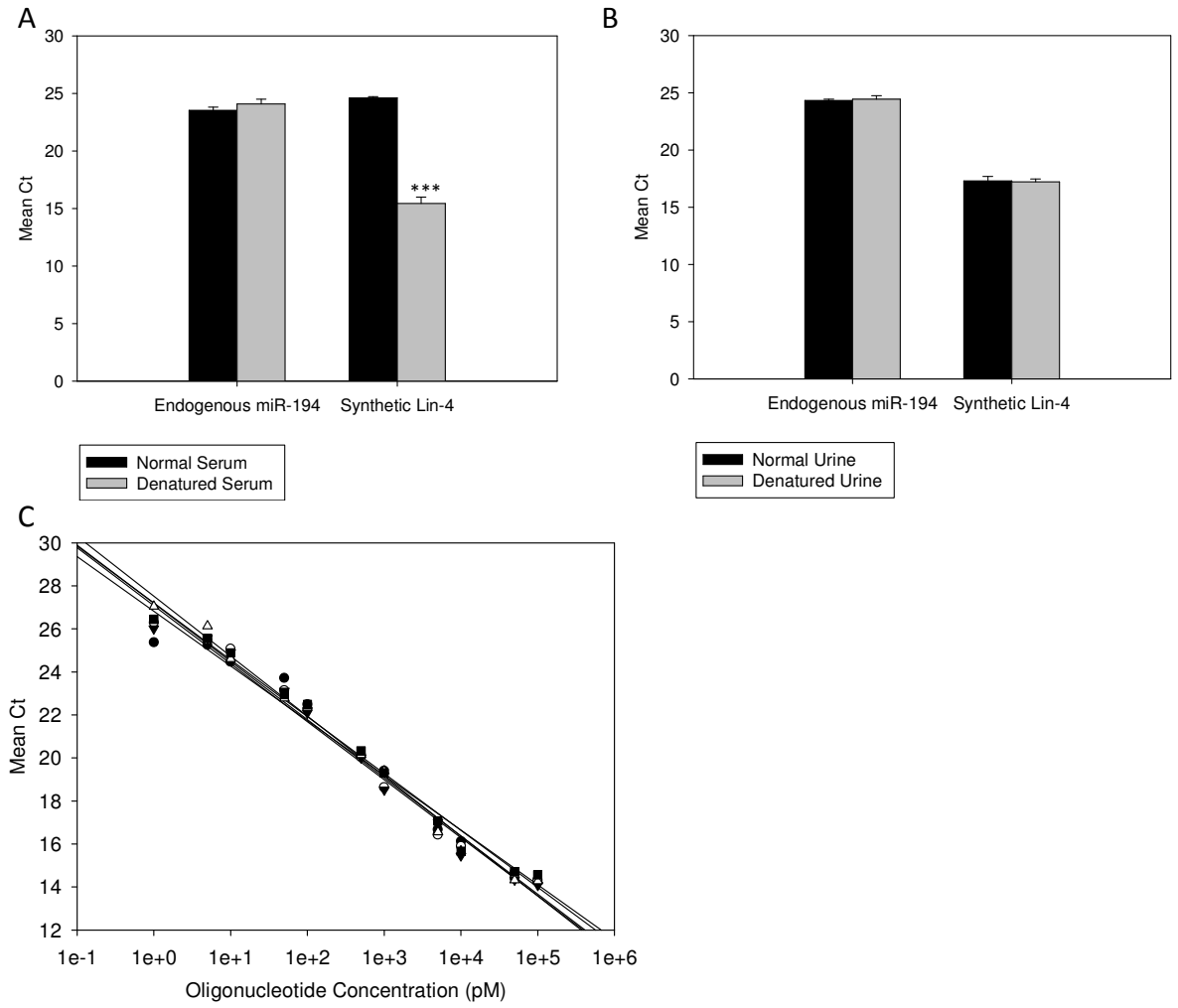

Figure 5:- Quantification of both the endogenous miR-194 and the exogenous c.elegans lin-4 oligonucleotide in both normal and denatured serum **(A)** and urine **(B)**. Each bar represents the mean of three independent experiments and each experiment is the mean of three technical replicates. Error bars represent to SEM of three independent experiments. **(C)** Stability of the synthetic C.elegans Lin-4 oligonucleotide when spiked into denatured urine after various numbers of urinary freeze-thaw cycles. Freeze-thaw cycle one (circles), two (open circles), three (triangles), four (open triangles), five (squares). *** P<0.001.

The quantity of synthetic lin-4 oligonucleotide is much greater when spiked into denatured serum than into normal serum as shown by the large difference in mean Ct value while not affecting the quantification of an endogenous miRNA (figure 5A). Very little variation between experiments was observed as shown by the error bars. A similar scenario in urine was observed in that the level of the endogenous miR-194 oligonucleotide was not affected by the denaturing however the exogenous synthetic lin-4 oligonucleotide is also not affected and remains constant in the denatured and normal urine (figure 5B).

To further assess whether the spiking of on exogenous internal standard would remain stable in urine, a synthetic oligonucleotide (identical to C.elegans Lin-4) was spiked into denatured control urine after a number of freeze-thaw cycles, the miRNAs isolated and purified, the product diluted with RNase free water and Lin-4 quantified to generate standard curves (figure 5C). There was no significant difference between any of the freeze-thaw cycles.

4 - **Discussion**

MiRNAs represent attractive developmental organ specific targets as candidate biomarkers for drug safety. However, techniques involving the isolation, purification and quantification of various miRNA species from various biological samples are relatively recent developments. Therefore, it is essential that these techniques are validated and a robust and repeatable standard of operation for their isolation and quantification is developed before being used for further evaluation as a candidate biomarker. This current investigation reports the validation of methods used to isolated and quantify potential miRNA biomarkers present in non-invasive biofluids that could be indicative of renal dysfunction.

4.1 - Precision of the techniques used to quantify miRNA species

In recent years methods have been developed to allow the quantification of miRNA species through qPCR where in the past techniques such as northern blotting were the principal method of miRNA detection which only allowed semi-quantification. Due to the expanding knowledge surrounding miRNAs and the increasing focus of research into the potential of them to serve as diagnostic markers of various disease states or progression, it is essential that the techniques used to quantify them are reliable. This body of work set out to determine the precision of the quantitative PCR technique through the determination of the inter-assay and intra-assay variability of the qPCR through the generation of a series of standard curves performed on the same day and over a number separate of days respectively. The results for the intra-assay variation show that the coefficients of variation are all less than 10 % except at the highest oligonucleotide concentration of 1 nM (11.44 %). The results for the inter-assay variation also tell a similar story with all coefficients of

variation falling below 10 % except at the lowest oligonucleotide concentration (0.1 pM). Together these results show that the qPCR procedure is highly precise in detecting miRNA species over a range of concentrations.

4.2 - MiRNA recovery from QIAGEN miRNeasy and minELUTE cleanup kits

Recovery parameters for an analyte in an assay, defined by the detector response obtained from an amount of the analyte added to and extracted from the biological matrix compared to the detector response obtained for the true concentration of the pure authentic standard, are important to define for new methodologies. As long as the recovery of the analyte is consistent, precise and reproducible this need not be 100%. This study found that there were significant differences in the fraction of a synthetic miRNA recovered from the miRNA isolation kits. Significant differences were found not only when different starting concentrations of miRNA were loaded into the kits but also between independent experiments at the same concentrations (as indicated by large error bars) (figure 2). This could have dramatic effects on the interpreted quantity of the miRNAs present in a study. One possible solution to this variation in recovery from the miRNA isolation and purification kits is to normalise results to an internal standard which is spiked into the biological sample matter prior to the extraction of miRNAs to correct for variations in miRNA recovery from the kits.

4.3 - Endogenous miRNA stability in urine

Previous studies have shown that miRNAs are incredibly stable in serum (Mitchell *et al.*, 2008) however there is little literature regarding miRNA stability in urine. This study showed that three kidney enriched miRNA species were stable when isolated from urine immediately after collection and stored frozen in RNase free water for up to eight days

(figure 3A). It was also shown that the endogenous kidney enriched miR-26b seems to begin to degrade if urine is subjected to multiple freeze-thaw cycles prior to miRNA isolation; highlighting the need for immediate sample processing to avoid miRNA degradation (figure 3B).

4.4 - Synthetic miRNA stability

Having demonstrated that miRNA recovery is variable during the isolation and purification procedure the aim was then to develop a method of normalisation using a spiked in synthetic miRNA standard. With this in mind, the stability of synthetic miRNA oligonucleotides over a range of conditions was investigated. It was demonstrated that synthetic miRNA oligonucleotides are stable in RNase free water over a number of freeze-thaw cycles (figure 4A). They were also found to be incredibly stable in RNase free water when stored at 4 ˚C over three weeks (table 2) indicating that miRNA species are extremely stable when stored at various conditions in RNase free water.

However, it was also clearly demonstrated that when synthetic miRNA oligonucleotides are spiked directly into various biofluids their quantification is interfered with which is most likely due to degradation of the oligonucleotides by endonuclease activity in the biofluid (figure 4B). This is largely in agreement with the findings of *Mitchell et al. 2008* who showed similar results for serum. However *Mitchell et al. 2008* detected synthetic miRNAs in plasma whereas this study could not (synthetic or endogenous) at any concentration. This can most likely be attributed to the heparinised plasma which was used in the present study which has been previously reported to interfere with PCR (Kroh *et al.*, 2010). This investigation also demonstrates that a loss in stability also occurs when spiked into urine, as indicated by

larger error bars, although not to the same extent to that of in serum, possibly due to the greater endonuclease activity in serum.

4.5 - Development of a method to prevent the degradation of synthetic miRNAs in biofluids

This investigation demonstrates that synthetic miRNAs are degraded if spiked directly into serum, plasma and urine, so the biofluids were first denatured to examine whether this would prevent their degradation. Endogenous miR-194 was also measured in the same samples to investigate whether this affected the levels of endogenous miRNA species. Denaturing serum prior to the spiking of the exogenous oligonucleotide caused a significant increase in the quantity of the oligonucleotide in the product when compared to normal serum (figure 5A). Again, it was not possible to detect miRNA, synthetic or otherwise, in plasma samples as previously described. Both the synthetic oligonucleotide and the endogenous miR-194 were detectable in urine however there were no significant differences in the levels of the endogenous or exogenous miRNA in both normal and denatured urine. This may indicate that the differences observed when spiking miR-194 into urine described previously were a result of losses from the isolation and purification procedure rather than RNase activity in the urine, although it is known that urine has a degree of RNase activity (Weickmann *et al.*, 1982).

To further investigate the stability of synthetic miRNA oligonucleotides in urine the synthetic c.elegans Lin-4 oligonucleotide was spiked into denatured control urine after a number of urinary freeze-thaw cycles (Figure 5C). There were no significant differences in the standard curves and they all showed the expected level of linearity with very little variation between experimental repeats indicating a stable synthetic miRNA in the purified product.

This project has assessed the validity of techniques used to isolate, purify and quantify miRNA species from tissue samples, techniques which are sure to be used more frequently as the interest and understanding of the complex role of miRNAs increases. The qPCR procedure used to quantify miRNA species has been shown to be highly specific and can repeatedly measure precisely to at least an fM level. Endogenous miRNAs isolated from urine have been shown to be stable in RNase free water after a number a freeze-thaw cycles indicating that storage in RNase free water is suitable without risk of degradation of miRNAs. Conversely this data suggests that endogenous miRNAs can begin to degrade if not isolated from urine prior to being subjected to freeze thawing. This research into the stability of synthetic miRNAs has shown that they are incredibly stable in RNase free water after a number of freeze-thaw cycles and when stored at 4 °C. However synthetic miRNA is rapidly degraded in serum and to a lesser extent, urine. The findings in serum are in agreement with other research which suggested that they are rapidly degraded by RNases (Mitchell *et al.*, 2008). This research also suggests that miRNAs cannot be detected in heparinised plasma. A method to spike in synthetic miRNA species into serum and urine without their degradation was developed to serve as exogenous internal standards to help correct for losses of miRNAs during the isolation and purification procedure. These findings warrant further investigation and it is essential that a standard, widely accepted method of normalisation is developed if miRNAs are to be subject to continued investigation. Finally this study shows that miRNAs can be isolated from urine and are incredibly stable if isolated immediately and stored in RNase free water. This holds promise for future studies using urinary miRNAs as potential biomarkers of disease. The techniques validated and developed in this study will form the basis of future studies involving miRNA species in this thesis.

Chapter 3

MicroRNAs as Potential Biomarkers of Kidney Injury in Vivo

Contents

1 - Introduction

A biomarker is any measurable parameter in the body which can indicate a biological, pathological or pharmacological process. The FDA defines a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacological responses to therapeutic intervention". Biomarkers are usually used to indicate damage or to predict the likelihood of damage although they can be used to diagnose disease or injury, to indicate the status of a disease or injury (extent, severity) and to predict or monitor the outcome of an intervention. Prior to the marketing of a new compound and its approval by the FDA, MHRA or EMEA It must first be subjected to rigorous testing to ensure its suitability (Ozer *et al.*, 2008).

Acute Kidney Injury (AKI) is caused by many chemical agents, including amine antibiotics and chemotherapeutics and causes problems in clinical settings. In a drug development setting AKI causes the cessation of development of approximately 30 % of compounds from the stage of identification of a potential compound to marketed products and in a clinical setting nephrotoxicity is estimated to be responsible for 19 – 25 % of all cases of AKI (Bonventre *et al.*, 2010). Currently AKI is diagnosed using quantification of serum creatinine (SCr) and/or blood urea nitrogen (BUN) however SCr and BUN are known to be insensitive biomarkers for kidney injury due to elevations only being observed after loss of approximately half of the functional kidney capacity (Bonventre *et al.*, 2010; Vaidya *et al.*, 2010). For this reason there is a real need for more sensitive biomarkers of kidney injury to allow earlier, more reliable diagnosis of kidney injury. (Bonventre *et al.*, 2010; Ozer, 2010; Ozer *et al.*, 2010).

This chapter focuses on the potential of urinary miRNAs to be used as biomarkers of kidney injury in an ischemic reperfusion induced model of kidney. Ischemic reperfusion injury involves occlusion of the blood supply to a particular organ or tissue for a period of time followed by reperfusion which leads to generation of reactive oxygen species (ROS) which contribute to dysfunction (Mishra *et al.*, 2003; Renolleau *et al.*, 1998). This work was preceded by the reproduction of a recently published method to quantify circulating serum miRNAs in a model of APAP induced hepatotoxicity to confirm similar results could be obtained in this laboratory (Girard *et al.*, 2008; Mitchell *et al.*, 2008; Wang *et al.*, 2009).

Acetaminophen (APAP) is a well-established hepatotoxin in pre-clinical species and in man where it causes primarily centrilobular hepatic necrosis. At therapeutic doses APAP is primarily detoxified by conjugation with sulphate or a glucuronic acid and a small amount is excreted as a mercapturic acid after metabolism via CYP P450 (primarily CYP2E1) and conjugation with glutathione. At toxic doses the glucuronidation and sulphation pathways become rapidly saturated so metabolism by CYP enzymes becomes the primary route which forms a chemically reactive intermediate N-acetyl-p-benzoquinoneimine (NAPQI) (Amacher, 2002; Antoine *et al.*, 2008). NAPQI is detoxified through conjugation with glutathione (GSH) however at large doses, GSH becomes rapidly depleted. Once GSH has been depleted by ~80 % NAPQI covalently binds to cellular proteins, cause oxidative stress and oxidises protein sulphydryl groups as well as causing lipid peroxidation.

The hypothesis of this chapter is that kidney enriched miRNA species can be used as preclinical biomarkers of kidney injury. This will be examined through the following aims:

- Replication of a published method of using miRNAs as biomarkers of APAP induced DILI.

- Quantification of four kidney enriched miRNA species in the urine of mice subjected to either 10 or 30 minutes of kidney ischemia followed by reperfusion.
- Quantification of the same miRNAs in the serum of an APAP induced DILI.

The investigations involving miRNAs in this chapter will be undertaken using the techniques and methods of normalisation which were validated in chapter two.

2 - Materials and Methods

RNA oligonucleotides (synthetic miRNAs) were purchased from integrated DNA technologies (IDT). RNA isolation and purification kits were purchased from QIAGEN (miRNeasy mini kit, RNeasy minElute cleanup kit). Reverse transcription specific and qPCR specific miRNA primers (TaqMan® MicroRNA Assays) were obtained from Applied Biosystems. Reagents for reverse transcription and for qPCR were also obtained from Applied Biosystems (TaqMan® MicroRNA Reverse Transcription Kit and TaqMan® Universal PCR Master Mix). All other reagents were purchased from Sigma (Poole, UK) unless otherwise stated.

2.1 - Experimental animals

The protocols described were in accordance with criteria outlined in a licence granted under the Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Animal Ethics Committee. CD1 male mice for the APAP study were purchased from Charles River laboratories and had a five day acclimatisation period prior to experimentation. Animals were maintained in a 12 hour light/dark cycle and had access to food and water Ad libitum.

2.2 - Histological analysis

Tissue samples were isolated from animals and were fixed in neutral buffered formalin (10 %). To prepare for histological assessment 3 -5 µM paraffin sections were prepared and stained with haemotoxylin and eosin (H & E). Stained sections were examined for any histopathological features blinded.

2.3 - APAP animal dosing regime

Male CD1 mice (28-33 g) had access to food and water Ad libitum throughout the experimental procedures and throughout the acclimatisation period. Mice were

administered with a single IP injection of APAP (530 mg/kg) in 0.9 % saline for one, two, four or eight hours and control animals received 0.9 % saline for eight hours. Animals were euthanised with increasing concentrations of CO_2 and confirmed via cervical dislocation. Blood was collected via cardiac puncture and allowed to clot overnight at 4°C. Livers were removed and rinsed in ice cold 0.9 % saline.

2.4 - Induction of Ischemic Reperfusion in CD1 male mice

CD1 male mice were anaesthetised and incisions made on the flanks of the animals. The renal arteries of both kidneys were occluded for either 10 or 30 minutes followed by reperfusion. Mice were sutured and returned to individual metabolism cages. Mice were euthanized using increasing concentrations of CO_2 and confirmed via cervical dislocation. Animals were culled at 0, 3, 6, 9, 12, 18, 24, 48 and 72 hours post ischemic reperfusion and kidneys, blood and urine collected. Kidneys were fixed in 5 % formaldehyde.

2.5 - Determination of hepatic glutathione in CD1 male mice post APAP administration

A section of the major lobe from the liver of each mouse (30 – 50 mg) was homogenised in 5-sulfosalicyclic acid (200 µl; 6.5 % w/v) and GSH stock buffer (800 µl; 143 mM NaH_2PO_4, 6.3 mM EDTA, pH 7.4). The protein was allowed to precipitate on ice and was then centrifuged (14 000 rpm, 5 min). Liver protein samples were then dissolved in 1 M NaOH prior to protein content determination via methods described by Bradford *et al (Bradford, 1976)*. Total GSH content was determined spectrophotometrically (412 nm) using a kinetic reaction between GSH and GSH reductase on a Dynatech Laboratories UV-visible absorbance MRX microtiter-plate reader as previously described by Vandeputte *et al* (Vandeputte *et al.*, 1994). Results were expressed as µmol GSH per milligram of cellular protein.

2.6 - Determination of serum ALT levels in CD1 male mice post APAP administration

Serum ALT levels were determined by a kinetic enzymatic reaction in a 96 well plate. Serum samples were diluted appropriately in 0.9 % saline solution (1:5, 1:10 or 1:20). Sample (30 µl) was added to wells in triplicate followed by ALT liquid stable reagent-based kinetic assay (Thermo Scientific, Waltham, MA) (300 µl) at 37 ˚C according to manufacturer's instructions.

2.7 - miRNA isolation and purification

miRNA isolation and purification was carried out according to manufacturer's instructions. Briefly, 50 µl miRNA containing sample (serum, urine) were mixed with Qiazol reagent (700 µl). 100 % chloroform was added (140 µl) and mixed vigorously. Samples were centrifuged for 15 minutes (12 000 g, 4°C) and the supernatant transferred to a separate tube. An equal volume of ethanol (70 %) was added to the supernatant and mixed well. Sample was transferred (700 µl) at a time to RNeasy mini spin columns and centrifuged for 15 seconds (8,000 g, RT). The flow through was transferred to a separate tube and 0.65 volumes of ethanol (100 %) was added and mixed well. Samples were transferred (700 µl at a time) to RNeasy MinElute spin columns and centrifuged for 15 seconds (8,000 g, RT). The flow through was discarded and RWT (a stringent washing buffer) buffer (700 µl) was added to columns before centrifuging for 15 seconds (8,000 g, RT). Flow through was again discarded before the same being repeated with RPE buffer (500 µl). Ethanol (80 %) was added and centrifuged for two minutes (8,000 g, RT) and flow through discarded. Columns were the centrifuged for five minutes with their caps open (8,000 g, RT) followed by the addition of RNase-free water (14 µl) to the column membrane and final centrifugation for one minute (8,000g, RT).

2.8 - Reverse transcription of miRNA to cDNA

Reverse transcription was carried out according to manufacturer's instructions in a total reaction volume of 15 µl. Briefly a master mix was made up in RNase free water according to instructions from the manufacturer. MiRNA was added to non-skirted PCR plates (2 µl) followed by RNase free water (3 µl). RT master mix was added to each well (7 µl) followed by the miRNA RT specific primer (3 µl).

The following primer sequences were used:

miR-122: UGGAGUGUGACAAUGGUGUUUG

miR-1: UGGAAUGUAAAGAAGUAUGUAU

miR-218:UUGUGCUUGAUCUAACCAUGU

miR-192: CUGACCUAUGAAUUGACAGCC

Lin-4: UCCCUGAGACCUCAAGUGUGA

U6snRNA:
GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATACAGAGAAGATTAGCATGGCCCCTGC
GCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTT

miR-26b: UUCAAGUAAUUCAGGAUAGGU

miR-30b: UGUAAACAUCCUACACUCAGCU

miR-30c: UGUAAACAUCCUACACUCUCAGC

The plates were then sealed using PCR plate caps and centrifuged briefly (1 500 g). The plate was then run on a GeneAmp PCR system 9700 using the following parameters: 30 minutes at 16 °C, 30 minutes at 42 °C, 5 minutes at 85 °C and then held at 4 °C. The cDNA product was then stored at -80°C until required.

2.9 - Quantification of cDNA by qPCR

Amplification and quantification of cDNA by qPCR was carried out according to manufacturer's instructions at a total reaction volume of 20 µl. The cDNA product from

reverse transcription (1.33 µl) was added to separate wells in a skirted 96 well PCR plate in duplicate. qPCR master mix was then made up as shown in table 2 and scaled up appropriately. Master mix (17.67 µl) and miRNA specific primer (1 µl) was then added to each well in duplicate. Plates were then sealed and centrifuged briefly prior to running on the ABI PRISM 7000 sequence detection system for 50 cycles using the following parameters: 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds.

2.10 - Normalisation of qPCR data

Quantified miRNA species were normalised in two separate ways as previously described using the ΔΔCt method. The first method involved the measurement of the endogenous small nuclear ribonuclearprotein, U6 RNA, in all samples alongside miRNA measurements. Each individual miRNA species measured was then normalised to its corresponding U6snRNA measurement in the corresponding sample.

The second method of normalisation was implemented following investigations described in chapter two. A synthetic oligonucleotide was spiked into serum and/or urine samples prior to the isolation and purification of miRNA species to allow for correction to variations in miRNA recovery during the isolation and purification procedure. To prevent the degradation of the synthetic miRNA species by RNase activity in sample matrices the protocol to isolate and purify miRNAs was modified slightly. The procedure was carried out as described above except the Qiazol reagent was added to the sample and incubated for five minutes (room temperature) prior to the addition of the lin-4 oligonucleotide to ensure thorough denaturation to prevent subsequent denaturation of the exogenous standard. The lin-4 (5 µl) oligonucleotide was added to the solution at a final concentration of 50 fM.

2.11 - Quantification of Serum Creatinine and Urinary Kim-1

Serum creatinine and Urinary Kim-1 were quantified in the Bonventre laboratory (Harvard, Boston, Massachusetts), according to standard laboratory practice.

2.12 - Statistical analysis

Each experiment involved duplicate qPCR repeats of the reverse transcription cDNA product. Each independent experiment was repeated to generate at least an n of three. Error bars represent standard error of the means (SEM) of the three independent experiments. Shapiro Wilk test was used to test all data for normality followed by the Kruskal Wallis test to assess statistical significance.

3 - Results

3.1 - Quantification of miRNAs in the serum of APAP dosed mice

Hepatic glutathione levels and serum ALT levels were assessed in the livers and the serum of the APAP dosed mice respectively to assess both the metabolic status and the degree of liver injury in the mice (figure 1, 2). Hepatic glutathione levels were found to decrease significantly one and two hours post APAP administration. This significant decrease was followed by increases back towards the basal (control) glutathione levels at four and eight hours post APAP administration. No significant elevations in serum ALT levels were observed up until four hours post APAP administration. It should be noted however that two mice in the eight hour time group were culled prior to the scheduled culling time so were not used in the study.

Four different miRNA species were quantified in the serum of control and APAP dosed CD1 male mice. MiR-122 and miR-192 were quantified as they have been previously been reported to be elevated in the serum of mice with APAP induced liver injury (Wang *et al.*, 2009). MiR-1 and miR-218 were quantified as they are found to be highly expressed in organs other than the liver (heart and kidney respectively) (Girard *et al.*, 2008; Mitchell *et al.*, 2008). All four miRNA species quantified in the serum were normalised to the endogenous U6snRNA (figure 3, 4, 5, 6). No significant elevation in miR-1 or miR-218 was observed from the control at any time point measured post APAP administration. However significant elevations in serum levels of miR-192 and miR-122 were observed at both four and eight hours post APAP administration.

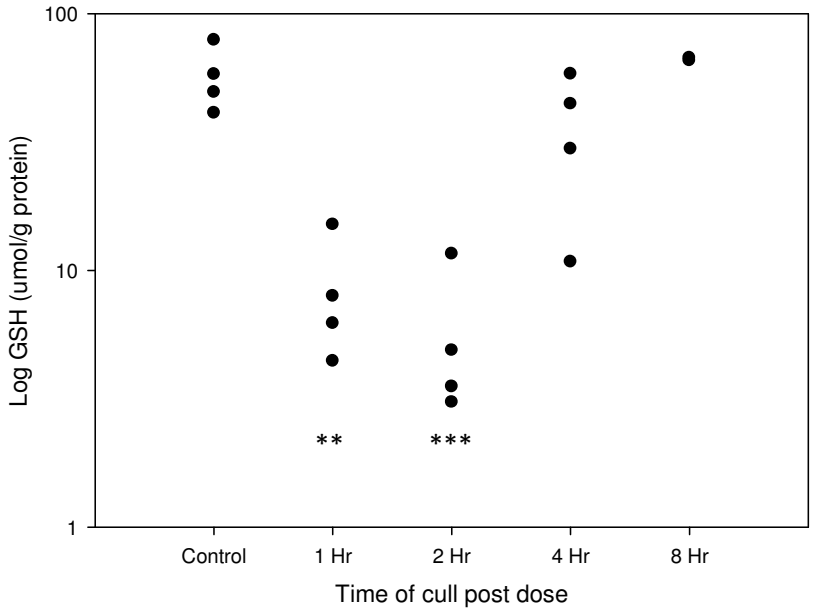

Figure 1:- Hepatic glutathione levels in the livers of control and APAP dosed CD1 male mice. Glutathione levels are expressed as µmol glutathione per gram of protein. **P<0.01, ***P<0.005.

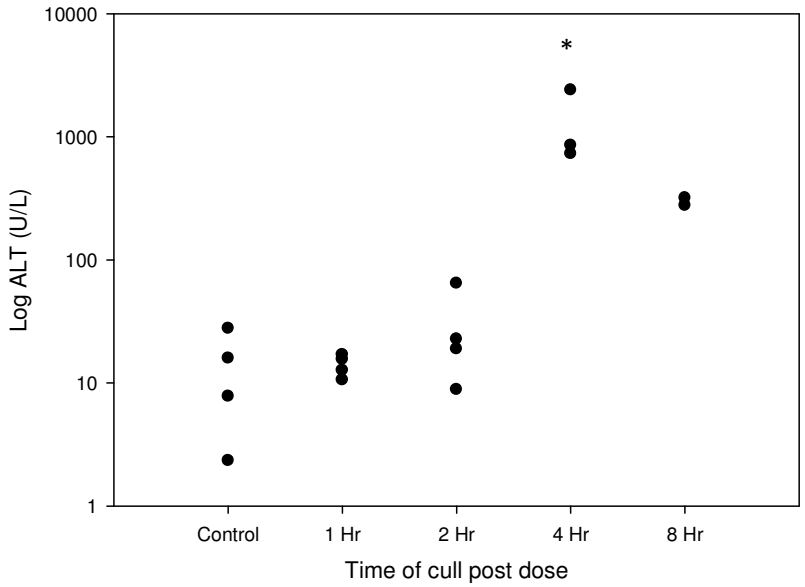

Figure 2:- ALT levels quantified in the serum of control and APAP dosed CD1 male mice. *P<0.05.

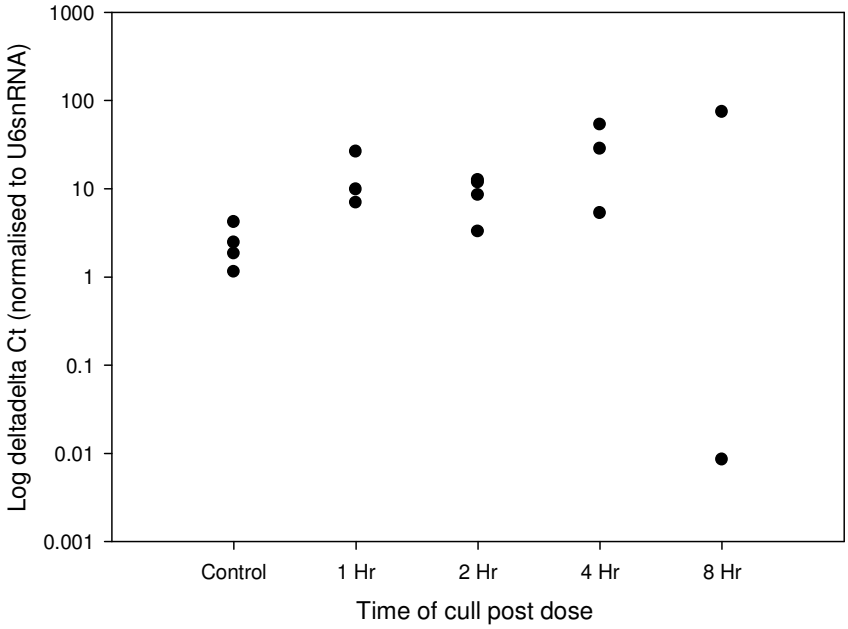

Figure 3:- miR-1 quantified in the serum of control and APAP dosed CD1 male mice normalised via the ΔΔCt method to U6snRNA.

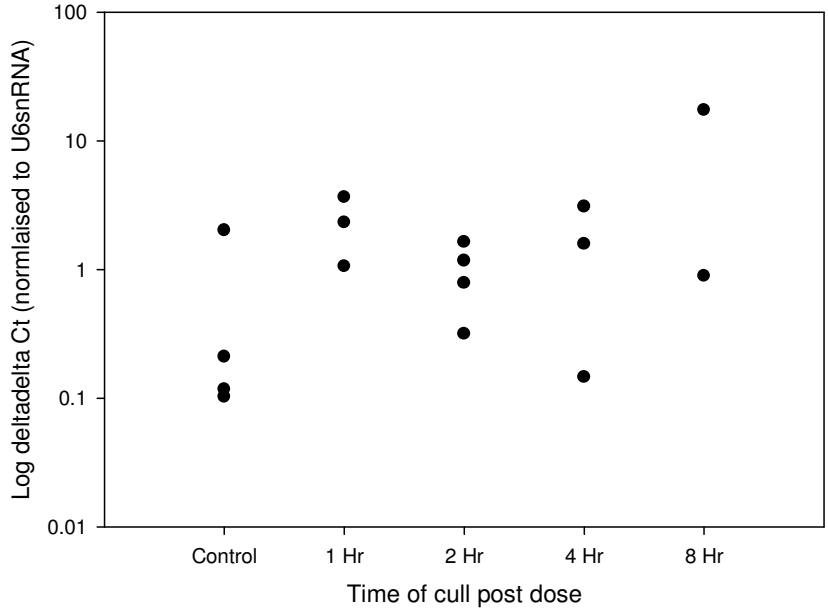

Figure 4:- miR-218 quantified in the serum of control and APAP dosed CD1 male mice normalised via the ΔΔCt method to U6snRNA.

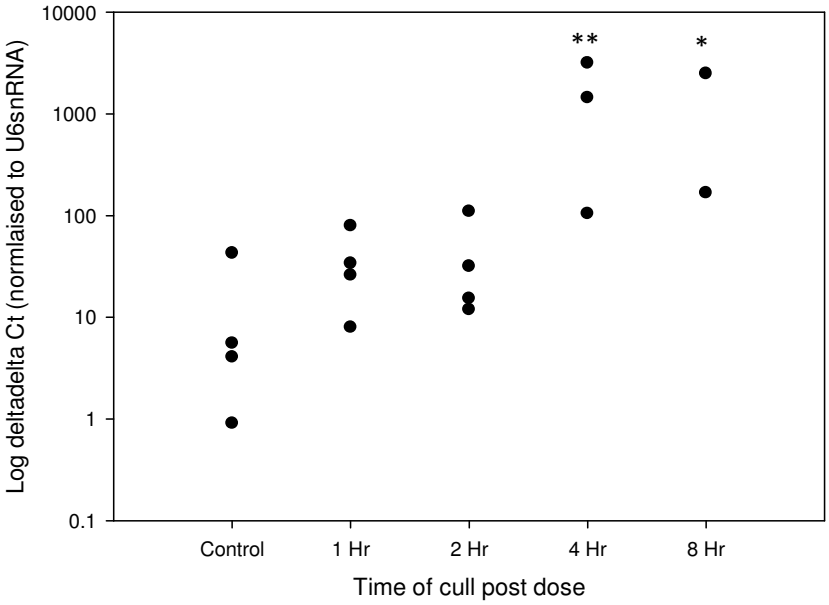

Figure 5:- miR-192 quantified in the serum of control and APAP dosed CD1 male mice normalised via the ΔΔCt method to U6snRNA. *P<0.05, **P<0.01.

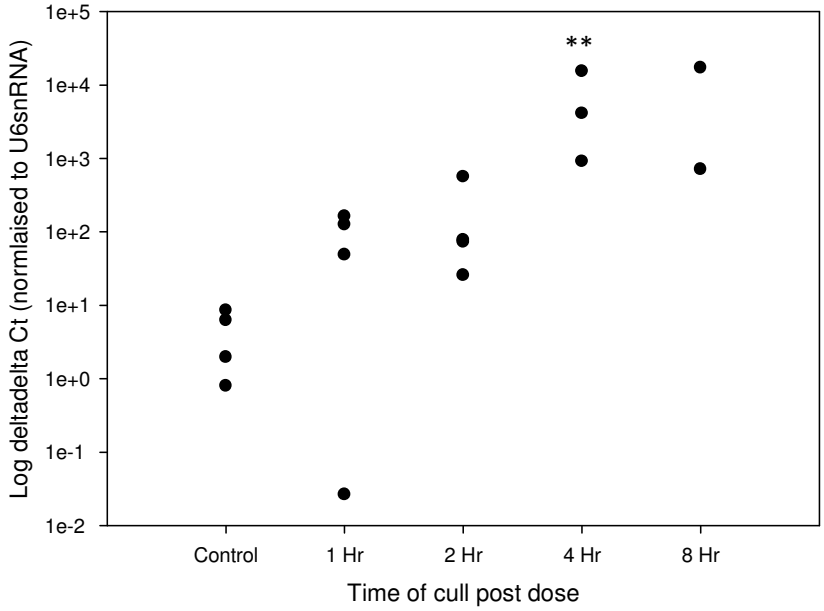

Figure 6:- miR-122 quantified in the serum of control and APAP dosed CD1 male mice normalised via the ΔΔCt method to U6snRNA. **P<0.01

3.2 - Quantification of kidney enriched miRNAs in an ischemic reperfusion mouse model of kidney injury

Creatinine was quantified in the urine of mice which had undergone various periods (10 or 30 minutes) of ischemic reperfusion induced kidney injury (figure 7). Urinary creatinine initially decreased sharply in both the 10 and 30 minute ischemic reperfusion models of kidney injury (significantly so in the 30 minute model only). This was followed by an increase back towards baseline from 12 hours post ischemic reperfusion in both models although levels had not returned back to baseline at 72 hours post ischemic reperfusion. Serum creatinine (figure 12, 13) and urinary Kim-1 (figure 14, 15) were also quantified in the animals. Serum creatinine showed a significant increase from the animals in the 10 minute group (figure 12) prior to the injury at nine hours which peaked at 24 hours followed a decrease back towards baseline. Urinary Kim-1 (figure 14) was found to be significantly elevated at three hours which peaked at 12 hours followed by a decrease back towards baseline which was still found to be statistically significant at 72 hours. The 30 minute group showed significant increases in serum creatinine (figure 13) as early as three hours post injury which peaked at 12 hours followed by a decrease back to baseline. However in this group the serum creatinine values were still significantly elevated at 72 hours. Urinary Kim-1 (figure 15) was also found to be significantly elevated at three hours and peaked at 12 hours before a slight decrease which was still significantly elevated from the control animals. Histopathological analysis of the proximal tubules of the animals revealed that in both groups all control animals had a histopathology grade of zero. The 10 minute group had a greatest histopathology grade of three at nine and 12 hours. The 30 minute group however had peak histopathology grades of five which were observed as early as nine hours.

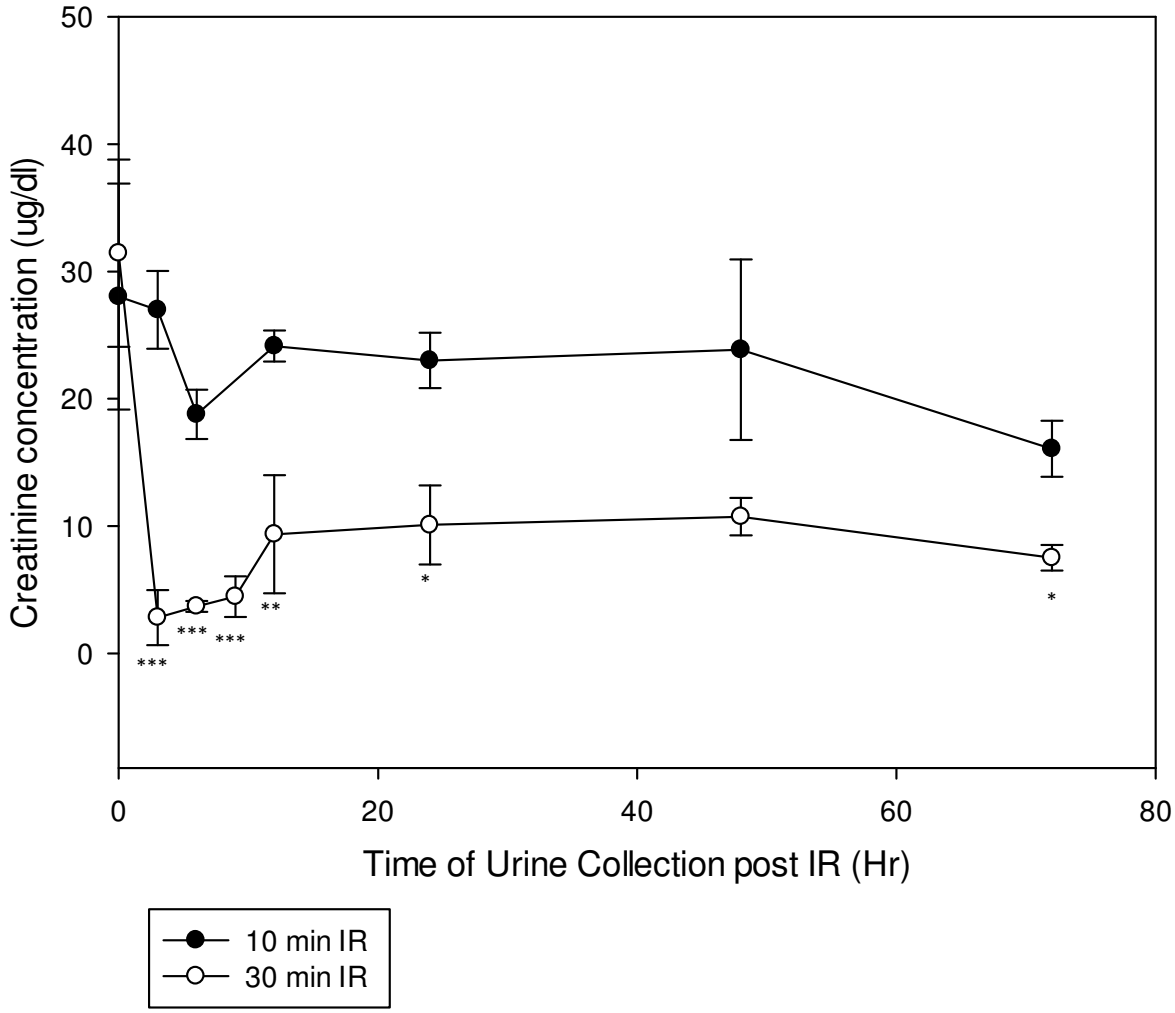

Figure 7:- Mean urinary creatinine for each time point for both 10 minute IR injury (closed circles) and 30 minute IR injury (open circles). Error bars represent the SEM. *** P<0.005, **P<0.01, *P<0.05

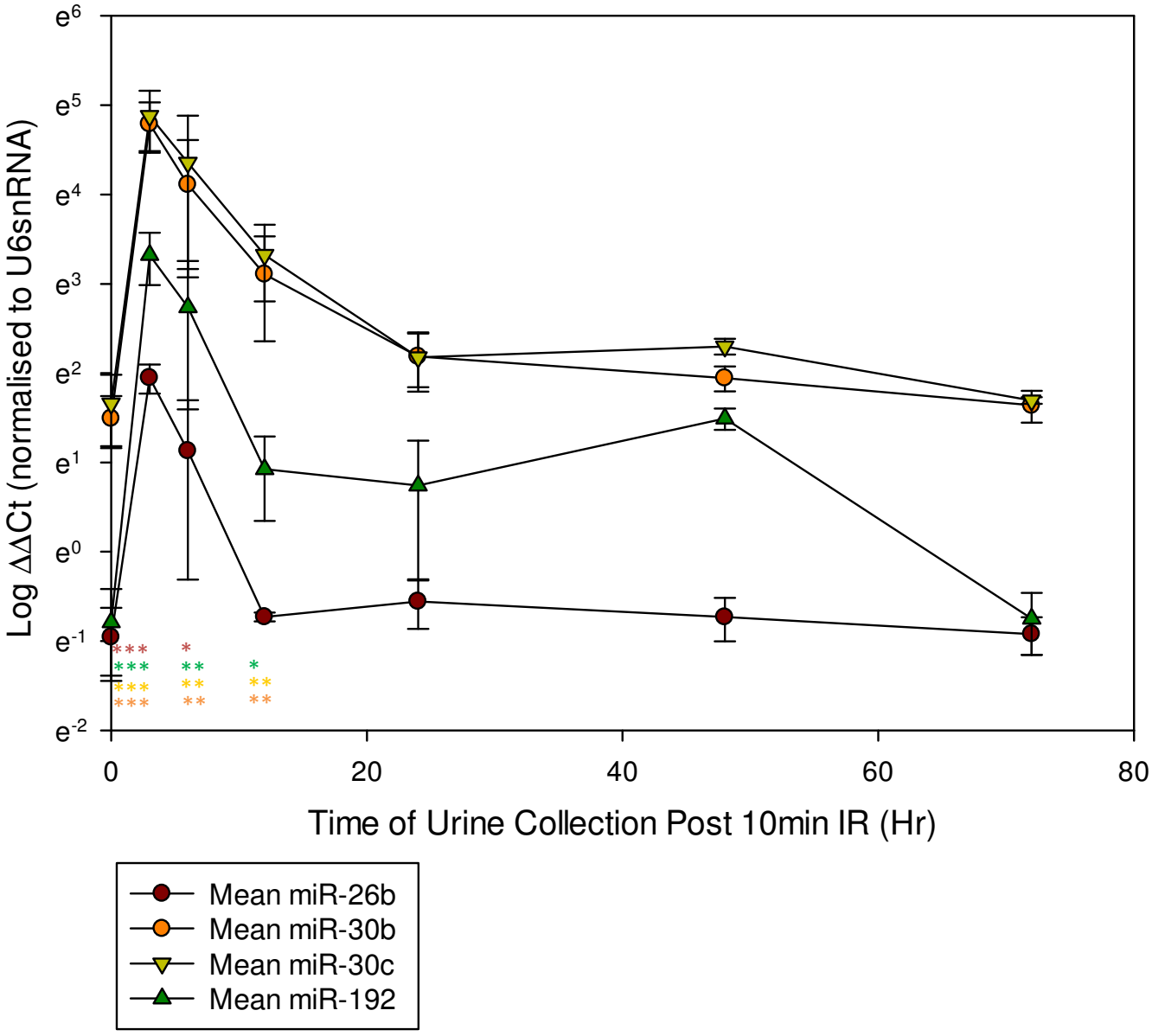

Figure 8:- Mean of quantified miRNA species in urine of 10 minute IR mouse model. miR-26b (blue), miR-30b (red), miR-30c (green), miR-192 (purple). Each miRNA species has been normalised to the endogenous U6snRNA. Error bars represent the SEM of each group.*** P<0.005, **P<0.01, *P<0.05

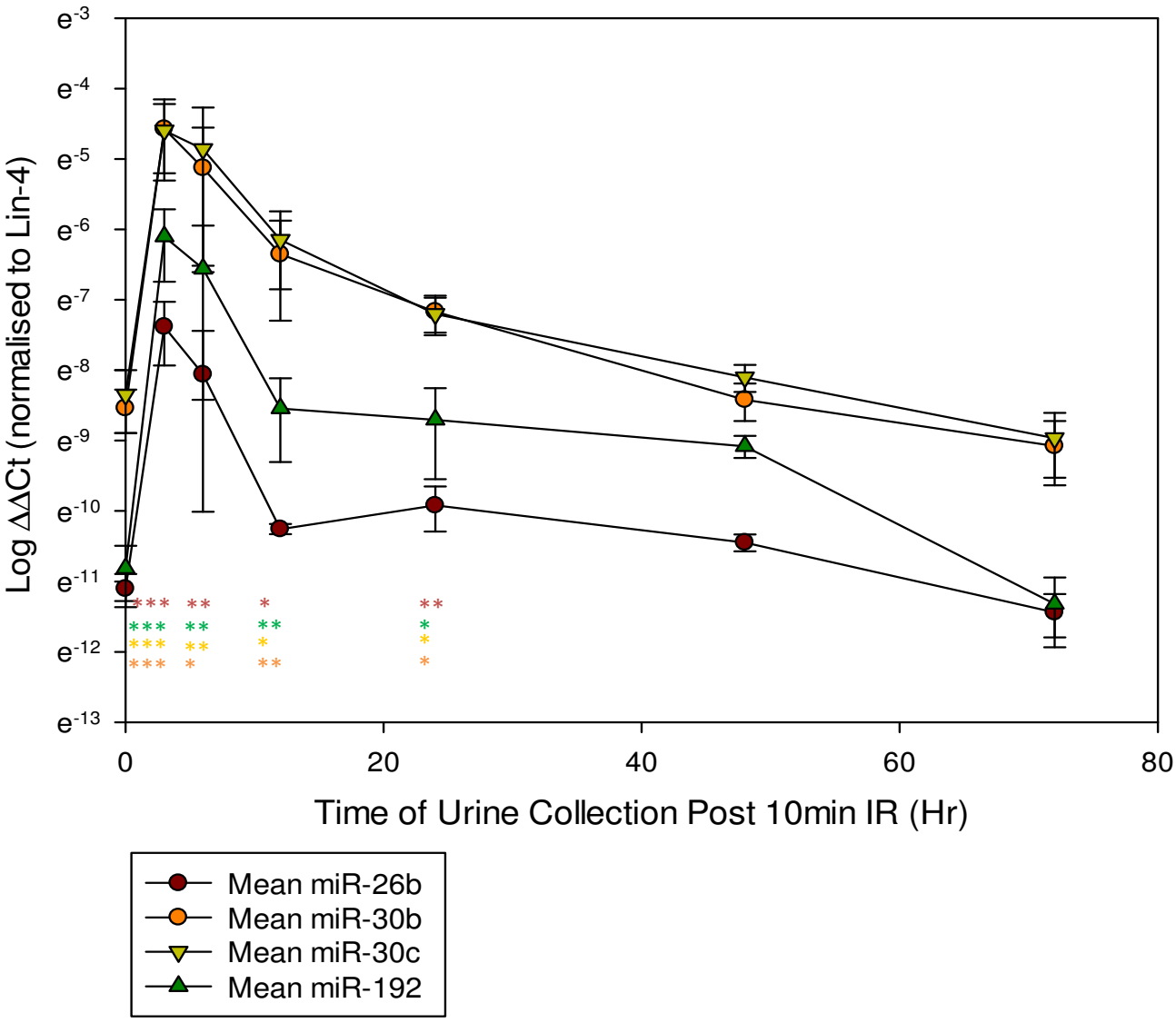

Figure 9:- Mean of quantified miRNA species in urine of 10 minute IR mouse model. MiR-26b (blue), miR-30b (red), miR-30c (green), miR-192 (purple). Each miRNA species has been normalised to the exogenous Lin-4. Error bars represent the SEM of each group. *** P<0.005, **P<0.01, *P<0.05

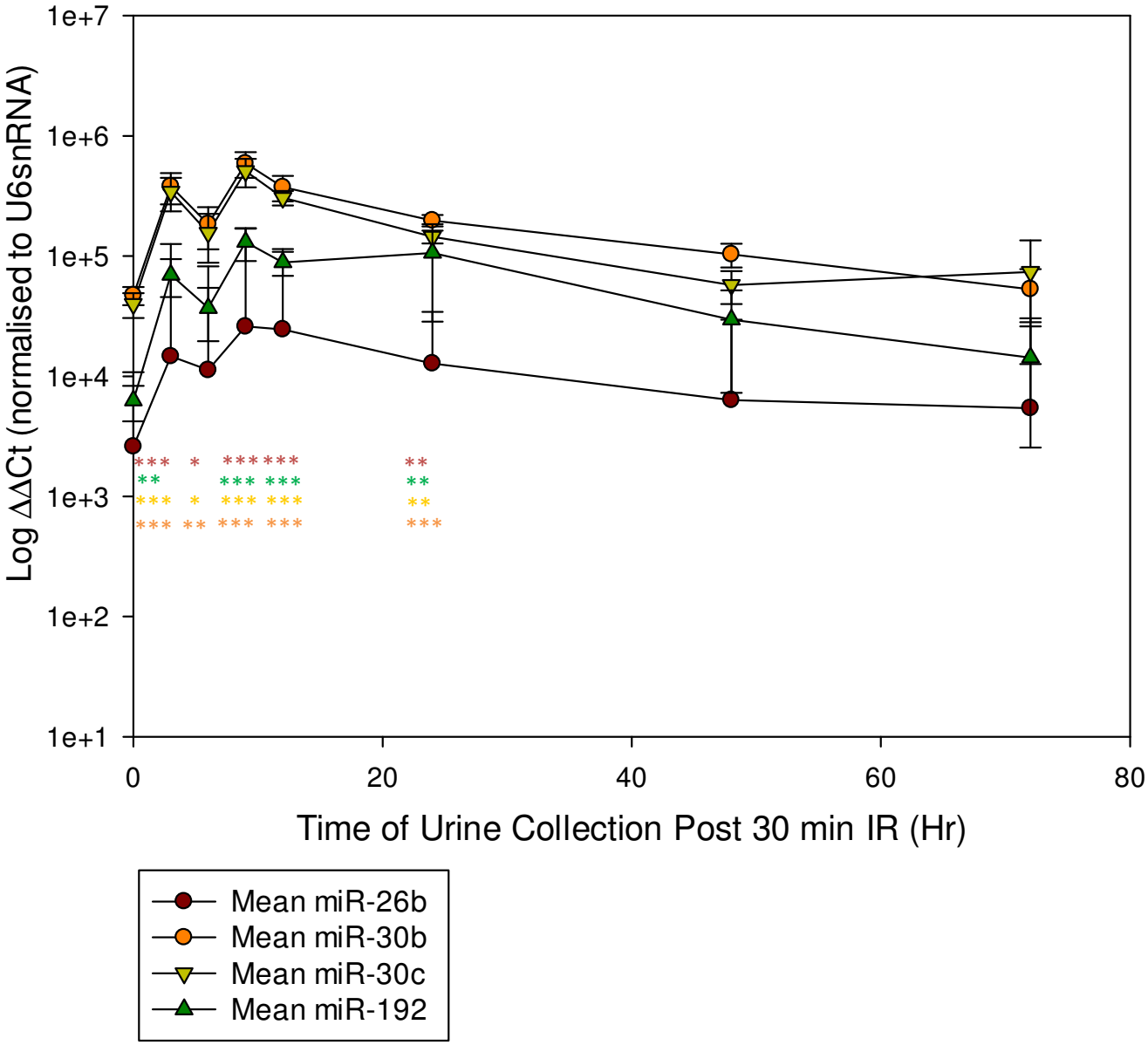

Figure 10:- Mean of quantified miRNA species in urine of 30 minute IR mouse model. MiR-26b (blue), miR-30b (red), miR-30c (green), miR-192 (purple). Each miRNA species has been normalised to the endogenous U6snRNA. Error bars represent the SEM of each group. *** P<0.005, **P<0.01, *P<0.05

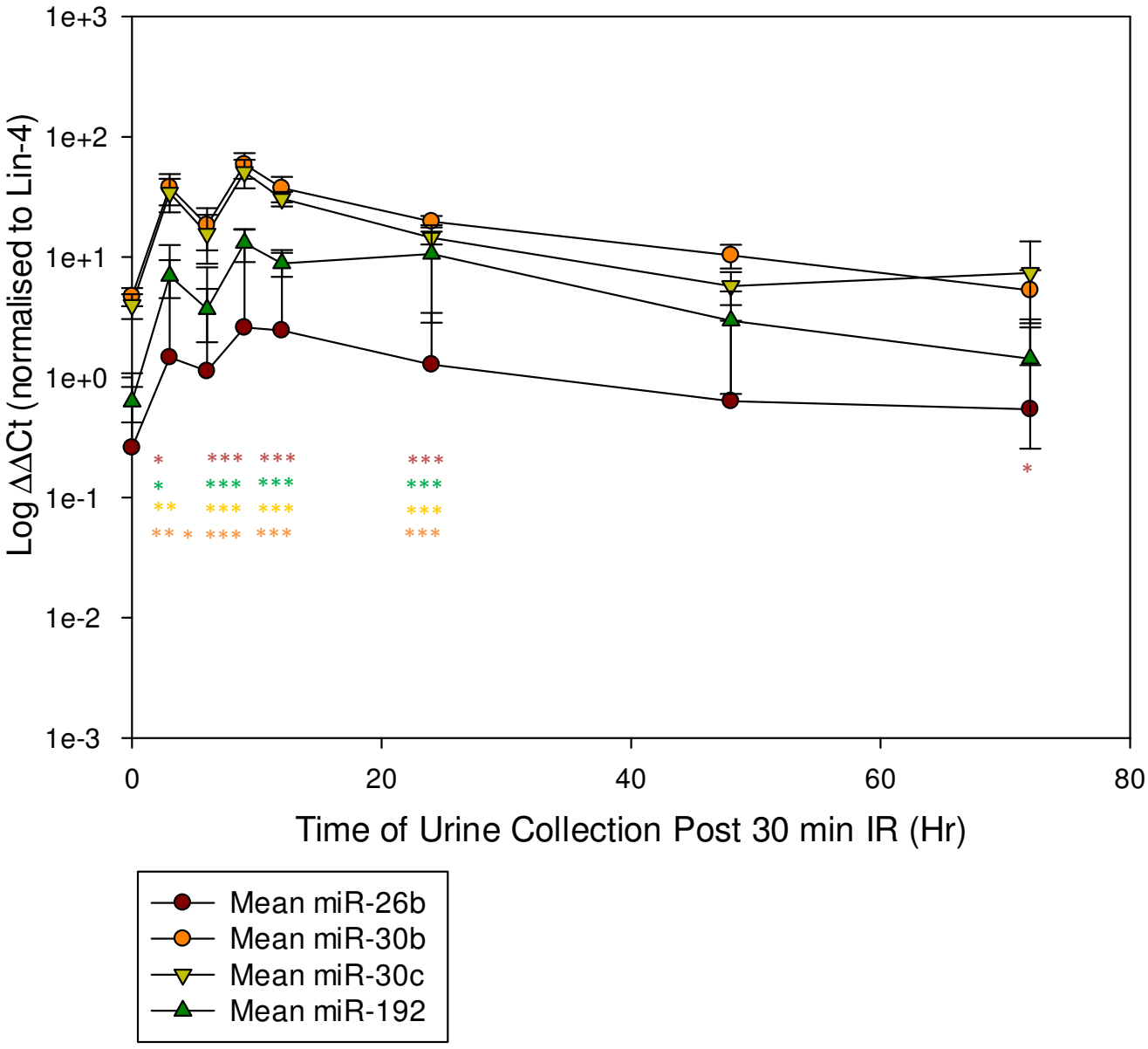

Figure 11:- Mean of quantified miRNA species in urine of 30 minute IR mouse model. miR-26b (blue), miR-30b (red), miR-30c (green), miR-192 (purple). Each miRNA species has been normalised to the exogenous Lin-4. Error bars represent the SEM of each group. *** P<0.005, **P<0.01, *P<0.05

Four miRNA species which have been reported in literature to be enriched in the kidney were also quantified in urine samples of two ischemic reperfusion mouse models (10 and 30 minutes IR). Figures 8 and 10 show the U6snRNA normalised levels of miR-26b, miR-30b, miR-30c and miR-192 respectively vary in the urine of the 10 minute and 30 minute IR model mice at varying periods of time post IR respectively. All four of the miRNA species measured were detectable in the urine of the IR mice at varying levels. Figures 9 and 11 show the lin-4 normalised levels of the four kidney enriched miRNA species in the 10 minute and 30 minute IR model respectively. The two different methods of normalisation show similar trends in the levels of miRNA species in the urine following IR injury. Each miRNA species shows a similar trend in their levels in any given treatment group however the quantities of each miRNA in the urine vary. Each miRNA species showed significant increases from control animals as early as three hours in both methods of normalisation which peaked at three hours or 12 hours for the 10 and 30 minute models respectively. These increases in urinary miRNAs then decreased back towards baseline however only the 10 minute group recovered back to baseline whereas miR-26b in the 30 minute group was still significantly elevated at 72 hours. Figures 16 and 17 show how miR-30b correlated with histological analysis in both models of IR.

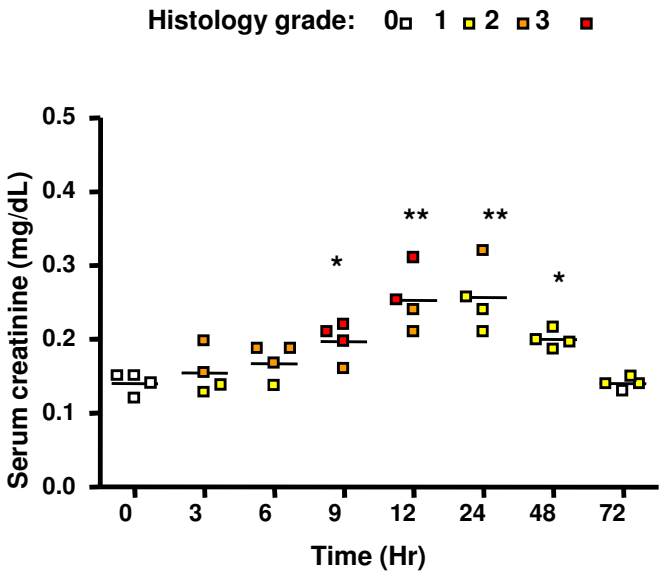

Figure 12:- Serum creatinine quantified in the urine of 10 minute IR mouse model and the histopathological score of the proximal tubules from each animal. Severity of the damage to the proximal tubule is colour coded; Histology grade zero (white), one (yellow), two (orange), three (red), four (purple), and five (blue). Error bars represent the SEM of each group. **P<0.01, *P<0.05

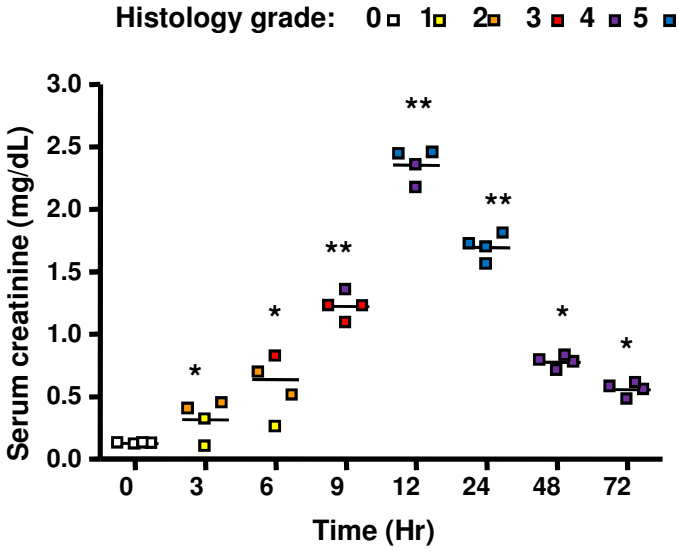

Figure 13:- Serum creatinine quantified in the urine of 30 minute IR mouse model and the histopathological score of the proximal tubules from each animal. Severity of the damage to the proximal tubule is colour coded; Histology grade zero (white), one (yellow), two (orange), three (red), four (purple), and five (blue). Error bars represent the SEM of each group. **P<0.01, *P<0.05

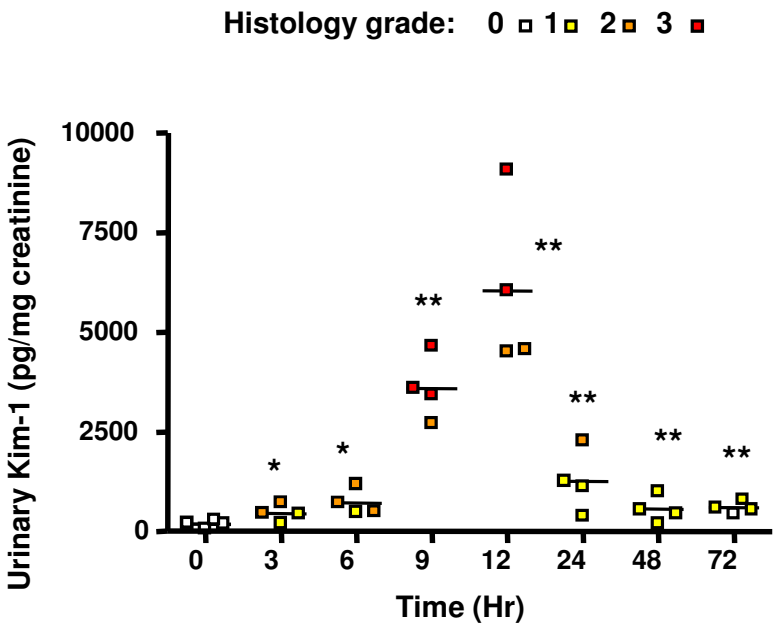

Figure 14:- Urinary Kim-1 (normalised to urinary creatinine) quantified in the urine of 10 minute IR mouse model and the histopathological score of the proximal tubules from each animal. Severity of the damage to the proximal tubule is colour coded; Histology grade zero (white), one (yellow), two (orange), three (red), four (purple), and five (blue). Error bars represent the SEM of each group. **P<0.01, *P<0.05

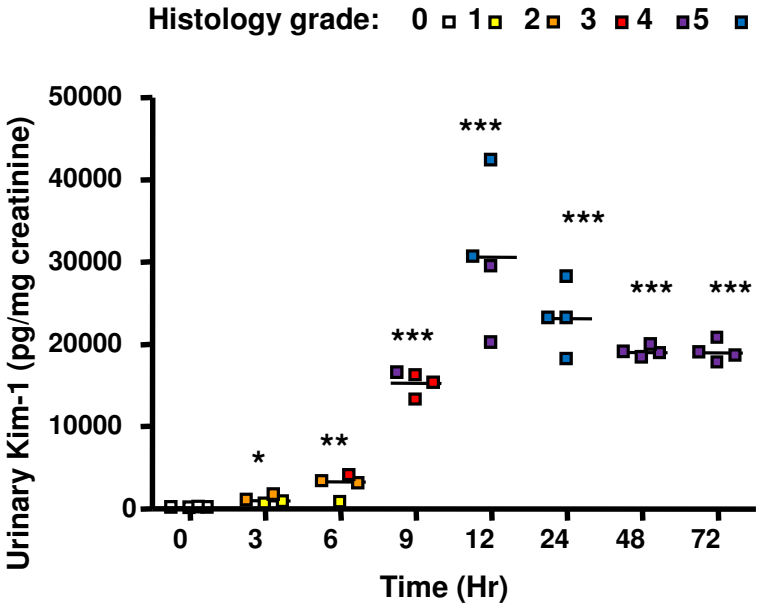

Figure 15:- Urinary Kim-1 (normalised to urinary creatinine) quantified in the urine of 30 minute IR mouse model and the histopathological score of the proximal tubules from each animal. Severity of the damage to the proximal tubule is colour coded; Histology grade zero (white), one (yellow), two (orange), three (red), four (purple), and five (blue). Error bars represent the SEM of each group. *** P<0.005, **P<0.01, *P<0.05

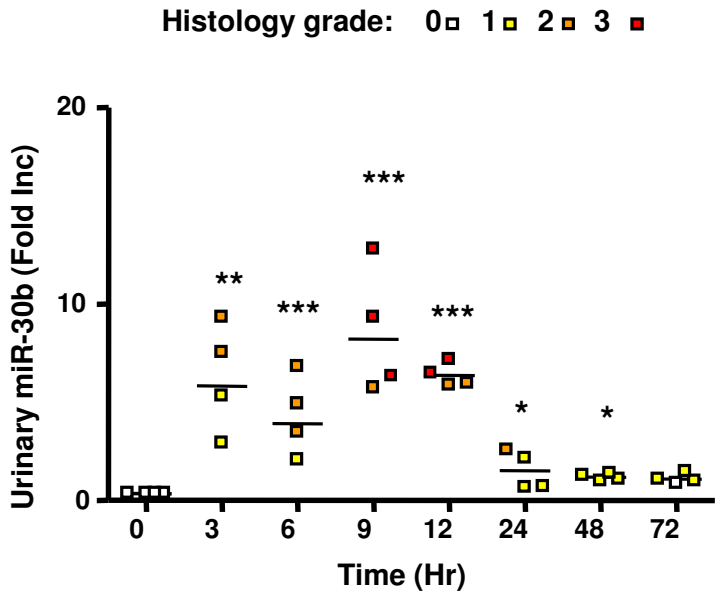

Figure 16:- Urinary miR-30b (expressed as fold increase) quantified in the urine of 10 minute IR mouse model and the histopathological score of the proximal tubules from each animal. Severity of the damage to the proximal tubule is colour coded; Histology grade zero (white), one (yellow), two (orange), three (red), four (purple), and five (blue). Error bars represent the SEM of each group. *** P<0.005, **P<0.01, *P<0.05

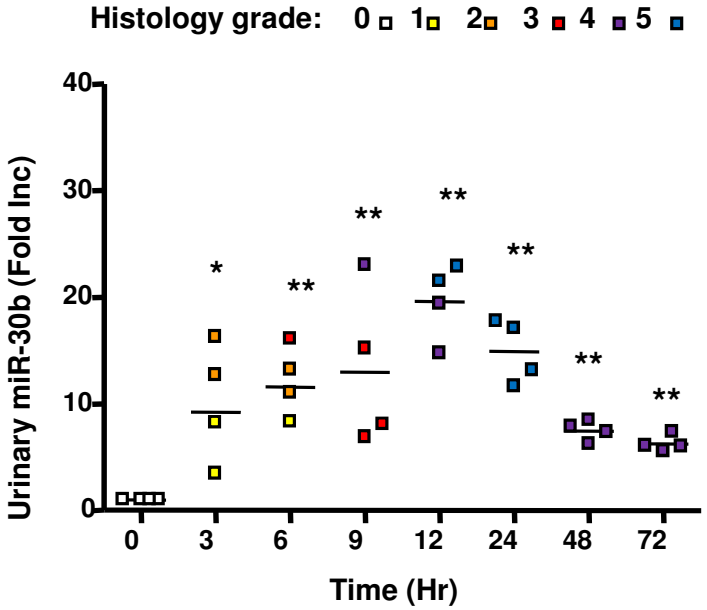

Figure 17:- Urinary miR-30b (expressed as fold increase) quantified in the urine of 30 minute IR mouse model and the histopathological score of the proximal tubules from each animal. Severity of the damage to the proximal tubule is colour coded; Histology grade zero (white), one (yellow), two (orange), three (red), four (purple), and five (blue). Error bars represent the SEM of each group. **P<0.01, *P<0.05

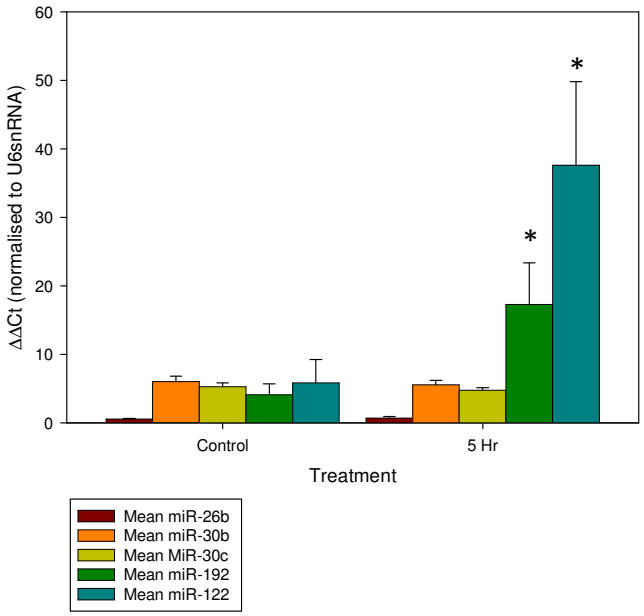

Figure 18:- Four kidney enriched miRNAs and the liver specific miR-122 quantified in the serum of control and APAP (540 mg/kg) dosed CD1 male mice after five hours. *P<0.05

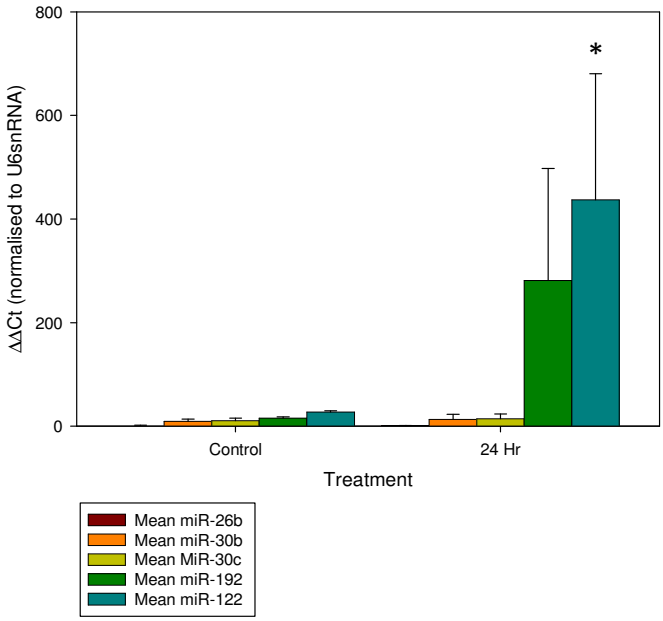

Figure 19:- Four kidney enriched miRNAs and the liver specific miR-122 quantified in the serum of control and APAP (540 mg/kg) dosed CD1 male mice after 24 hours. *P<0.05

3.3 - Quantification of kidney enriched miRNAs in the serum of APAP dosed mice

The four kidney enriched miRNAs quantified in the urine of ischemic reperfusion induced kidney injury were also quantified, along with miR-122 in the serum of mice treated with APAP. The miRNAs were quantified in control animals (0.9% saline) and APAP dosed animals (540 mg/kg) five and 24 hours post APAP administration (Figure 18, 19). Both miR-192 and miR-122 were elevated after five and 24 hour APAP administration, significantly so in the five hour group but only miR-1 significantly so in the 24 hour group.

4 - Discussion

The aims of this study were to quantify specific miRNA species in various animal models of organ injury. Firstly, to reproduce a published method of quantifying various miRNA species in the serum of APAP dosed (530 mg/kg) CD1 male mice to confirm the technique in this laboratory. This was followed by the quantification of miRNA species in the urine of an in vivo model of kidney injury to elucidate whether there is potential for urinary miRNAs to be used as biomarkers of kidney injury.

4.1 - Quantification of miRNAs in the serum of APAP dosed CD1 male mice

Quantification of hepatic glutathione and serum ALT levels were carried out along with quantification of the serum miRNA levels to provide an insight into the redox status of the liver and the degree of hepatotoxicity respectively. ALT is one of the current gold standard serum markers of liver injury however it is not entirely liver specific and is only released from hepatocytes upon loss of membrane integrity; a certain degree of hepatoctye necrosis is required before significant elevations in serum ALT levels are observed (Antoine *et al.*, 2008; Lee, 2003; Nathwani *et al.*, 2005). Glutathione is one of the most important protective molecules in the body which is particularly abundant in the liver. It serves its protective function through phase II conjugation reactions of electrophillic molecules due to the nucleophillic sulphydryl group provided by the cysteine molecule. GSH levels in the APAP dosed CD1 male mice were found to decrease significantly from controls one and two hours post-dose as would be expected given the large dose of APAP which they were administered (figure 1). This would rapidly saturate the sulphation and glucuronidation pathways, resulting in large amounts of NAPQI being produced which rapidly depletes GSH. Four hours post-dose there was large degree of variation in the GSH levels of the dosed mice and

certain animals had similar GSH levels to the controls and by eight hours post-dose the GSH levels in the livers had returned to control levels indicating recovery in GSH levels via reduction of the oxidised form by glutathione reductase. It should however be noted that two animals in the eight hour group were culled prior to scheduled culling so no GSH data is available for these animals. The mice which were culled early would be expected to have had extensively depleted liver GSH levels. However the loss of these animals decreases the statistical power of the data in the eight hour time group. In both the ALT and GSH measurements there was quite a large variation in the baseline levels which may reflect physiological differences in animals due to food intake or genetic variation.

There was no significant increase in serum ALT levels from the control levels observed in animals culled one and two hours post-dose which is consistent with the findings of Wang et al (Wang *et al.*, 2009). This was followed by a significant rise in serum ALT in animals culled at four hours. It is important to note that no elevations in serum ALT were observed until four hours post APAP administration. This is as would be expected as significant cell death does not occur until GSH becomes depleted allowing it to cause oxidative stress, covalent binding to cellular proteins and immune mediated cell death in the hepatocytes. This cell death (initially apoptosis and necrosis followed by extensive necrosis upon depletion of ATP) leads to release of ALT from cells, which then enters the circulation. Perhaps surprisingly serum ALT levels in the eight hour group were found to decrease from the four hour time group, yet were still elevated from the control. This could be explained somewhat by the death of two mice prior to culling which would be expected to have had extremely high serum ALT levels.

Recent research by Wang et al suggested the potential use of miRNAs as serum biomarkers of drug induced hepatocyte injury (Wang *et al.*, 2009). Two liver enriched miRNAs (miR-122 and miR-192) and two miRNAs which are enriched in other organs (miR-1 (heart) and miR-218 (kidney)) were measured in the serum by qPCR to assess their potential as blood based biomarkers of liver injury and to compare them to the currently used validated biomarker, ALT. MiR-1 (figure 3) and miR-218 (figure 4) levels were found to slightly increase in the serum of the dosed mice upon APAP administration but no statistically significant increases were observed. This would be expected given that neither of which are enriched in the liver. MiR-192 (figure 5) was found to be significantly increased from the control animals for animals culled four and eight hours post dose to a maximum of 117 fold from control at four hours. Animals culled one and four hours post dosing only yielded 1.4 and 1.6 fold changes from control values. This suggests that miR-192 is no more sensitive as a biomarker of liver injury than ALT levels. MiR-122 (figure 6) has been previously shown to be the most abundant miRNA in the liver and has been investigated by many groups for its potential as a biomarker of cancer (Starkey Lewis *et al.*, 2011; Wang *et al.*, 2009). This study found that miR-122 increased far more greatly than any other miRNA investigated with fold changes of 2046 from the control eight hours post-dosing. Mean fold changes of 19 and 42 were observed in the serum one and two hours post-dosing which indicates that miR-122 is an earlier biomarker of APAP induced liver injury than ALT given that ALT levels were not found to change from the control until four hours post dosing. Although mean fold changes of 19 and 42 were observed from the control one and two hours post dosing for miR-122 the large variation in the mice in these groups reduces the statistical significance. This variation is most likely due to the degree of liver damage in these mice at these early time points and could reflect the GSH status of the animals.

It is important to try and understand why miRNAs can be detected in the serum earlier than ALT if they have real potential as serum biomarkers. It is known that ALT is released from hepatocytes upon loss of membrane integrity allowing their passive release and the same is most likely true for miRNA species upon this loss of integrity. What is interesting is that there is a mean 19 fold increase in miR-122 one hour post dosing yet no change in serum ALT levels. It is possible that the small size of miRNA species (~7.5KDa) relative to ALT (54KDa) allows their earlier release from hepatocytes losing membrane integrity. Perhaps more interestingly it is possible that miR-122 is released actively from hepatocytes as a signalling molecule upon toxic insult, however this is an ATP dependent process and APAP is known to deplete ATP after toxic insult. It is plausible that this active process may be important at earlier time points but decreases at later time points due to ATP depletion. At later time points active release would be overshadowed by passive release due to loss of membrane integrity. It has been shown by Valadi et al (Valadi *et al.*, 2007) that miRNAs are actively excreted in exosomes by mast cells as a method of genetic exchange and possibly communication. They have shown that other cells can take up exosomes containing miRNA species and that these miRNA species are functional. Although there is no research into hepatic transfer of miRNAs it is feasible and warrants further investigation.

This pilot study analysed the GSH levels, serum ALT levels and the serum levels of four distinct miRNA species in APAP (530 mg/kg) dosed CD1 male mice. It has been shown that miR-122 and miR-192 are both significantly elevated in the serum four and eight hours post APAP dosing. MiR-122 in particular showed extremely large increases from control levels and also yielded a 19 fold increase in the serum at one hour. This indicates that miR-122 has the potential to be an earlier biomarker of APAP induced hepatocellular injury than the

validated serum biomarker, ALT, which agrees with the findings of Wang *et al (Wang et al., 2009)*.

4.2 - Measurement of kidney enriched miRNAs in the urine of an ischemic reperfusion mouse model

In order to test the hypothesis that miRNAs have the potential to be used as biomarkers of kidney injury and to compare two routinely used methods of normalising quantified miRNA data, four kidney enriched miRNA species were measured in two models of mouse bilateral ischemic reperfusion induced kidney injury (10 and 30 minute ischemia). Ischemic reperfusion involves the occlusion of the blood supply to one (lateral) both (bilateral) kidneys which causes the generation of reactive oxygen species and oxidative stress. Upon reperfusion of the kidneys the cells which have been subjected to conditions of ischemia begin to undergo cell death. This model was used as it serves as a positive control for kidney injury due to its generalised location of damage. Four miRNA species were assessed in the urine of IR induced mice which have been shown to be enriched in the kidneys. MiR-30b and miR-30c have been reported to be expressed in the kidneys at levels which are at least twice that of other organs (Chung *et al.*, 2010; He *et al.*, 2004; Ho *et al.*, 2008; Sempere *et al.*, 2004). MiR-26b has been shown to represent approximately five per cent of kidney miRNA species and miR-192 has been shown to be highly upregulated in the diseased kidney and has been shown to be enriched preferentially in the cortex rather than the medulla (Chung *et al.*, 2010; Liang *et al.*, 2009; Sun *et al.*, 2004; Tian *et al.*, 2008; Wang *et al.*, 2009). The RNA of the small nuclear ribonucleoprotein, U6snRNA was quantified alongside each miRNA to provide an endogenous standard. A synthetic *c.elegans* Lin-4 oligonucleotide was spiked

into each sample as described in chapter two to provide an internal standard to try and correct for variations in recovery during miRNA isolation.

Importantly the two different methods of normalisation showed similar trends in miRNA levels in both models (figures 8, 9, 10, 11) improving the confidence of the data. The present results show a similar trend of initial significant increases in miRNA levels in the urine followed by a gradual fall back towards baseline miRNA levels as time from the IR increases. The 30 minute model showed a more prolonged increase in all four of the miRNA species measured in the urine when compared to the 10 minute model. Interestingly the 10 minute model showed increases in miRNA species which reached higher levels than the 30 minute model but persisted for a shorter period of time. It is important to note that all four kidney enriched miRNA species measured show the same trend.

Urinary creatinine was also quantified in the urine of the ischemic reperfusion induced mouse model of kidney injury to provide an insight into kidney function. The elevation in urinary miRNAs in the 30 minute IR mouse model samples correlate with a decrease in urinary creatinine levels as shown by figure 7. Creatinine levels in the urine fall sharply in the 30 minute model after IR injury reflecting a decrease in the ability of the glomerular filtration capacity of the kidney. This initial fall is followed by a slight increase in urinary creatinine possibly suggesting recovery in kidney function. The 10 minute IR samples show a slight decrease in urinary creatinine however the decreases are not significant. This may reflect a lesser degree of kidney damage than in the 30 minute IR samples. Interestingly, although there was no significant fall in creatinine there were significant increases in urinary miRNA species.

Serum creatinine is a commonly used clinical biomarker of kidney injury, although it is known to have limitations, mainly as a result of its lack of sensitivity. Urinary Kim-1 is a novel biomarker of kidney injury which a higher degree of sensitivity than more traditional biomarkers such as serum creatinine and blood urea nitrogen. Serum creatinine was significantly elevated at three hours post 30 minute ischemic reperfusion injury. The same was observed for urinary Kim-1 and for all four kidney enriched miRNA species quantified. Urinary Kim-1, serum creatinine and all miRNAs quantified peaked at 12 hours post injury. Histological analysis largely agrees with the data from the three different molecules quantified with the severity of damage increasing up to 12 hours post injury. However histological analysis doesn't show a recovery post 12 hours, instead the damage increases in severity indicating widespread and extreme proximal tubule damage. Due to the severity of the damage caused in the 30 minute ischemic reperfusion samples this sample set is possibly not as useful as the ten minute set as differences in the three different quantified molecules cannot be identified.

For the aforementioned reason the ten minute ischemic reperfusion samples provide more on an insight into the potential utility of miRNAs as biomarkers of kidney injury. Serum creatinine was found to increase significantly nine hours after 10 minutes of ischemic reperfusion injury whereas urinary Kim-1 was found to be significantly elevated at three hours post injury. This illustrates the lack of sensitivity of serum creatinine and the greater sensitivity of urinary Kim-1. Interestingly all four kidney enriched miRNA species followed a similar pattern to the urinary Kim-1 with significant elevations at three hours although they peaked at nine hours as opposed to 12 hours with Kim-1. This may indicate that measurement of urinary miRNA is a more sensitive marker of injury than serum creatinine.

Urinary Kim-1 and serum creatinine both peaked at 12 hours whereas the urinary miRNAs peaked at nine hours and began returning towards baseline levels more quickly than the other two biomarkers which may suggest a utility in identifying the time since injury more accurately than with Kim-1 or SCr.

The data from the ischemic reperfusion study shows that miRNAs can be successfully isolated and quantified from the urine of mice with varying degrees of kidney injury. It has also been shown that two different methods of normalisation can provide similar data, thus improving the confidence of the data. All four of the urinary miRNAs quantified here show a similar pattern to urinary Kim-1 and show elevations six hours earlier than serum creatinine. This data suggests that miRNAs may have potential utility as urinary biomarkers of kidney injury.

4.3 - Quantification of kidney enriched miRNAs in the serum of APAP dosed CD1 mice

The four kidney enriched miRNA species which were quantified in the ischemic reperfusion mouse model were also quantified in the serum of mice administered with APAP (540 mg/kg) or saline (0.9 %) to determine whether the increases observed in the urine of the ischemic reperfusion model of kidney injury were specific to kidney injury. The liver specific miRNA, miR-122 was also quantified alongside the kidney enriched miRNAs to serve as a positive control for liver injury as miR-122 has been previously been shown to be significantly elevated in the serum of mice post APAP induced liver injury in a number of studies. The miRNAs were quantified five and 24 hours post APAP administration and normalised to the endogenous U6snRNA as previously described.

Significant increases from control animals were only observed in miR-192 and miR-122 in the five hour animals. No significant increases were observed in any other of the quantified

miRNA species. In the 24 hour samples there were increases in miR-192 and miR-122 however only miR-122 was found to be significant. There were no increases observed in any of the other quantified miRNA species. It would be expected that miR-122 would be increased in the APAP administered animals due to its liver specificity and the fact that it has been shown in previous studies. The same likely applies to miR-192 as it is highly expressed in the liver as well as the kidney so increases in the APAP administered animals would also be expected in this case. Importantly none of the other kidney enriched miRNAs were found to increase in the serum of the APAP administered animals indicating a degree of kidney specificity over the liver. This is important if these miRNAs were to go on to be developed as potential biomarkers of kidney injury.

The investigations in this chapter suggest a potential role for kidney enriched miRNAs as biomarkers of kidney injury in an IR model of kidney injury. This damage caused by IR injury is severe and widespread throughout the nephron. For this reason it is necessary that similar studies using less severe models of kidney injury are carried out to determine whether miRNAs have utility in more subtle scenarios. The next chapter will investigate the use of miRNAs as biomarkers in Cisplatin and Adriamycin models of kidney injury.

Chapter 4

MicroRNA Profiling of Kidney tissue, Serum and Urine in Models of DIKI in vivo

Contents

1 – Introduction

Work in the previous chapter has shown that the quantification of kidney enriched miRNA species in urine can indicate the presence of kidney injury earlier than SCr and at least as early as urinary Kim-1 in a murine model of kidney IR. This chapter will focus on continuing to test the hypothesis that miRNAs can be used as biomarkers of kidney injury in a less severe, more subtle location specific model of DIKI. This will be evaluated using two nephrotoxic agents, Cisplatin and Adriamycin.

1.1 - Cisplatin

Cisplatin is an anticancer agent which is successful in treating a variety of different cancers including testicular, ovarian, lung and glioblastomas. However its use in these clinical situations is limited due to renal proximal tubular toxicity which occurs in 25 to 30 % of patients after a single dose (Pabla *et al.*, 2008; Zhang *et al.*, 2006). The kidney is a target of the toxic effects of Cisplatin due to the concentration of Cisplatin in the proximal tubules which can be to up to five times greater than in the serum (Hanigan *et al.*, 2003). Cisplatin nephrotoxicity causes tubulointerstitial lesions with mitochondrial swelling which in animal models are primarily in the S3 segment of the proximal tubule. Its pharmacological mechanism of action involves binding of Cisplatin to DNA causing Cisplatin-DNA cross links and eventually cell death in rapidly dividing cells such as cancer cells (Yao *et al.*, 2007). The toxicological mechanism was originally thought to be the same as the pharmacological mechanism however it is now known that proximal tubular cells are well differentiated and do not divide which makes the pharmacological mechanism unlikely to kill these cells. This theory is also supported by the fact that other DNA damaging agents do not kill proximal tubular cells. Cisplatin is transported into proximal tubular cells via the copper transporter,

Ctr1, chlorine dissociates from the platinum and the platinum ion binds to nucleophiles such as DNA (Hanigan *et al.*, 2003). Its toxicological mechanism generally involves the formation of reactive oxygen species (ROS), caspase activation and cell death via necrosis and apoptosis which can induce inflammation (Liu *et al.*, 2006). It is now believed that a reactive metabolite of Cisplatin is responsible for its toxicity as inhibition of either γ-glutamyltranspeptidase (GGT) or pyridoxal 5'-phosphate (PLP) dependent enzymes abolishes toxicity in vitro and in vivo, however inhibition of GGT also abolishes its pharmacological action (Pabla *et al.*, 2008; Yao *et al.*, 2007).

1.2 - Adriamycin

Adriamycin (Doxorubicin) is a quinine containing antibiotic which is has been used as a treatment for cancer with a high efficacy since the late 1960's. It has been particularly used to treat leukaemias and lymphomas however its use is restricted by the onset of various forms of toxicity including cardiac, pulmonary, testicular, haematological and renal. There are a number of cytotoxic mechanisms which have been shown to be caused by Adriamycin including enzyme inhibition, DNA intercalation, ROS generation and apoptosis induction(Ayla *et al.*, 2011; Yang *et al.*, 2009b). The toxicity of Adriamycin is a result of REDOX imbalances which leads to the generation of free radicals, lipid peroxidation and oxidative stress which results in tissue injury although the exact mechanism of toxicity is unknown. In the kidney Adriamycin targets the glomerulus where it increases glomerular permeability and causes tubular atrophy. This nephropathy has been well characterised in rodent models and the pathology includes albuminuria, impaired glomerular function, glomerulosclerosis and morphological changes to the glomerulus. Podocytes are the primary cells of the glomerulus and maintain the filtration barrier to proteins getting into the urine

and maintain the basement membrane of the glomerulus. Podocytes are the primary cell type to be affected by Adriamycin although a number of other cell types have been reported to be damaged which include tubular epithelial cells although it is unclear whether this damage is primary or secondary (Ayla *et al.*, 2011; Yang *et al.*, 2009b).

The hypothesis of this chapter is that profiling miRNA species expressed in the kidney tissue, serum and urine of a Cisplatin or Adriamycin animal model will provide potential biomarkers of DIKI which outperform current "gold standard" biomarkers of kidney injury. This will be investigated through the following aims:

- Development of a Cisplatin model of kidney injury and subsequent quantification of markers of kidney injury at various timepoints post dose. MiRNAs will be profiled in the kidney urine and serum of this model and compared to saline administered animals.
- MiRNA profiling of mice administered with either Adriamycin or saline.
- Finally the Cisplatin and Adriamycin models will be compared which may provide a set of miRNAs which may be locational markers of kidney injury.

2 - Materials and Methods

RNA oligonucleotides (synthetic miRNAs) were purchased from integrated DNA technologies (IDT). RNA isolation and purification kits were purchased from QIAGEN (miRNeasy mini kit, RNeasy minElute cleanup kit). Reverse transcription specific and qPCR specific miRNA primers (TaqMan® MicroRNA Assays) were obtained from Applied Biosystems. Reagents for reverse transcription and for qPCR were also obtained from Applied Biosystems (TaqMan® MicroRNA Reverse Transcription Kit and TaqMan® Universal PCR Master Mix). All other reagents were purchased from Sigma (Poole, UK) unless otherwise stated.

2.1 - Experimental animals

The protocols described were in accordance with criteria outlined in a licence granted under the Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Animal Ethics Committee. CD1 male mice for the Cisplatin study were purchased from Charles River laboratories and had a five day acclimatisation period prior to experimentation. Animals were maintained in a 12 hour light/dark cycle and had access to food and water Ad libitum.

2.2 - Histological analysis

Tissue samples were isolated from animals and were fixed in neutral buffered formalin (10 %). To prepare for histological assessment 3 -5 µM paraffin sections were prepared and stained with haemotoxylin and eosin (H & E). Stained sections were examined for any histopathological features blinded.

2.3 - Induction of Cisplatin induced nephrotoxicity in CD1 male mice

Male CD1 mice (28-33 g) were administered on day one of the study with either 0.9 % saline or Cisplatin (20 mg/kg) IP. Mice were allowed access to food and water Ad libitum throughout the study and during acclimatisation. Mice were grouped into four groups, each containing eight animals (four saline and four Cisplatin dosed). Each group were housed individually in metabolism cages for 24 hours prior to being culled by increasing concentrations of CO_2 either 24, 48, 72 and 96 hours post administration of either saline or Cisplatin. Urine was collected from the metabolism cages and immediately stored at -80 °C. Tissue samples were collected and either snap frozen or fixed in neutral buffered formalin (10 %). Blood was collected via cardiac puncture and allowed to clot before isolation of serum which was stored at -80 °C. For histological assessment tissue was prepared into 3 -5 µm paraffin sections and stained with haemotoxylin and eosin (H&E) and examined for any histopathological features.

2.4 - Induction of Adriamycin induced nephrotoxicity in BALB mice

BALB mice with severe combined immunodeficiency (SCID) (7 – 8 weeks old) were administered with a single dose of Adriamycin (6.3 – 6.5 mg/kg) intravenously while awake and restrained (tail vein). Mice were culled via terminal anaesthesia using Phenobarbiton (200 µl) intraperitoneally 2 weeks after Adriamycin administration. Blood was isolated via cardiac puncture. Kidneys were removed and either fixed in 10 % formaldehyde for histopathological analysis or snap frozen. Serum was isolated as previously described.

2.5 - miRNA isolation and purification

miRNA isolation and purification was carried out according to manufacturer's instructions. Briefly, 50 µl miRNA containing sample (serum, urine) were mixed with Qiazol reagent (700

µl). 100 % chloroform was added (140 µl) and mixed vigorously. Samples were centrifuged for 15 minutes (12 000 g, 4°C) and the supernatant transferred to a separate tube. An equal volume of ethanol (70 %) was added to the supernatant and mixed well. Sample was transferred (700 µl) at a time to RNeasy mini spin columns and centrifuged for 15 seconds (8,000 g, RT). The flow through was transferred to a separate tube and 0.65 volumes of ethanol (100 %) was added and mixed well. Samples were transferred (700 µl at a time) to RNeasy MinElute spin columns and centrifuged for 15 seconds (8 000 g, RT). The flow through was discarded and RWT buffer (a stringent washing buffer) (700 µl) was added to columns before centrifuging for 15 seconds (8 000 g, RT). Flow through was again discarded before the same being repeated with RPE buffer (500 µl). Ethanol (80 %) was added and centrifuged for two minutes (8 000 g, RT) and flow through discarded. Columns were the centrifuged for five minutes with their caps open (8 000 g, RT) followed by the addition of RNase-free water (14 µl) to the column membrane and final centrifugation for one minute (8 000g, RT).

2.6 - Quantification of Urinary Creatinine

Urinary creatinine was quantified using a plate based colourimetric assay. Urine samples were diluted appropriately with distilled water and added to separate wells of a 96 well plate in triplicate (25 µl). A standard curve was generated using an aqueous creatinine solution (1 mg/ml). Reagents A and B were created (Reagent A: 0.5 M NaOH, 0.1 M Na_2HPO_4, 0.56 M $Na_2B_4O_7{\cdot}10H_2O$, $NaC_{12}H_{25}SO_4$, Picric acid and DMSO) (Reagent B: Acetic acid, H_2SO_4). Reagent A was added to each well (125 µl) and incubated for 2 minutes. The reaction was terminated using reagent B (5 µl) and plate was incubated on a plate shaker for 10 minutes. Absorbance was read at 490 nm.

2.7 - Quantification of blood urea nitrogen (BUN)

BUN was quantified using a plate based colourimetric assay (QuantiChrom Urea Assay Kit, BioAssay Systems). The assay uses a chromogenic reagent which forms a coloured complex when it reacts specifically with Urea. Assay was carried out according to manufacturer's instructions. Briefly, a standard curve was generated using a urea solution (50 mg/dl). Serum samples were diluted appropriately and added to separate well of a 96 well plate in triplicate. Working reagent was added (200 µl) to each well and incubated at room temperature for 20 minutes. The plate was read at 520 nm.

2.8 - Quantification of urinary N-acetyl-β-glucosaminidase (NAG)

NAG was quantified in a plate based colourimetric assay (NAG detection kit, Roche) according to the manufacturer's instructions. Briefly, working solutions were made by adding appropriate volumes of distilled water to reagents. Urine samples were diluted appropriately and added to separate wells of a 96 well plate in triplicate (5 µl). Substrate solution (100 µl) was added to each well and incubated for 15 minutes (37 °C) followed by stop reagent (200 µl). Plate was incubated at room temperature for 10 minutes followed by the absorbance being read at 580 nm.

2.9 - Preparation of cDNA and carrying out miRNA arrays

Complimentary DNA was prepared for the miRNA arrays in a similar way as previously described but using Megaplex Rodent Primer Pool sets (Applied Biosystems) rather than individual primers and also by carrying out an additional pre amplification step to ensure sufficient amounts of cDNA. All procedures were carried out according to manufacturer's instructions. Briefly reverse transcription was carried out through the addition of mastermix (Megaplex rodent primer pool, dNTPs, reverse transcriptase, RT buffer, $MgCl_2$, RNase

inhibitor and dH_2O) to appropriate wells of a 96 well PCR plate (4.5 µl). Total RNA isolated from each mouse sample (urine, serum or kidney tissue) was added to each well (3 µl) and the plate was mixed and centrifuged. Reverse transcription was carried out using the following parameters: 40 cycles of 16 °C for 2 minutes, 42 °C for 1 minute and 50 °C for 1 second followed by 85 °C for 5 minutes. Pre-amplification was then carried out by combining Taqman PreAmp Master Mix, Megaplex PreAmp Primers (Applied Biosystems) and dH_2O and adding this to appropriate wells of a 96 well PCR plate. RT product was added to appropriate wells of the PCR plate (2.5 µl) and the plate was sealed and ran on a thermal cycler using the following parameters: 95 °C for 10 minutes, 55 °C for 2 minutes, 72 °C for 2 minutes, 12 cycles of 95 °C for 15 seconds and 60 °C for 4 minutes and 99 °C for 10 minutes. The pre-amplification product was combined with TaqMan Universal PCR MasterMix and dH_2O added to Rodent Taqman Low Density Array (TLDA) cards (Applied Biosystems) which were then run on a 7900HT qPCR system (Applied Biosystems) using the following parameters: 95 °C for 10 minutes, 40 cycles of 95 °C for 15 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. The TLDA cards contained 384 rodent miRNA species, and a number of control species. The baseline for each assay was automatically defined by expression suite software and the threshold was set for each individual assay (the same for each sample in a study). All analysis was carried out using Expression Suite software (Applied Biosystems). This software was also used to generate heat maps, correlation plots and volcano plots for array data. Relative miRNA levels were determined using the ΔΔCt method and results were normalised to the means of miR-99b, 203 and 218 for urine samples, miR-126-5p, 139-5p and 484 for serum samples and miR-195, 350 and 148b for kidney tissue samples. Normalisation miRNAs were chosen using Expression Suite

normalisation tool and were chosen based on smallest variation in across all samples in a study. Results were considered significant if $P < 0.05$ and fold changes were >2.

2.10 - Statistical analysis

Each experiment involved duplicate qPCR repeats of the reverse transcription cDNA product. Each independent experiment was repeated to generate at least an n of three. Error bars represent standard error of the means (SEM) of the three independent experiments. Shapiro Wilk test was used to test all data for normality followed by the Kruskal Wallis test to assess statistical significance.

3 - Results

3.1 - Quantification of biomarkers of kidney injury in an in vivo model of Cisplatin induced nephrotoxicity

In order to determine whether miRNAs were elevated in other models of kidney injury than the ischemic reperfusion model examined earlier in this thesis a second model was developed. This model was developed to be a more subtle model of kidney injury which affected a specific area of the kidney rather than global ischemia. Cisplatin was used as it has been widely studied and is a known proximal tubule specific toxin. Cisplatin (20 mg/kg) (n=4) or saline (0.9 %) (n=4) was administered to CD1 male mice via IP injection and they were culled 24, 48, 72 or 96 hours post dose. Urine was collected for the final 24 hours of life and blood and kidney tissue collected immediately after culling. There were no significant changes in weight loss or urinary volume between the Cisplatin dosed and saline dosed mice at any timepoint (data not shown). Urinary creatinine was also found not to be significantly elevated in any of the time points although there were greater variations in the 72 and 96 hour mice than the earlier time points (figure 1). Urinary N-acetyl-glucosamine (NAG) was quantified in the urine of the mice and normalised to urinary creatinine (UCr) (figure 2). The mean NAG normalised to UCr was elevated from the time matched controls in each group however only significantly so in the 72 hour group (mean saline 122 mU/ml mg UCr, mean Cisplatin 313 mU/ml mg UCr). The mean BUN (figure 3) was elevated at every time point relative to its time matched control but only significantly so 96 hours post Cisplatin administration (mean saline 22 mg/dl, mean Cisplatin 117 mg/dl).

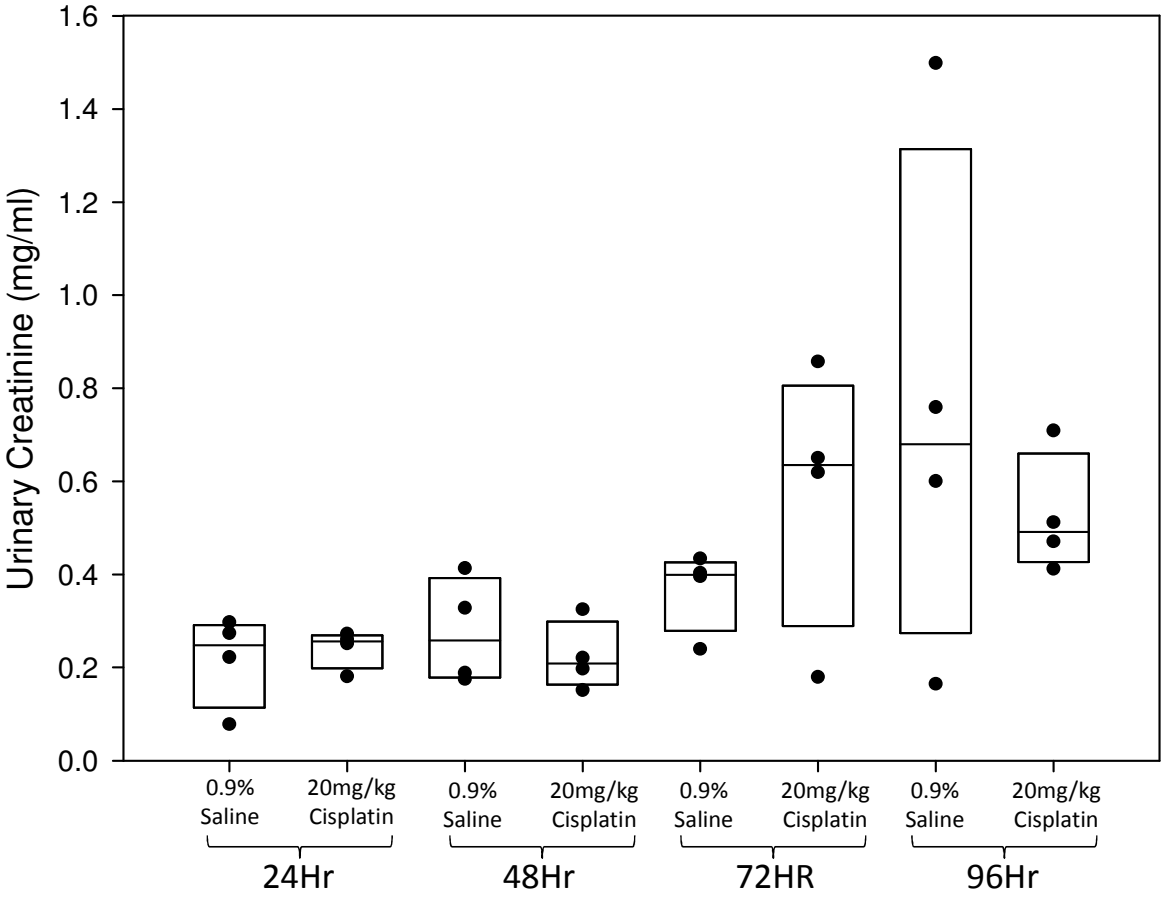

Figure 1: Urinary creatinine quantified in the urine of CD1 male mice administered with either Cisplatin (20 mg/kg) or saline (0.9 %) for 24, 48, 72 or 96 hours. Urine was collected for a 24 hour period prior to the culling of the animals. Each point represents an individual animal. Lines represent the mean and upper and lower quartiles.

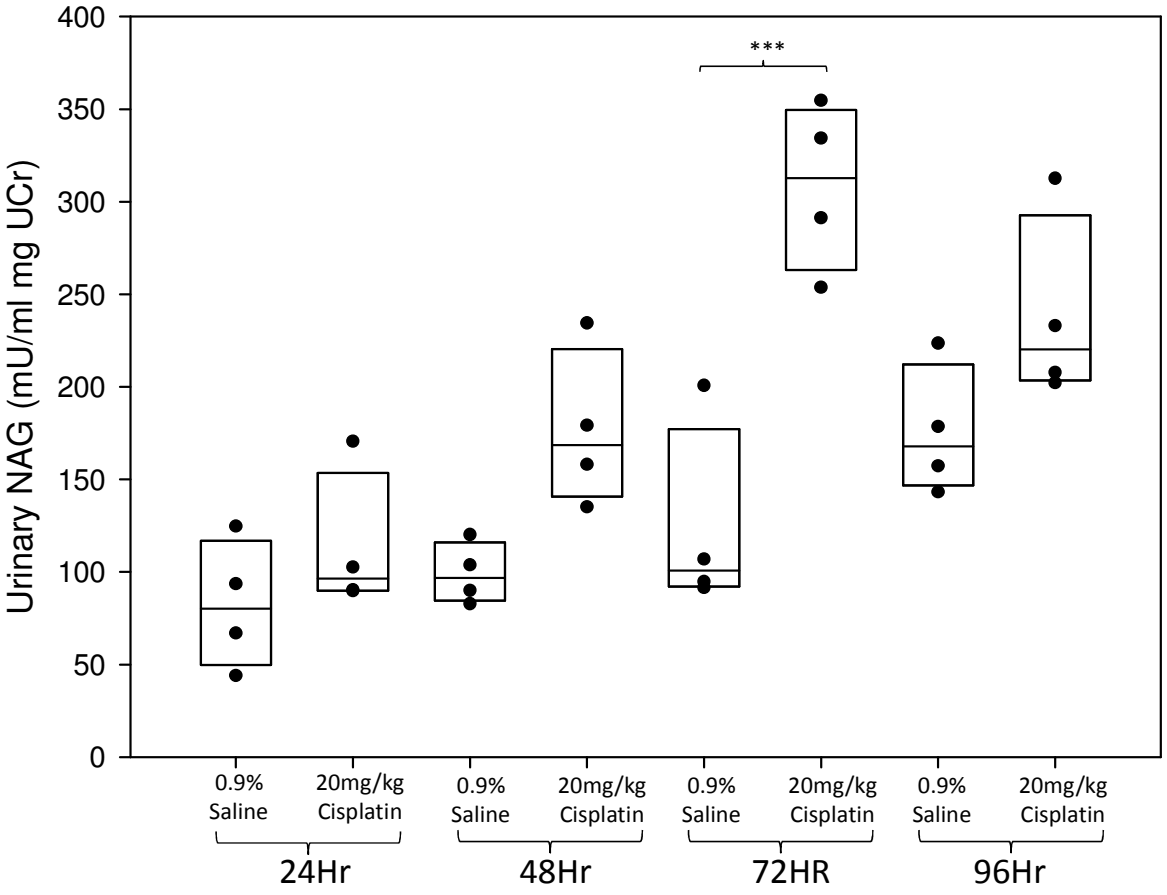

Figure 2: Urinary NAG quantified in the urine of CD1 male mice administered with either Cisplatin (20 mg/kg) or saline (0.9 %) for 24, 48, 72 or 96 hours and normalised to urinary creatinine. Urine was collected for a 24 hour period prior to the culling of the animals. Each point represents an individual animal. Lines represent the mean and upper and lower quartiles. *** P<0.005.

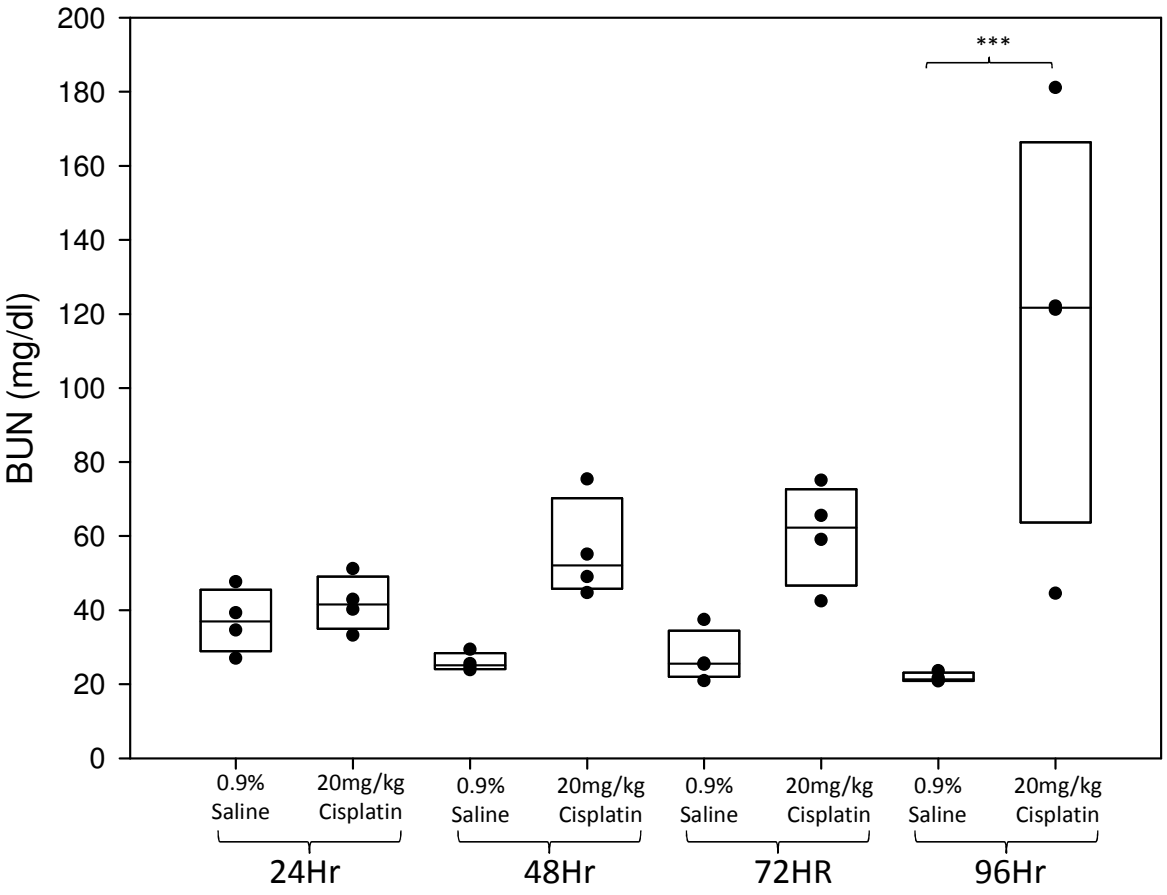

Figure 3: Blood urea nitrogen quantified in CD1 male mice administered with either Cisplatin (20 mg/kg) or saline (0.9 %) for 24, 48, 72 or 96 hours and normalised to urinary creatinine. Urine was collected for a 24 hour period prior to the culling of the animals. Each point represents an individual animal. Lines represent the mean and upper and lower quartiles. *** P<0.005.

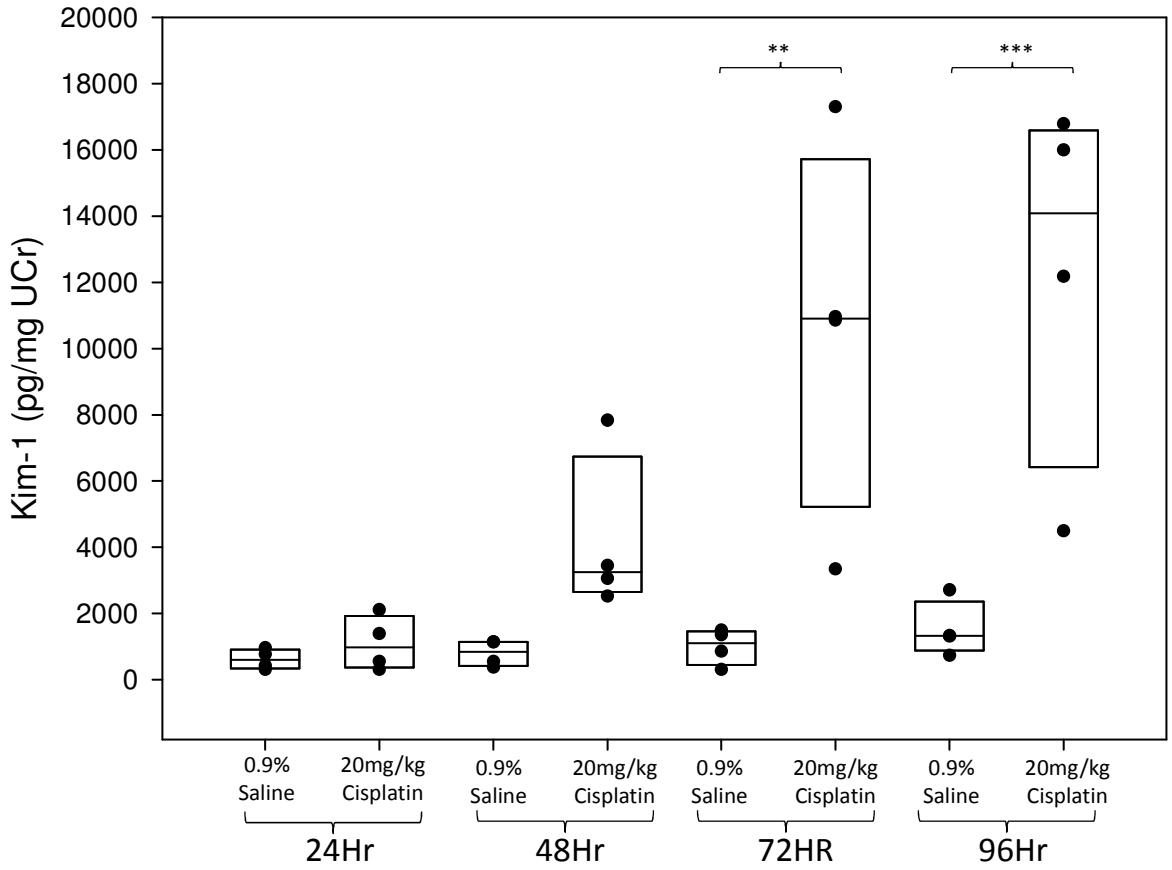

Figure 4: Kim-1 quantified in CD1 male mice administered with either Cisplatin (20 mg/kg) or saline (0.9 %) for 24, 48, 72 or 96 hours and normalised to urinary creatinine. Urine was collected for a 24 hour period prior to the culling of the animals. Each point represents an individual animal. Lines represent the mean and upper and lower quartiles. *** P<0.005, P<0.01

Mean urinary Kim-1 (figure 4) was also elevated at each time point in relation to its time matched control however only significantly so in the 72 (mean saline 1003 pg/mg UCr and mean Cisplatin 10617 pg/mg UCr) and 96 hour (mean saline 1521 pg/mg UCr and mean Cisplatin 12366 pg/mg UCr) time points.

There were no histological abnormalities in the proximal tubules of any of the saline administered mice or in the 24 hour Cisplatin dosed mice. 48 hour Cisplatin caused low/moderate scattered apoptotic and necrotic tubular epithelial cells. 72 hour Cisplatin caused low/moderate number of overall epithelial tubular cells with sloughed off and apoptotic and necrotic cells. 96 hour Cisplatin caused marked epithelial cell loss and the presence of variable numbers if apoptotic/necrotic cells.

3.2 - MiRNA array analysis of serum, urine and kidney tissue in Cisplatin and Adriamycin dosed mice

TLDA array cards were used to profile 384 different miRNA species in the urine, serum and kidney tissue of Cisplatin dosed mice. The 48 hour time point was chosen to be investigated due to non-significant elevations being observed in NAG, BUN and Kim-1. MiRNAs were profiled in the four saline and four Cisplatin dosed mice from the 48 hour group. MiRNAs were also profiled in 8 mice which had been administered with Adriamycin (n=5) or saline (n=3) for two weeks. Adriamycin serves as a second nephrotoxin which targets the podocytes rather than the proximal tubules and is also a chronic nephrotoxin rather than an acute one.

3.2.1 - Normalisation of array data

Expression suite software (Applied Biosystems) was used to determine the most appropriate internal control genes for each biological matrix examined. This is the most widely used

method of normalisation of array studies. The software determines the most stable miRNA species throughout the experimental samples and assigns them values based on their stability. The three most stable miRNA species across each biological matrix as determined by the software were used to normalise the array data. MiR-99b, 203 and 218 were used for urine samples, miR-126-5p, 139-5p and 484 for serum samples and miR-195, 350 and 148b for kidney tissue samples.

3.2.2 - Array data for Kidney tissue of saline, Cisplatin or Adriamycin dosed mice

A total of 40 miRNA species were significantly changed ($P < 0.05$) in the kidney tissue of saline administered CD1 mice when compared to saline administered BALB SCID mice. Of these 41 significantly changed miRNA species, 15 were elevated in the CD1 mice and 24 were reduced. Of the 15 elevated miRNAs, eight had a fold change of over two and of the 24 reduced, five were reduced by a fold change greater than two (figure 5, 6). The most greatly elevated included miR-467b, 187 and 467a (5.5, 3.3 and 2.8 fold respectively) and greatly reduced were miR-205, 802 and let-7a (0.1, 0.1 and 0.4 fold respectively).

A total of 21 miRNA species were significantly changed in the kidney tissue of the Cisplatin dosed CD1 mice when compared to saline administered CD1 mice (figure 7, 8). Of these 21, 16 were elevated and five were reduced significantly. Four of the 16 were elevated by over two fold; miR-685, 205, 34a and Y1 (117.2, 4.1, 2.9 and 2.8 fold respectively). None of the five reduced miRNA species were reduced by greater than two fold.

In the kidneys of Adriamycin (6.5 mg/kg) dosed BALB SCID mice compared to saline dosed BALB SCID mice there were 17 significantly changed miRNA species ($P < 0.05$), 17 of which were elevated and three reduced. Eight of the 17 were elevated by a fold change greater

than two including miR-380-5p, 376a and 34a (4.5, 3.5 and 3 fold respectively) (figure 9, 10). There were no miRNAs that were reduced by over two fold.

When the Cisplatin dosed CD1 mice were compared to the Adriamycin dosed BALB SCID mice (figure 11, 12) a total of 55 miRNA species were found to be significantly changed ($P < 0.05$). Of these 55, 20 were elevated in the Cisplatin dosed mice and 35 were reduced. Eight of the elevated had a fold change of over 2 fold; these included miR-685, 582-3p and 187 (7.9, 7.7 and 3.5 fold respectively). Of the 35 reduced miRNAs 12 were so by greater than two fold; these included miR-302b, 216b, 802 and 217 (0.03, 0.06, 0.09 and 0.2 fold respectively).

3.2.3 - Array data for serum from saline, Cisplatin or Adriamycin dosed mice

When the serum of saline administered CD1 mice was compared to saline administered BALB SCID mice (figure 13, 14) there were a total of 30 significantly ($P < 0.05$) changed miRNA species were found. Of these 30 species, 12 were elevated and 28 were reduced significantly. Of the 12 elevated species, 11 had a fold change of greater than 2; these included miR-182, 376b, 511 and 29b (6.9, 6.1, 5, and 4.4 fold respectively). Of the 28 reduced species, 12 were reduced by a fold change greater than two; these included miR-802, 122, 384-5p and 101a (0.02, 0.1, 0.3 and 0.3 fold respectively).

There were found to be eight significantly changed miRNA species ($P < 0.005$) in Cisplatin dosed CD1 mice compared to saline treated animals (figure 15, 16), six were elevated and two were reduced significantly. Five of the elevated species were by greater than two fold; these included miR-429, 200a, 182 and 221 (6.5, 3.2, 3 and 2.9 fold respectively). Both of the significantly reduced species were reduced by greater than two fold; miR-340-5p and 652 (0.002 and 0.03 fold respectively).

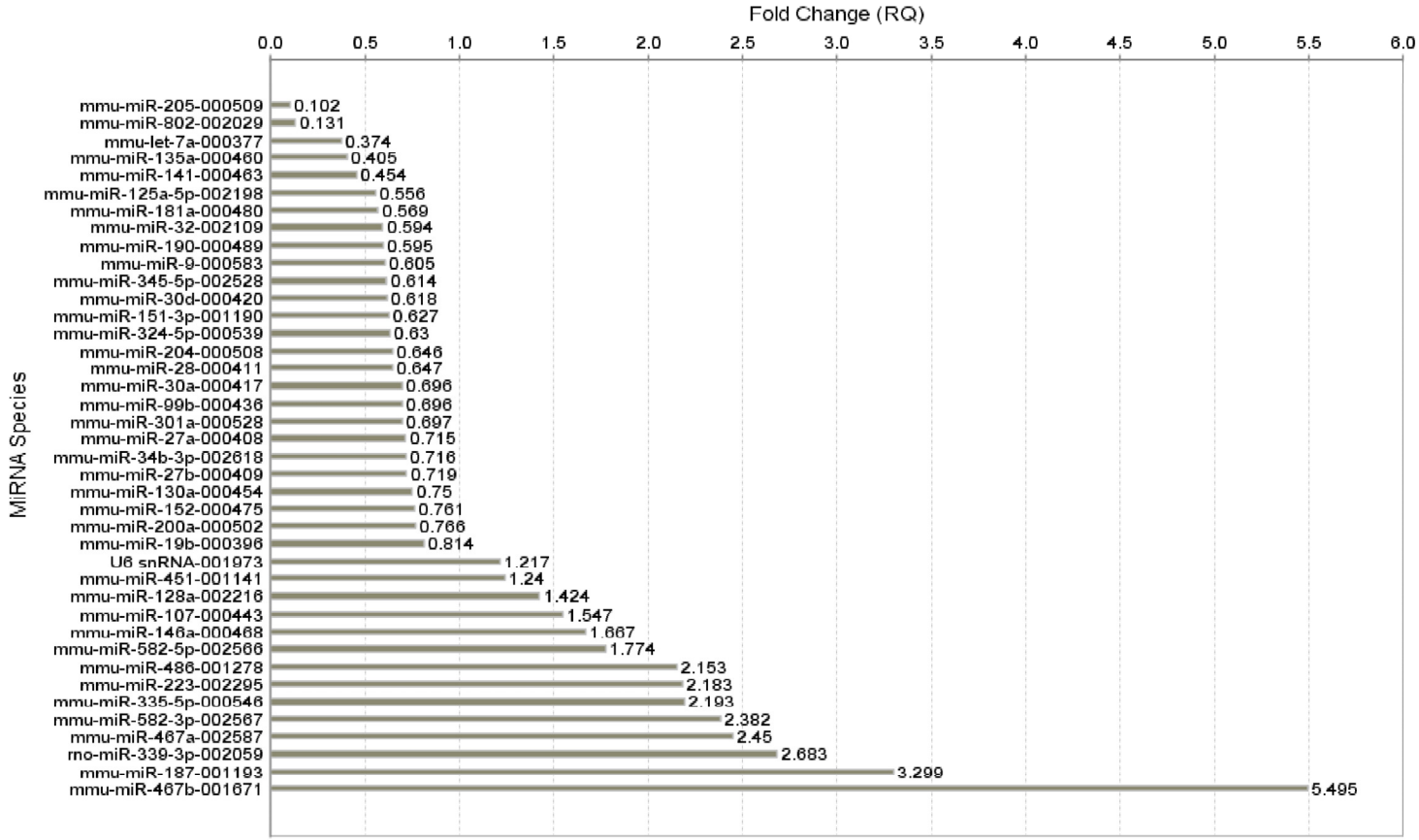

Figure 5: Expression Suite software was used to determine the miRNA species which were significantly ($P < 0.05$) changed in the kidneys of saline (0.9 %) administered CD1 male mice and SCID BALB mice. Fold changes were calculated as $2^{[-(\text{mean of } \Delta Ct \text{ of treated samples - mean of } \Delta Ct \text{ values of control samples})]}$. The fold changes are displayed on the graph. The values are the averages of the independent animal samples

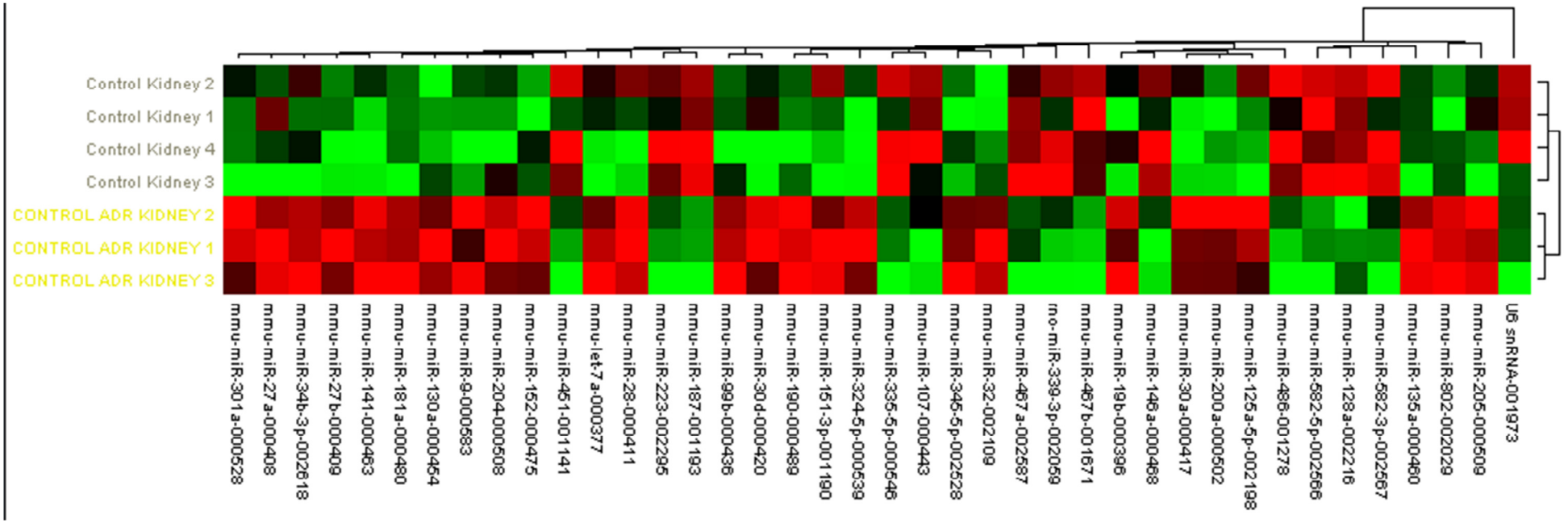

Figure 6: Hierarchical clustering analysis of all miRNAs which were found to be significantly changed between the kidneys of saline dosed BALB SCID mice and CD1 male mice. Expression Suite software was used to identify the significantly changed miRNA species ($P < 0.05$). The figure shows the relative miRNA concentration reported as $2^{-\Delta Ct}$. The progressively brighter shades of red indicate higher miRNA levels and the green indicates lower levels. The miRNA identities are listed on the bottom and the samples on the left.

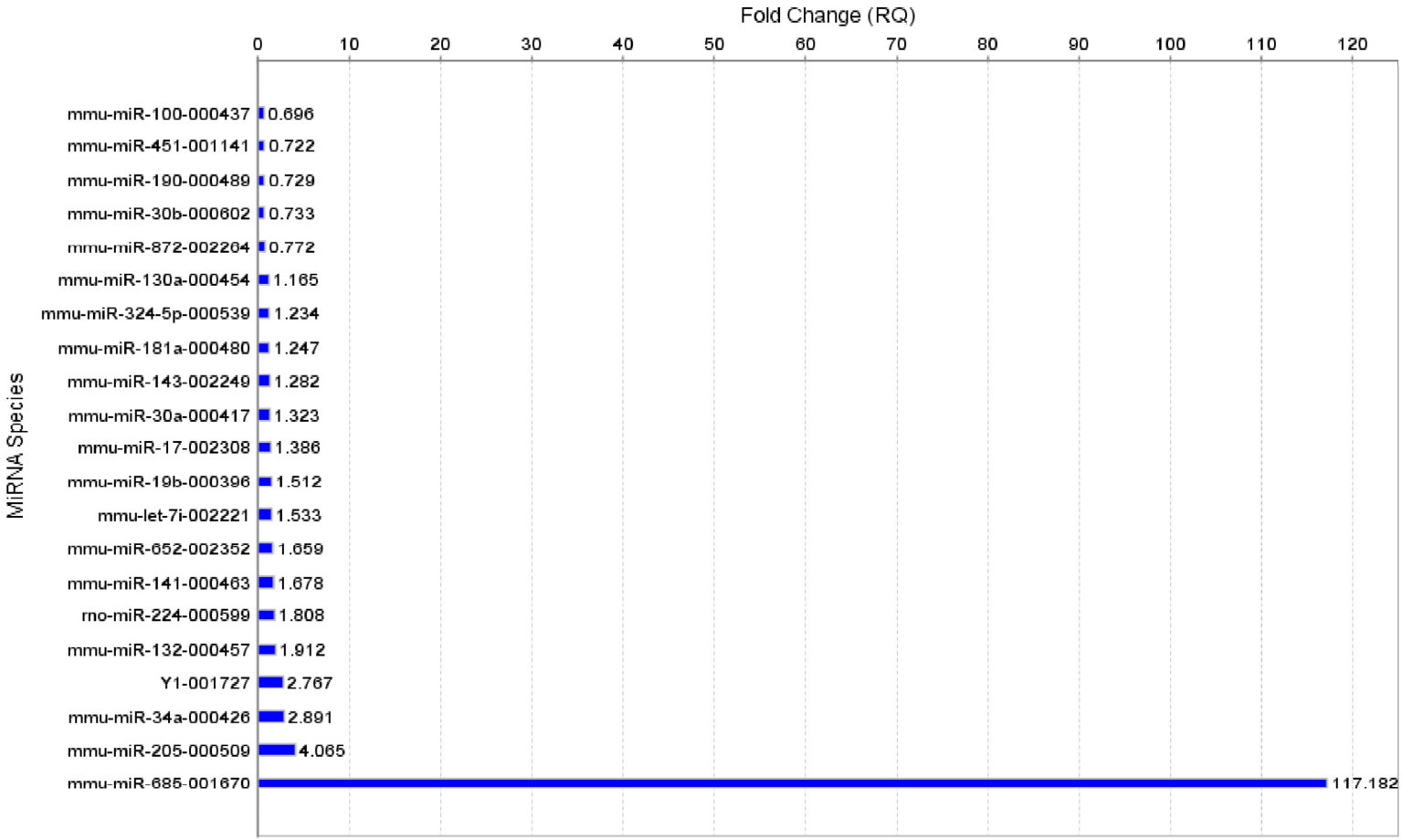

Figure 7: Expression Suite software was used to determine the miRNA species which were significantly ($P < 0.05$) changed in the kidneys of saline (0.9 %) administered CD1 male mice and Cisplatin (20 mg/kg) CD1 mice. Fold changes were calculated as $2^{[-(\text{mean of } \Delta Ct \text{ of treated samples - mean of } \Delta Ct \text{ values of control samples})]}$. The fold changes are displayed on the graph. The values are the averages of the independent animal samples

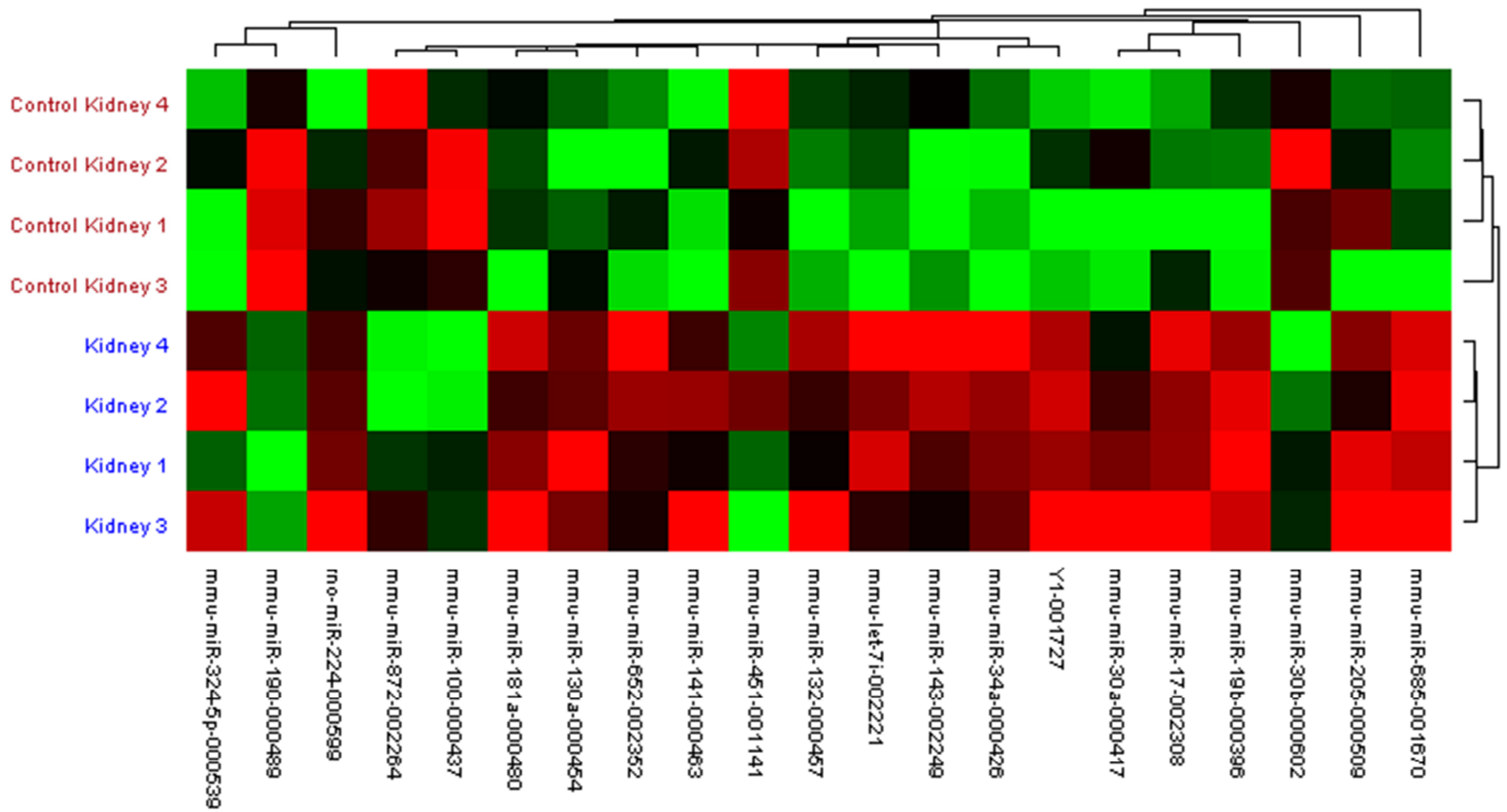

Figure 8: Hierarchical clustering analysis of all miRNAs which were found to be significantly changed between the kidneys of saline and Cisplatin (20 mg/kg) dosed CD1 male mice. Expression Suite software was used to identify the significantly changed miRNA species ($P < 0.05$). The figure shows the relative miRNA concentration reported as $2^{-\Delta Ct}$. The progressively brighter shades of red indicate higher miRNA levels and the green indicates lower levels. The miRNA identities are listed on the bottom and the samples on the left.

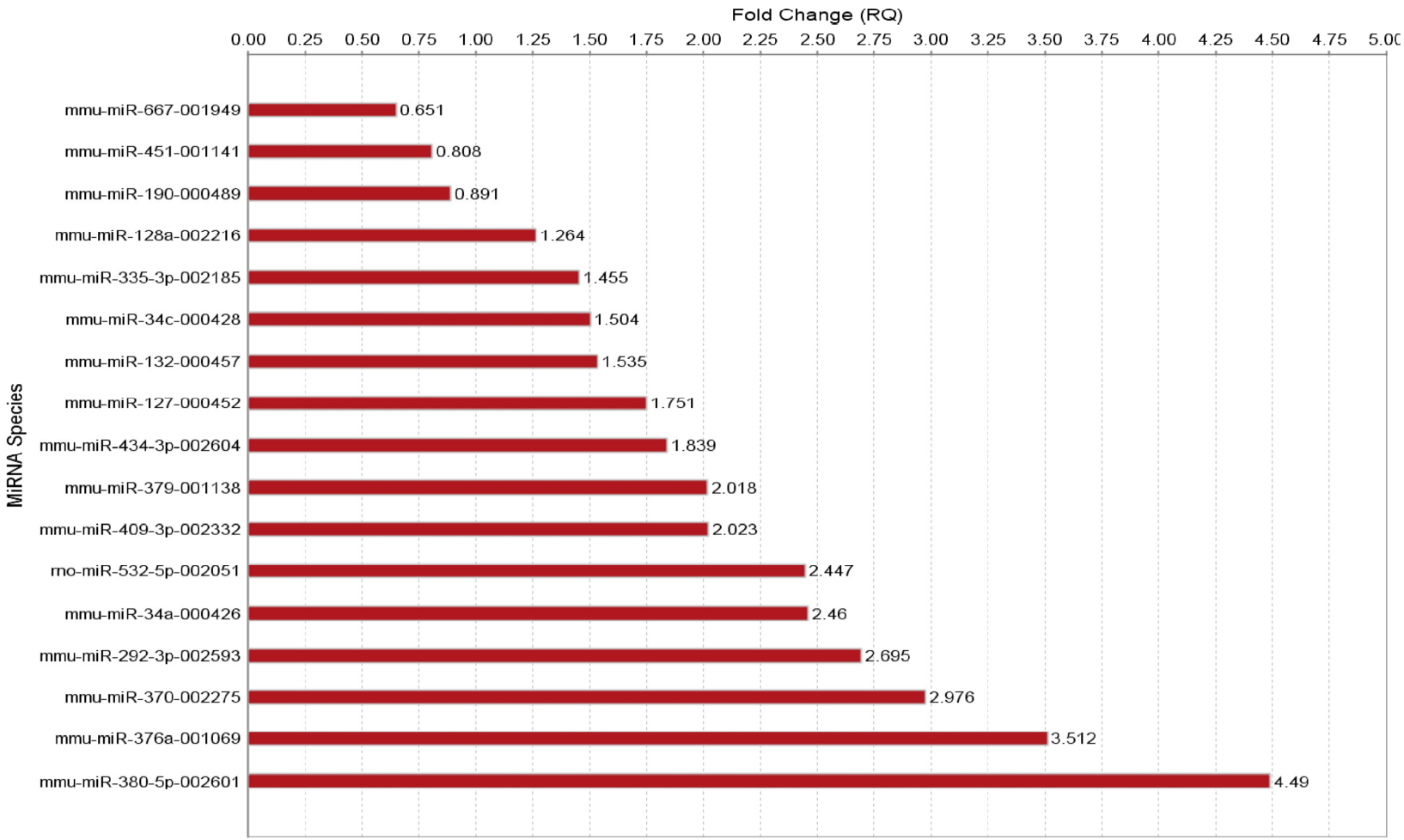

Figure 9: Expression Suite software was used to determine the miRNA species which were significantly ($P < 0.05$) changed in the kidneys of saline (0.9 %) administered BALB SCID mice and Adriamycin (6.5 mg/kg) BALB SCID mice. Fold changes were calculated as $2^{[-(\text{mean of } \Delta Ct \text{ of treated samples - mean of } \Delta Ct \text{ values of control samples})]}$. The fold changes are displayed on the graph. The values are the averages of the independent animal samples

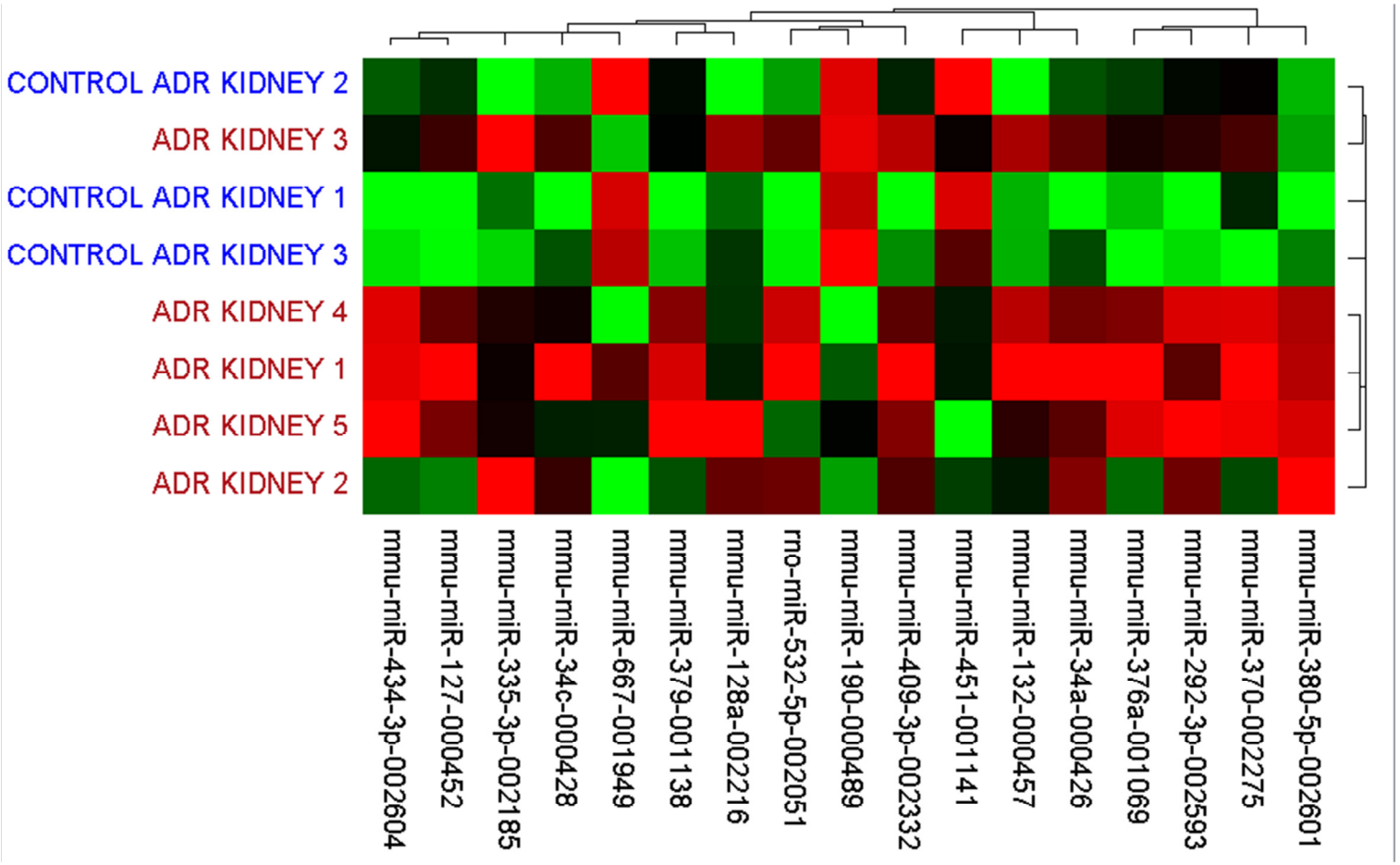

Figure 10: Hierarchical clustering analysis of all miRNAs which were found to be significantly changed between the kidneys of saline and Adriamycin (6.5 mg/kg) dosed BALB SCID mice. Expression Suite software was used to identify the significantly changed miRNA species ($P < 0.05$). The figure shows the relative miRNA concentration reported as $2^{-\Delta Ct}$. The progressively brighter shades of red indicate higher miRNA levels and the green indicates lower levels. The miRNA identities are listed on the bottom and the samples on the left.

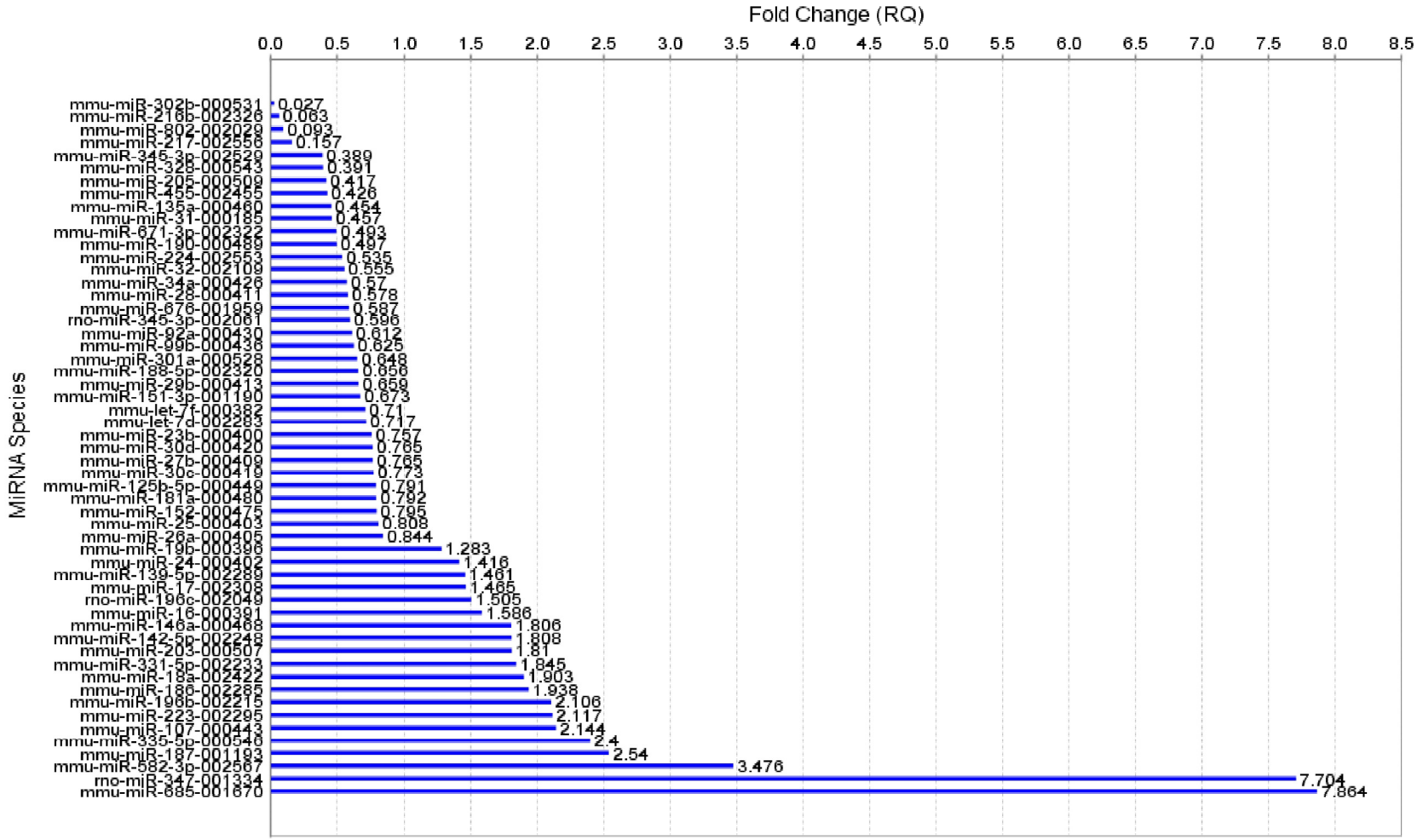

Figure 11: Expression Suite software was used to determine the miRNA species which were significantly ($P < 0.05$) changed in the kidneys of Cisplatin administered (20 mg/kg) CD1 mice and Adriamycin administered (6.5 mg/kg) BALB SCID mice. Fold changes were calculated as $2^{[-(\text{mean of } \Delta Ct \text{ of treated samples - mean of } \Delta Ct \text{ values of control samples})]}$. The fold changes are displayed on the graph. The values are the averages of the independent animal samples

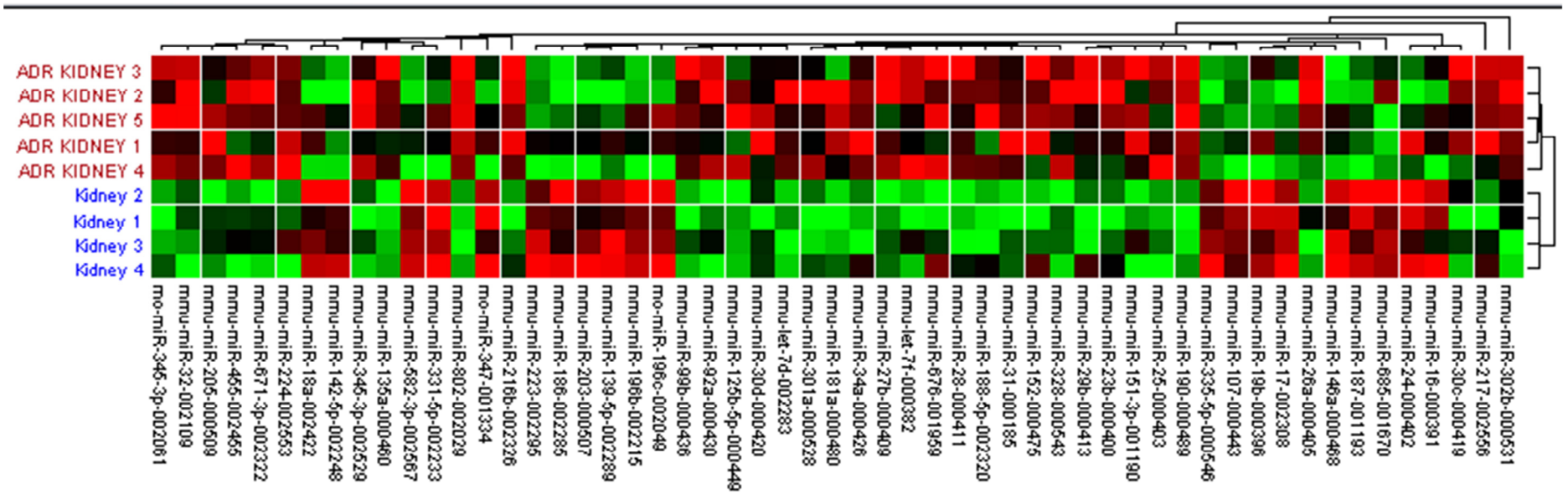

Figure 12: Hierarchical clustering analysis of all miRNAs which were found to be significantly changed between the kidneys of Cisplatin (20 mg/kg) dosed CD1 mice and Adriamycin (6.5 mg/kg) dosed BALB SCID mice. Expression Suite software was used to identify the significantly changed miRNA species ($P < 0.05$). The figure shows the relative miRNA concentration reported as $2^{-\Delta Ct}$. The progressively brighter shades of red indicate higher miRNA levels and the green indicates lower levels. The miRNA identities are listed on the bottom and the samples on the left.

In the Adriamycin dosed BALB SCID mice (figure 17, 18) there were 31 significantly changed miRNA species when compared to control BALB SCID mice. Of these 31, 29 were elevated and two were reduced significantly. Of the 31, 20 were elevated by a fold change greater than two; the greatest of which included miR-129-3p, 544, 224 and 495 (15.3, 5.9, 5.8 and 5.4 respectively). None of the significantly reduced miRNA species were reduced by greater than two fold.

When comparing the Cisplatin dosed mice to the Adriamycin dosed mice a total of 54 miRNA species were significantly altered ($P < 0.05$) (Figure 19, 20), six were elevated and 48 were reduced significantly. All six of the elevated miRNA species were so by greater than two fold; the greatest of which were miR-667, 429, 150 and 134 (21.8, 6, 4.3 and 3.4 fold respectively). Of the 48 reduced species, 32 were so by greater than two fold; the largest of which included miR-216a, 216b, 872 and 217 (0.02, 0.03, 0.04 and 0.05 fold respectively).

3.2.4 - Array data for urine from saline, Cisplatin or Adriamycin dosed mice

The urine of saline administered CD1 mice and BALB SCID mice was found to have five miRNA species which were significantly ($P < 0.05$) changed between the two groups, four were elevated and one reduced in the CD1 mice (Figure 21, 22). Of the five elevated, four were altered by a fold change of over two; these were miR125a, 222, 30e and 204 (11.9, 6.3, 5.5 and 4.8 respectively). The single reduced miRNA was so by greater than twofold (miR-328 – 0.4 fold).

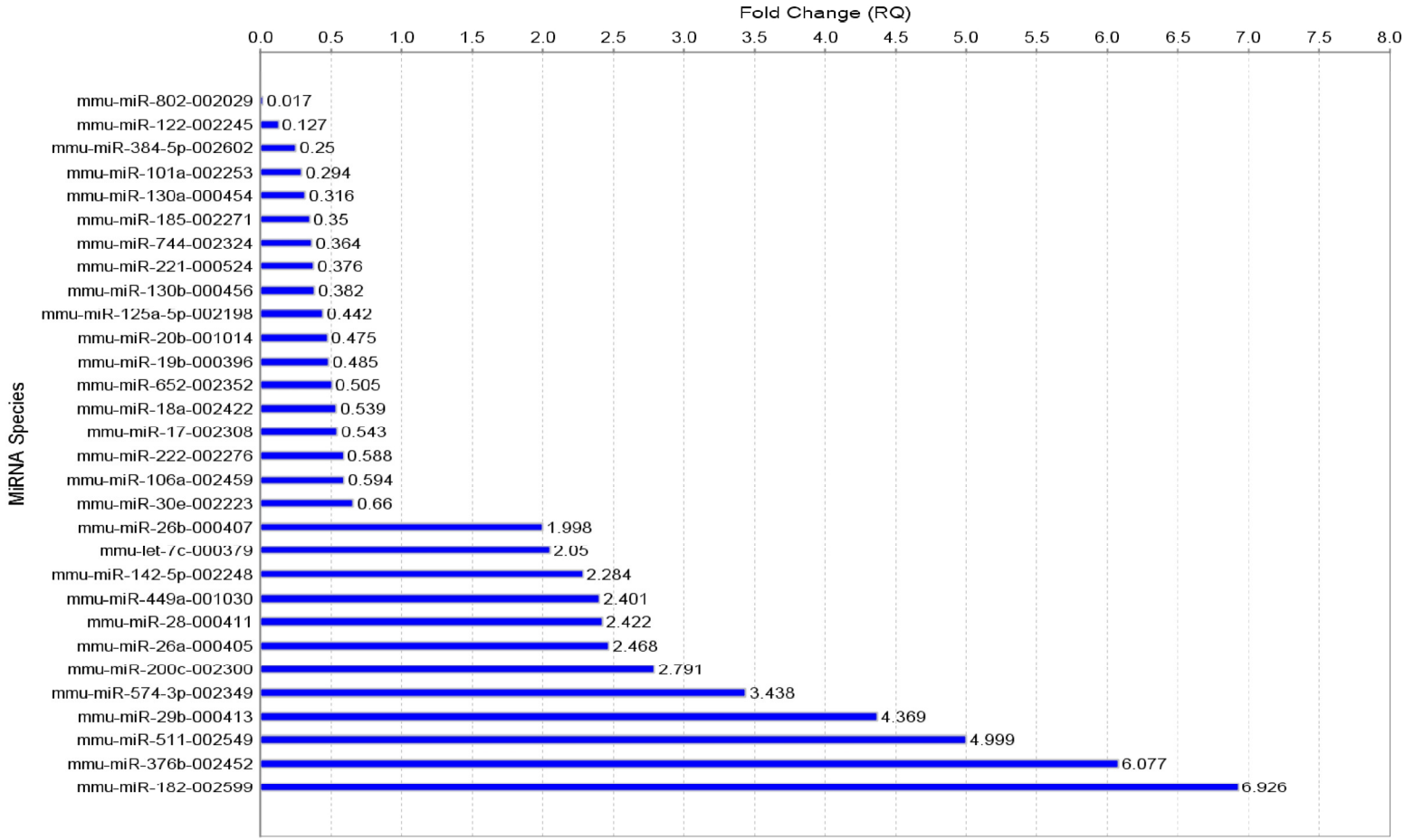

Figure 13: Expression Suite software was used to determine the miRNA species which were significantly ($P < 0.05$) changed in the serum of saline dosed CD1 and BALB SCID mice. Fold changes were calculated as $2^{[-(\text{mean of } \Delta Ct \text{ of treated samples - mean of } \Delta Ct \text{ values of control samples})]}$. The fold changes are displayed on the graph. The values are the averages of the independent animal samples

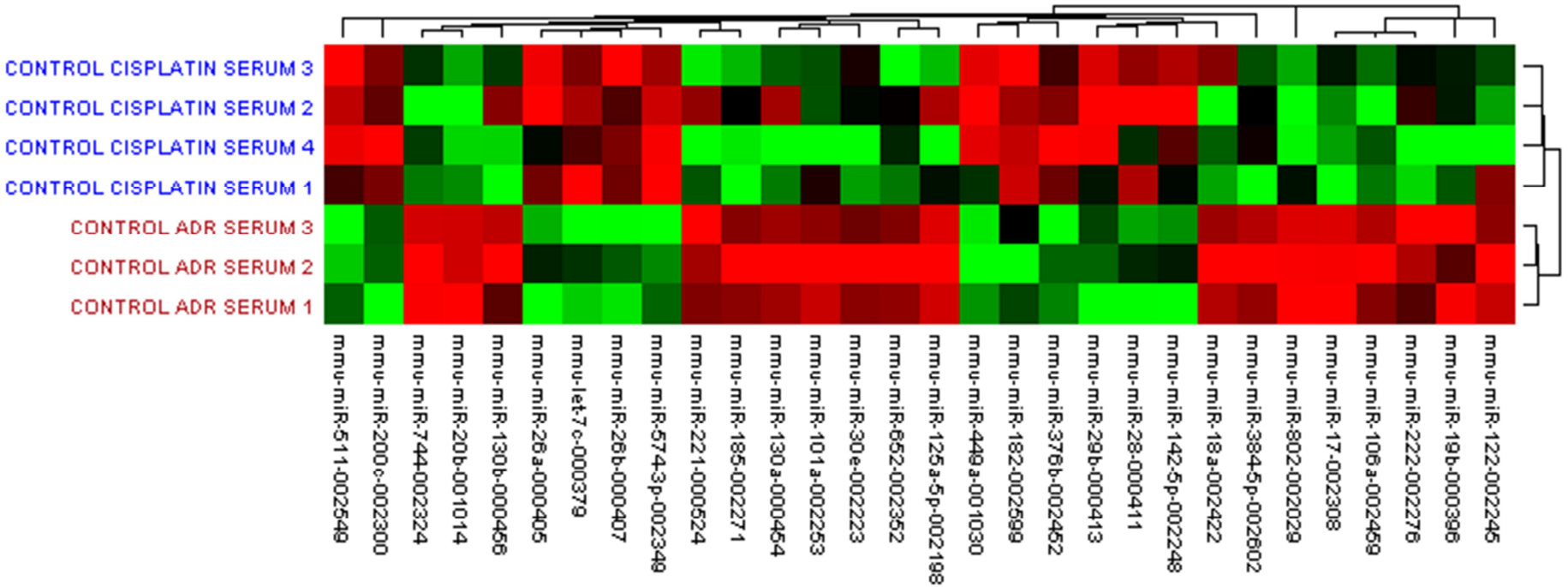

Figure 14: Hierarchical clustering analysis of all miRNAs which were found to be significantly changed between the serum of saline dosed CD1 mice and BALB SCID mice. Expression Suite software was used to identify the significantly changed miRNA species ($P < 0.05$). The figure shows the relative miRNA concentration reported as $2^{-\Delta Ct}$. The progressively brighter shades of red indicate higher miRNA levels and the green indicates lower levels. The miRNA identities are listed on the bottom and the samples on the left.

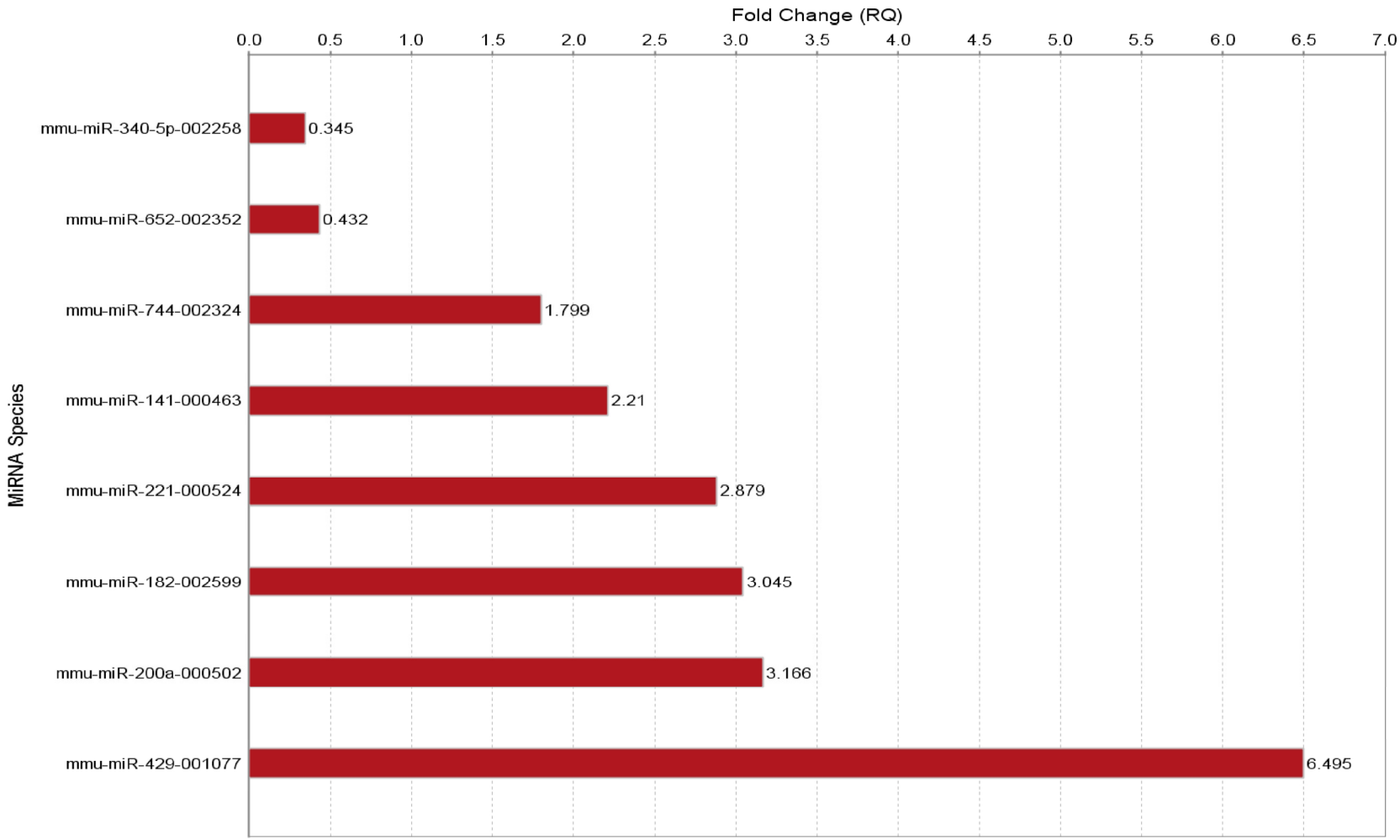

Figure 15: Expression Suite software was used to determine the miRNA species which were significantly ($P < 0.05$) changed in the serum of Cisplatin (20 mg/kg) dosed and saline dosed CD1 mice. Fold changes were calculated as $2^{[-(\text{mean of } \Delta Ct \text{ of treated samples - mean of } \Delta Ct \text{ values of control samples})]}$. The fold changes are displayed on the graph. The values are the averages of the independent animal samples

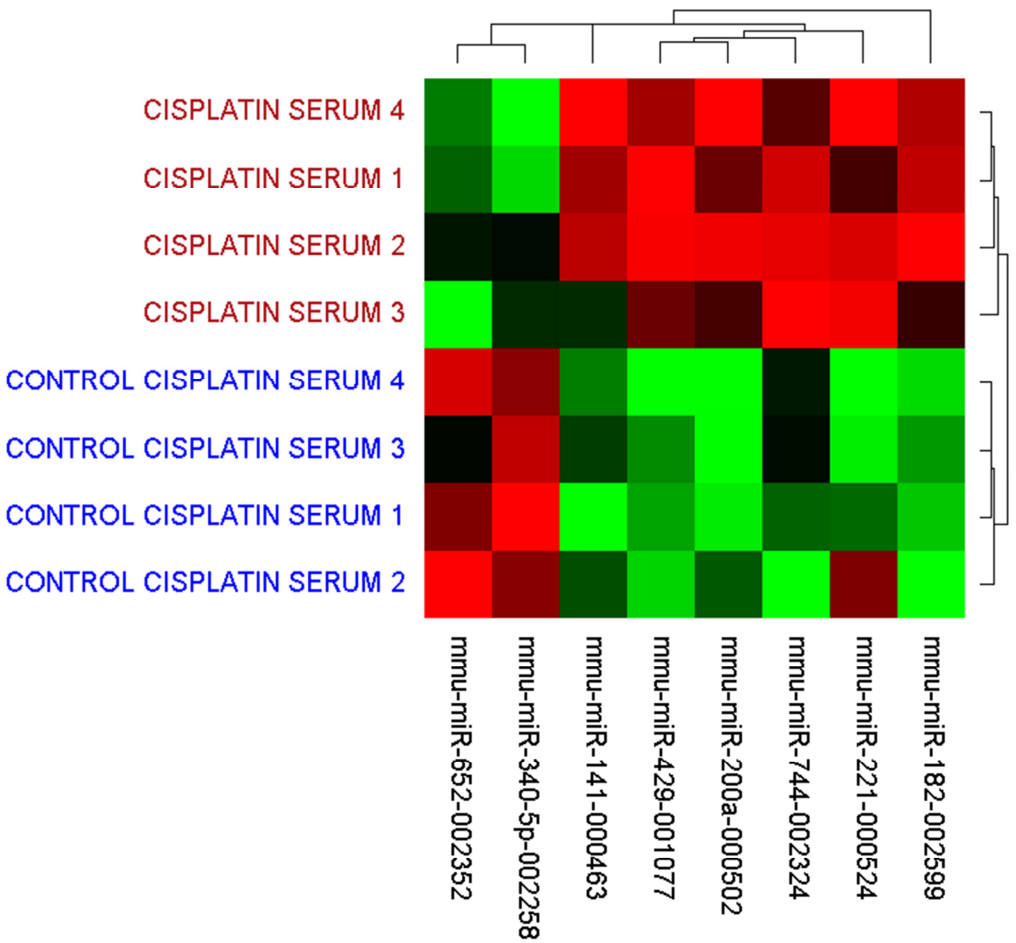

Figure 16: Hierarchical clustering analysis of all miRNAs which were found to be significantly changed between the serum of Cisplatin (20 mg/kg) and saline dosed CD1 mice. Expression Suite software was used to identify the significantly changed miRNA species ($P < 0.05$). The figure shows the relative miRNA concentration reported as $2^{-\Delta Ct}$. The progressively brighter shades of red indicate higher miRNA levels and the green indicates lower levels. The miRNA identities are listed on the bottom and the samples on the left.

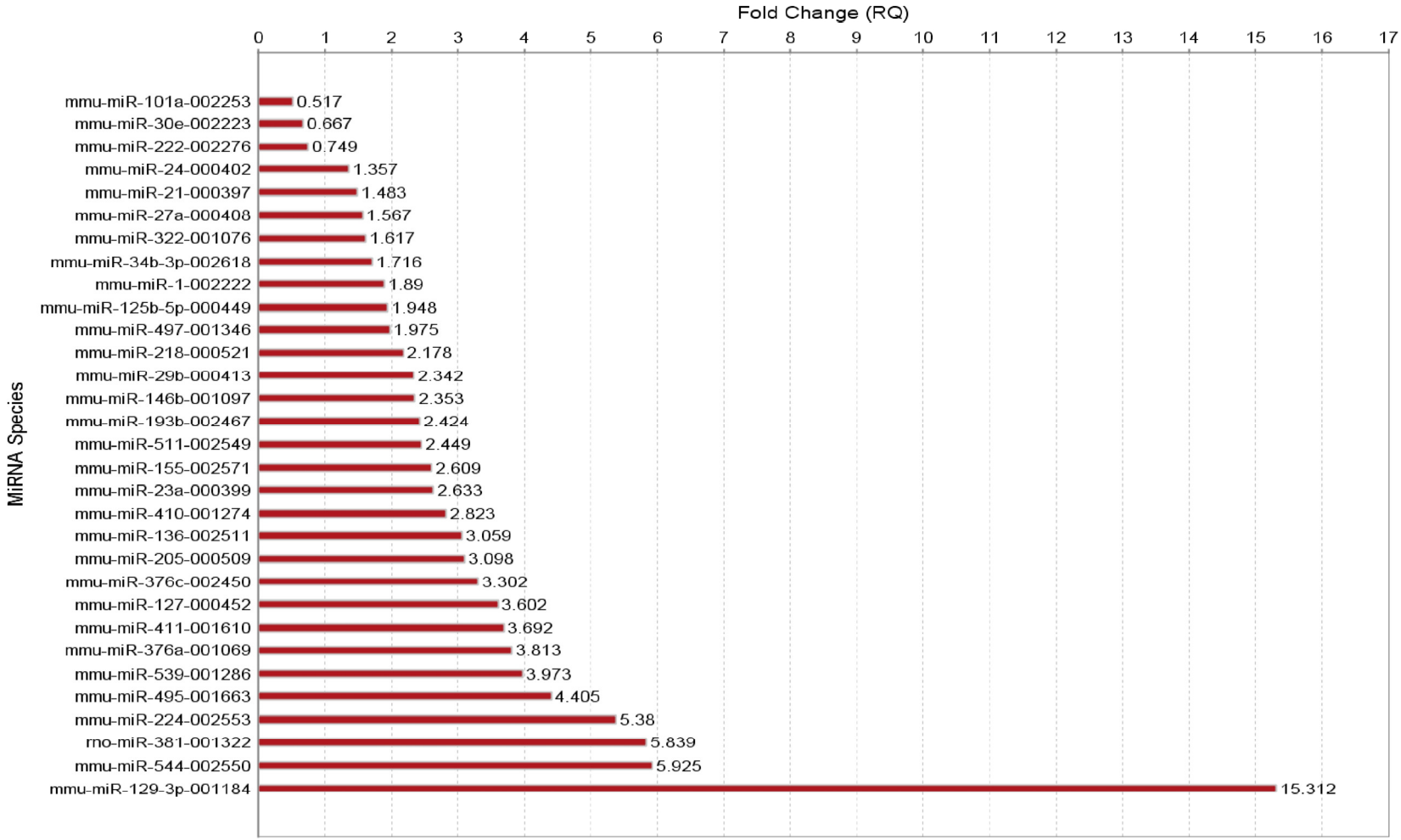

Figure 17: Expression Suite software was used to determine the miRNA species which were significantly ($P < 0.05$) changed in the serum of Adriamycin (6.5 mg/kg) and saline dosed BALB SCID mice. Fold changes were calculated as $2^{[-(\text{mean of } \Delta Ct \text{ of treated samples - mean of } \Delta Ct \text{ values of control samples})]}$. The fold changes are displayed on the graph. The values are the averages of the independent animal samples

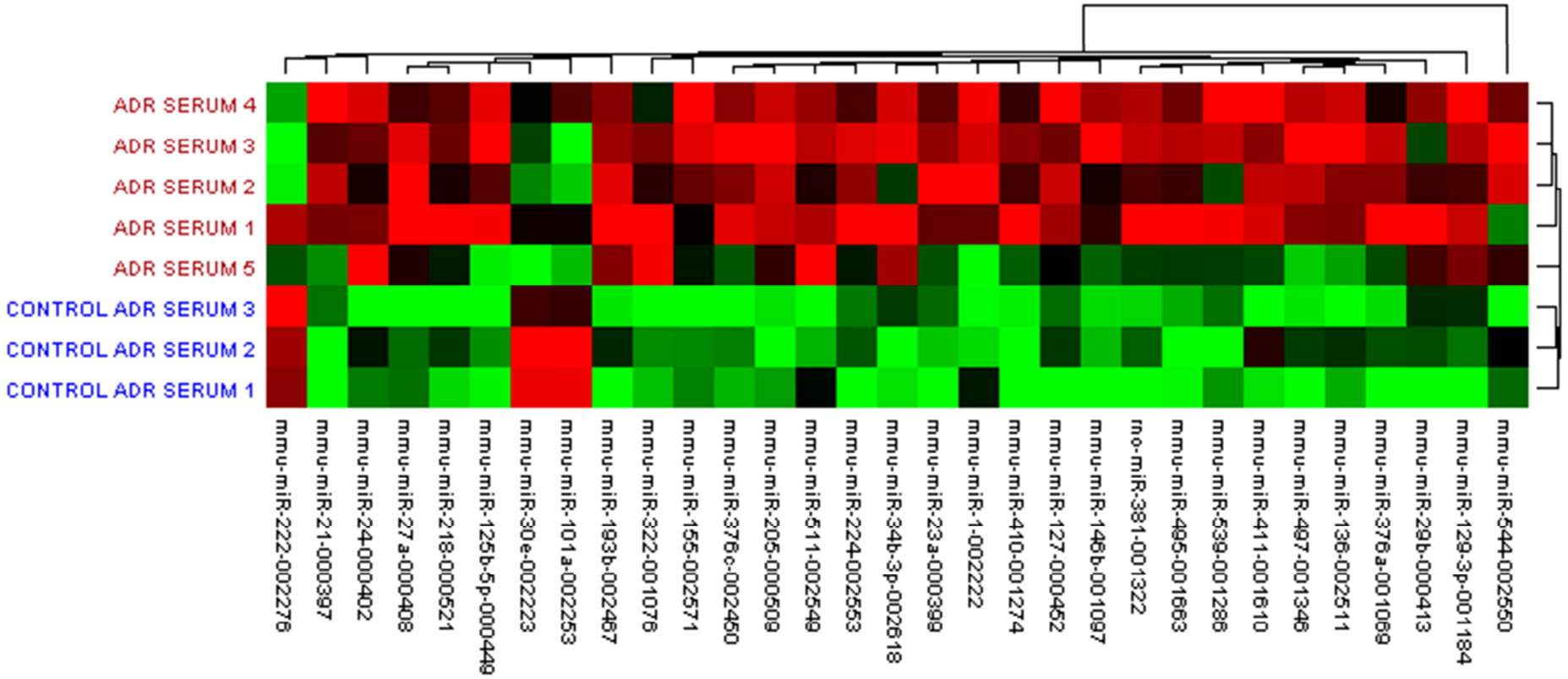

Figure 18: Hierarchical clustering analysis of all miRNAs which were found to be significantly changed between the serum of Adriamycin (6.5 mg/kg) and saline dosed BALB SCID mice. Expression Suite software was used to identify the significantly changed miRNA species ($P < 0.05$). The figure shows the relative miRNA concentration reported as $2^{-\Delta Ct}$. The progressively brighter shades of red indicate higher miRNA levels and the green indicates lower levels. The miRNA identities are listed on the bottom and the samples on the left.

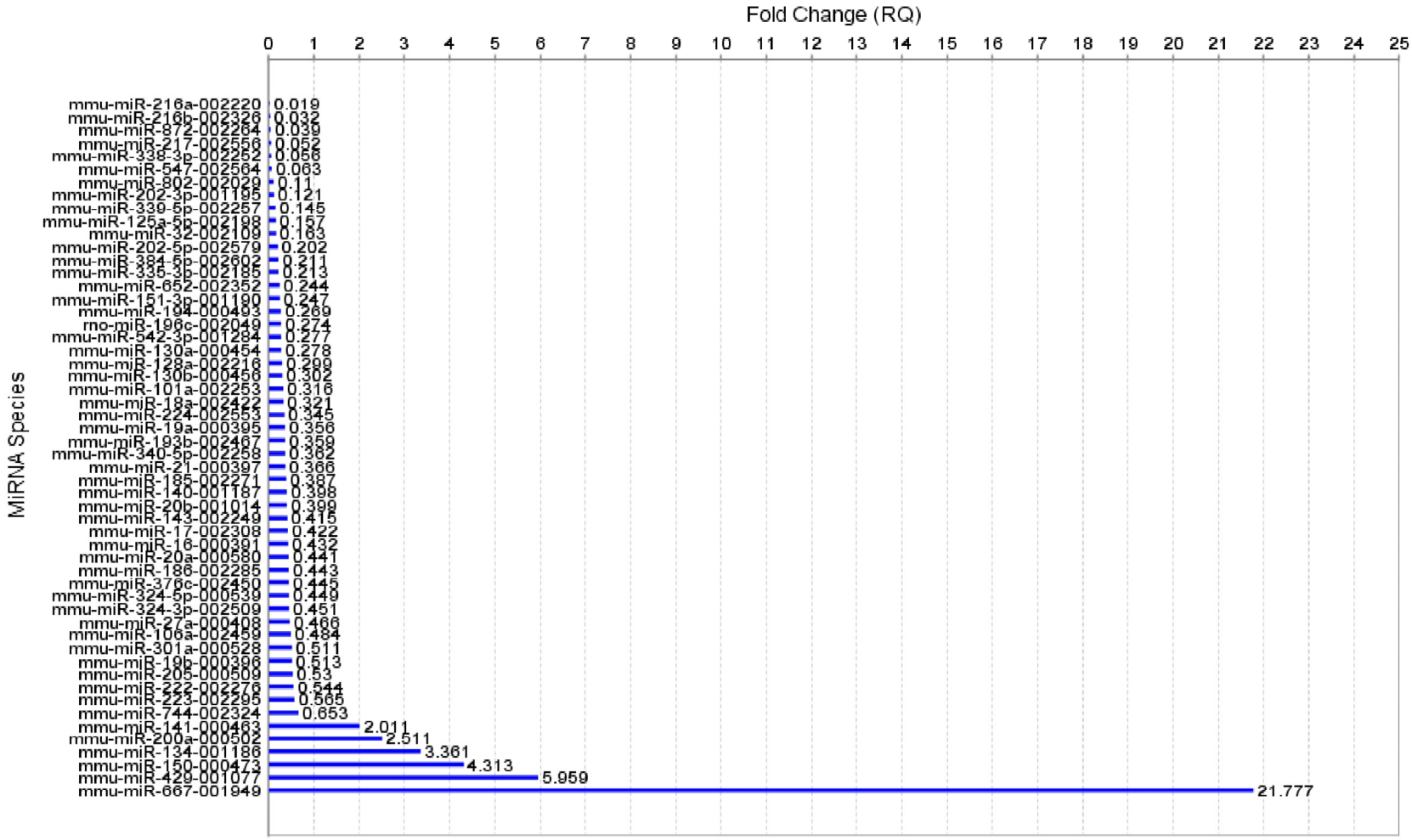

Figure 19: Expression Suite software was used to determine the miRNA species which were significantly ($P < 0.05$) changed in the serum of Cisplatin (20 mg/kg) dosed CD1 mice and Adriamycin (6.5 mg/kg) dosed BALB SCID mice. Fold changes were calculated as $2^{[-(\text{mean of } \Delta Ct \text{ of treated samples - mean of } \Delta Ct \text{ values of control samples})]}$. The fold changes are displayed on the graph. The values are the averages of the independent animal samples

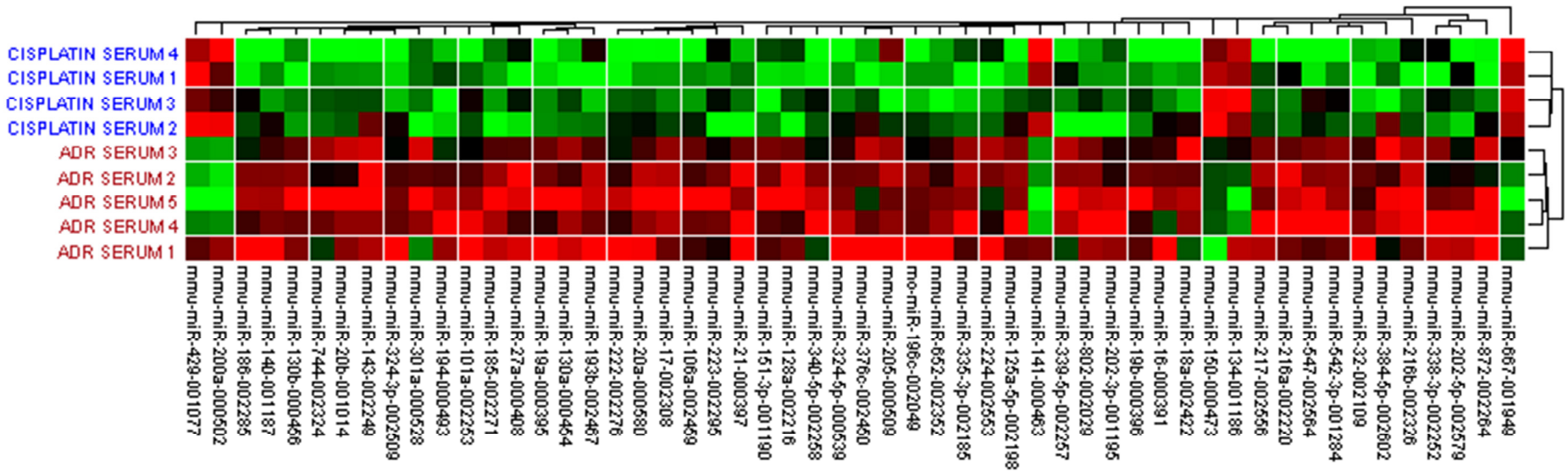

Figure 20: Hierarchical clustering analysis of all miRNAs which were found to be significantly changed between the serum of Cisplatin (20 mg/kg) dosed CD1 mice and Adriamycin (6.5 mg/kg) dosed BALB SCID mice. Expression Suite software was used to identify the significantly changed miRNA species ($P < 0.05$). The figure shows the relative miRNA concentration reported as $2^{-\Delta Ct}$. The progressively brighter shades of red indicate higher miRNA levels and the green indicates lower levels. The miRNA identities are listed on the bottom and the samples on the left.

The Cisplatin dosed CD1 mice contained 20 miRNA species which were significantly changed in the urine of Cisplatin administered compared to saline administered animals (figure 23, 24). Of this 20, 18 were had a fold changer greater than two and all 18 were expressed at greater levels in the urine of the Cisplatin dosed animals relative to the saline dosed ones. The greatest changes included miR15a, 744, 365 and let-7e (27, 21.4, 12.3 and 11.8 fold respectively).

In the Adriamycin dosed BALB SCID mice (figure 25, 26) seven miRNA species were significantly altered when compared to the saline administered mice of the same species, one was elevated while the other six were reduced significantly. The one elevated miRNA was miR-152 which was elevated by 2.4 fold in the Adriamycin dosed mice urine. Of the six significantly reduced miRNA species three were by greater than two fold; miR-9, 10a and 20a (0.04, 0.2, 0.4 fold respectively).

When comparing the Cisplatin dosed CD1 mice and the Adriamycin dosed BALB SCID mice 31 miRNA species were shown to be significantly altered ($P < 0.05$), 13 were elevated and 18 were reduced in the Cisplatin mouse urine significantly (Figure 27, 28). Of the elevated miRNA species 12 were by greater than two fold; the greatest of which were let-7e, miR-324-3p, Y1 let-7d and miR-26b (32.1, 16.9, 10.6, 9.1 and 8.6 fold respectively). Of the 18 reduced species 17 were done so by greater than two fold; the greatest of which were miR342-3p, 146b, 484 and 434-3p (0.01, 0.02, 0.05 and 0.06 fold respectively).

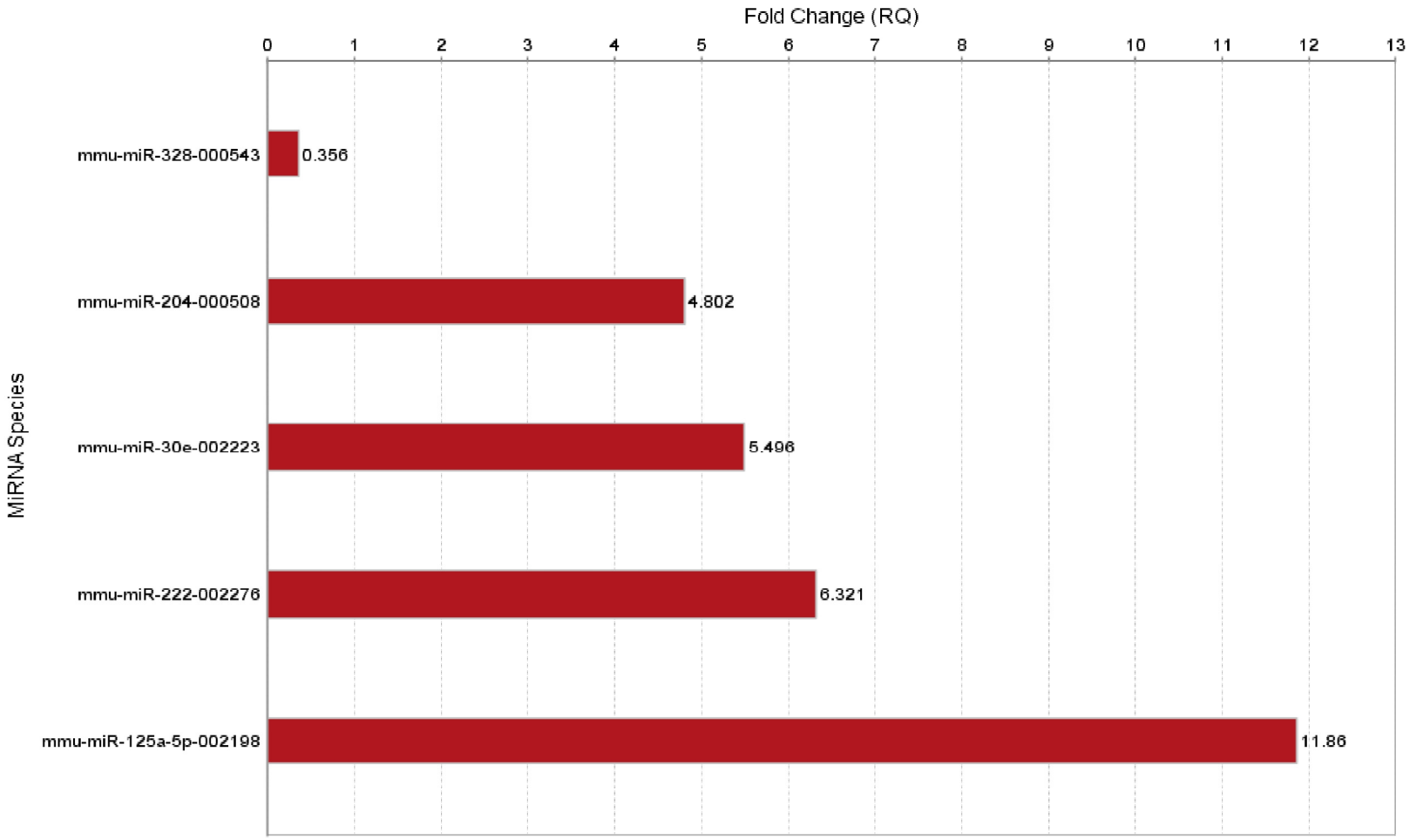

Figure 21: Expression Suite software was used to determine the miRNA species which were significantly ($P < 0.05$) changed in the urine of saline dosed CD1 mice and saline dosed BALB SCID mice. Fold changes were calculated as $2^{[-(\text{mean of } \Delta Ct \text{ of treated samples - mean of } \Delta Ct \text{ values of control samples})]}$. The fold changes are displayed on the graph. The values are the averages of the independent animal samples

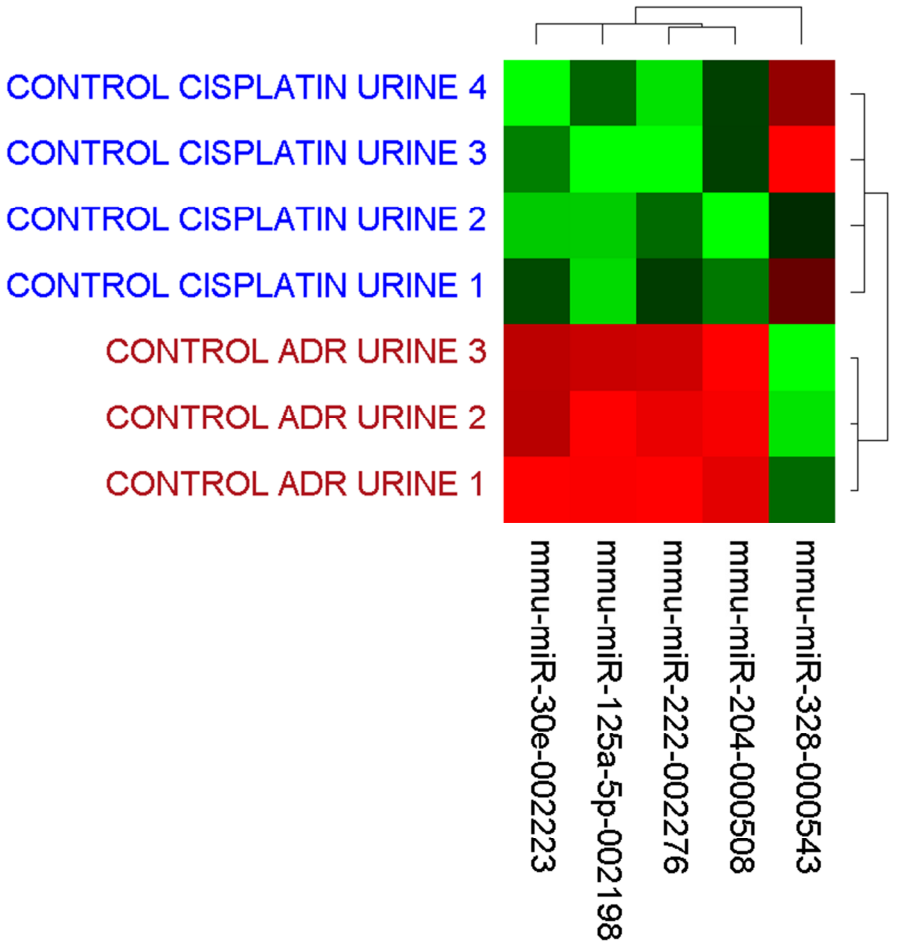

Figure 22: Hierarchical clustering analysis of all miRNAs which were found to be significantly changed between the urine saline dosed CD1 mice and saline dosed BALB SCID mice. Expression Suite software was used to identify the significantly changed miRNA species ($P < 0.05$). The figure shows the relative miRNA concentration reported as $2^{-\Delta Ct}$. The progressively brighter shades of red indicate higher miRNA levels and the green indicates lower levels. The miRNA identities are listed on the bottom and the samples on the left.

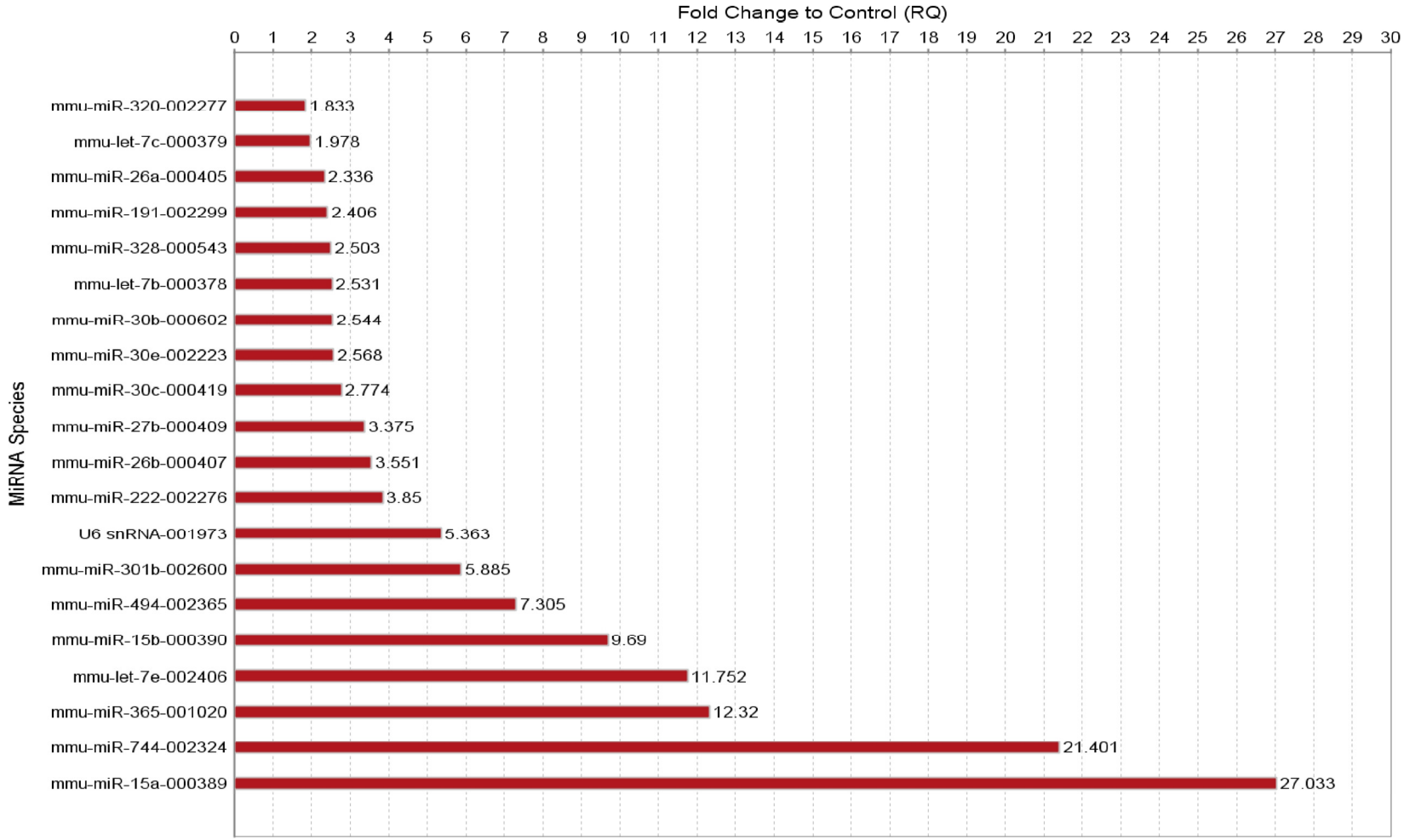

Figure 23: Expression Suite software was used to determine the miRNA species which were significantly ($P < 0.05$) changed in the urine of Cisplatin (20 mg/kg) dosed CD1 mice and saline dosed CD1 mice. Fold changes were calculated as $2^{[-(\text{mean of } \Delta Ct \text{ of treated samples - mean of } \Delta Ct \text{ values of control samples})]}$. The fold changes are displayed on the graph. The values are the averages of the independent animal samples

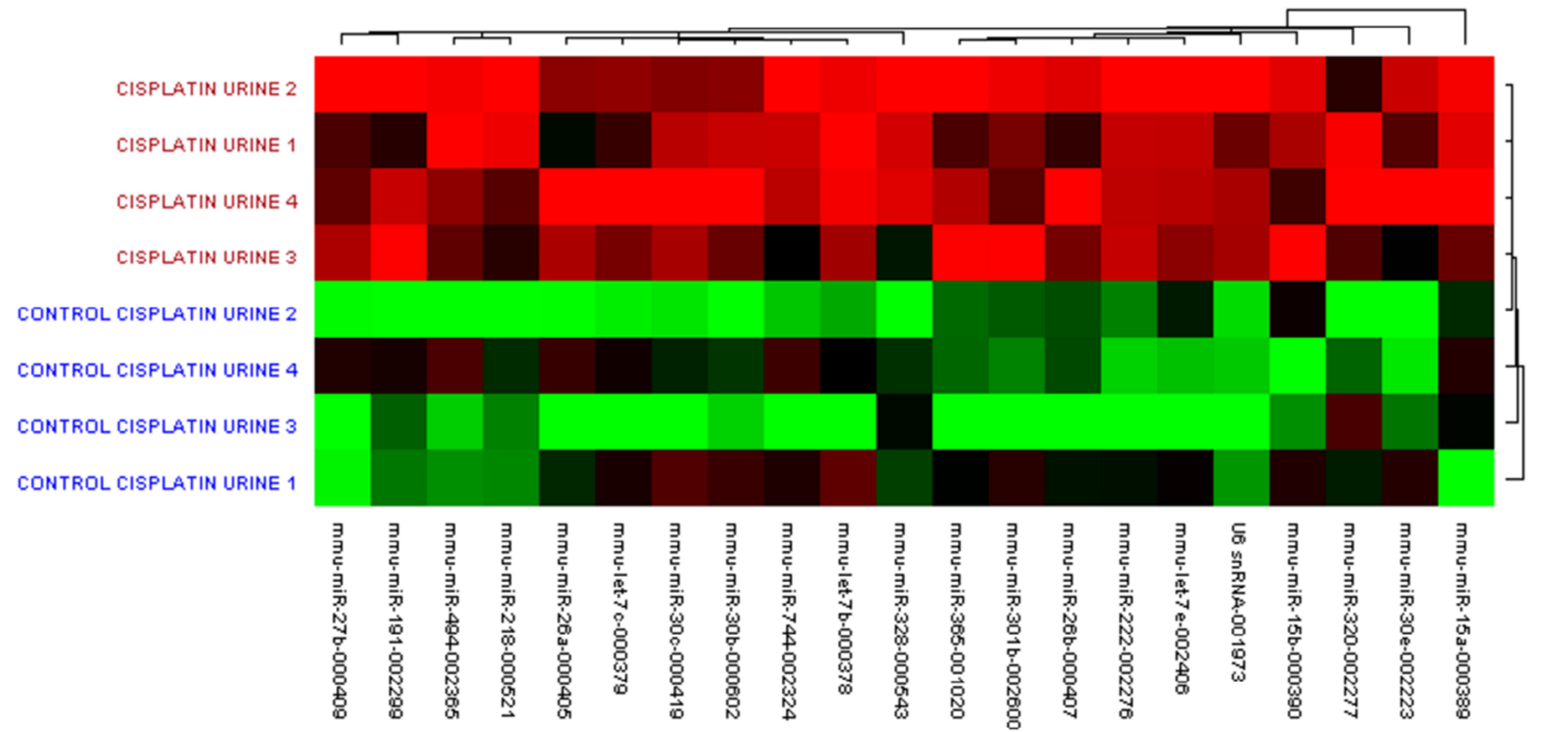

Figure 24: Hierarchical clustering analysis of all miRNAs which were found to be significantly changed between the urine of Cisplatin (20 mg/kg) and saline dosed CD1 mice. Expression Suite software was used to identify the significantly changed miRNA species ($P < 0.05$). The figure shows the relative miRNA concentration reported as $2^{-\Delta Ct}$. The progressively brighter shades of red indicate higher miRNA levels and the green indicates lower levels. The miRNA identities are listed on the bottom and the samples on the left.

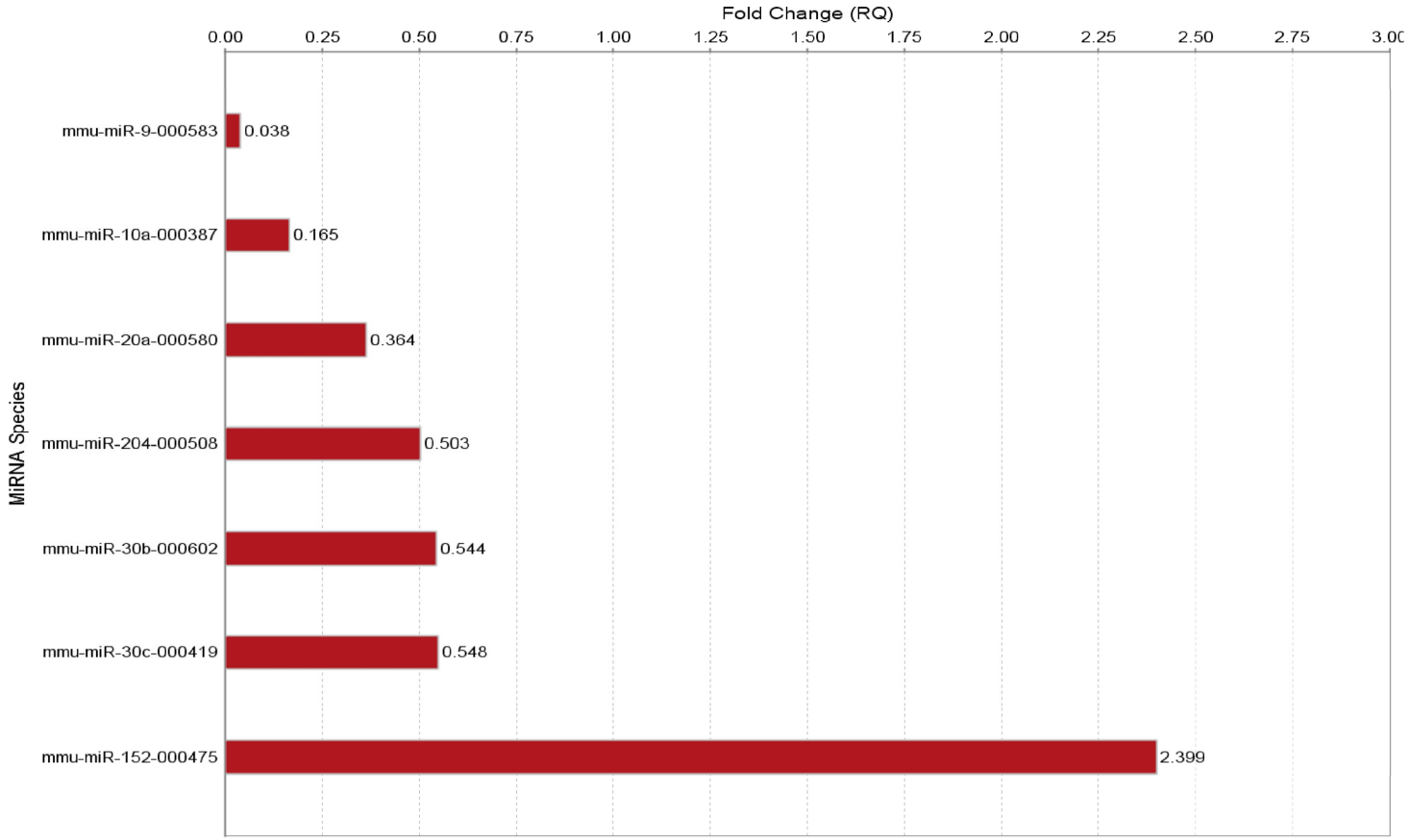

Figure 25: Expression Suite software was used to determine the miRNA species which were significantly ($P < 0.05$) changed in the urine of Adriamycin (6.5 mg/kg) and Saline dosed BALB SCID mice. Fold changes were calculated as $2^{[-(\text{mean of } \Delta Ct \text{ of treated samples - mean of } \Delta Ct \text{ values of control samples})]}$. The fold changes are displayed on the graph. The values are the averages of the independent animal samples

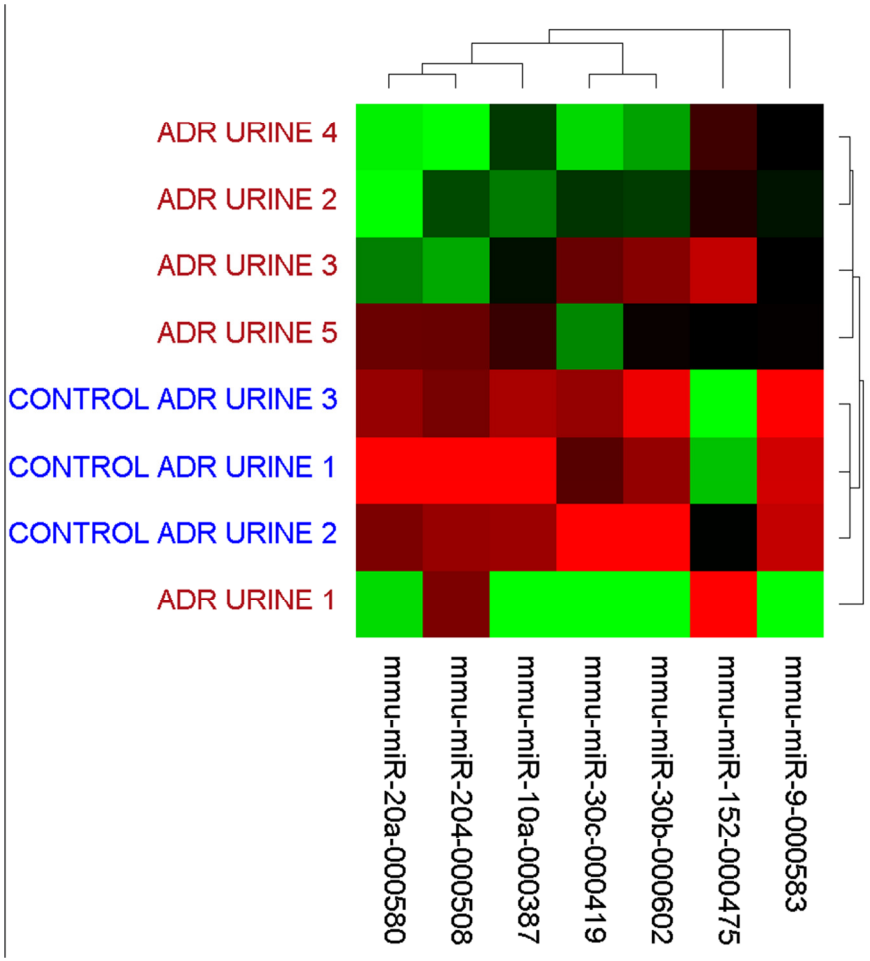

Figure 26: Hierarchical clustering analysis of all miRNAs which were found to be significantly changed between the urine of Adriamycin (6.5 mg/kg) and saline dosed BALB SCID mice. Expression Suite software was used to identify the significantly changed miRNA species ($P < 0.05$). The figure shows the relative miRNA concentration reported as $2^{-\Delta Ct}$. The progressively brighter shades of red indicate higher miRNA levels and the green indicates lower levels. The miRNA identities are listed on the bottom and the samples on the left.

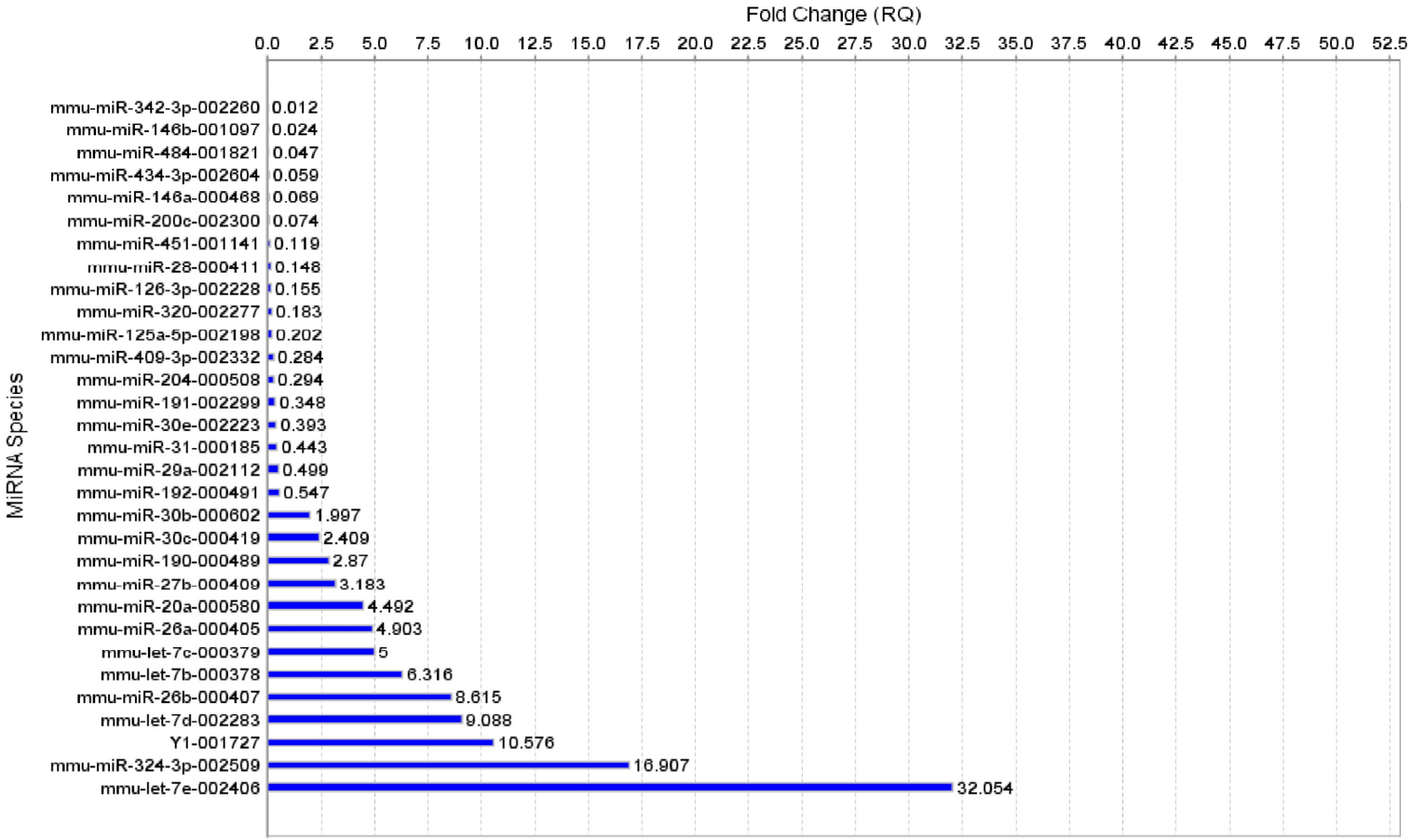

Figure 27: Expression Suite software was used to determine the miRNA species which were significantly ($P < 0.05$) changed in the urine of Cisplatin (20 mg/kg) dosed CD1 mice and Adriamycin (6.5 mg/kg) dosed BALB SCID mice. Fold changes were calculated as $2^{[-(\text{mean of } \Delta Ct \text{ of treated samples - mean of } \Delta Ct \text{ values of control samples})]}$. The fold changes are displayed on the graph. The values are the averages of the independent animal samples

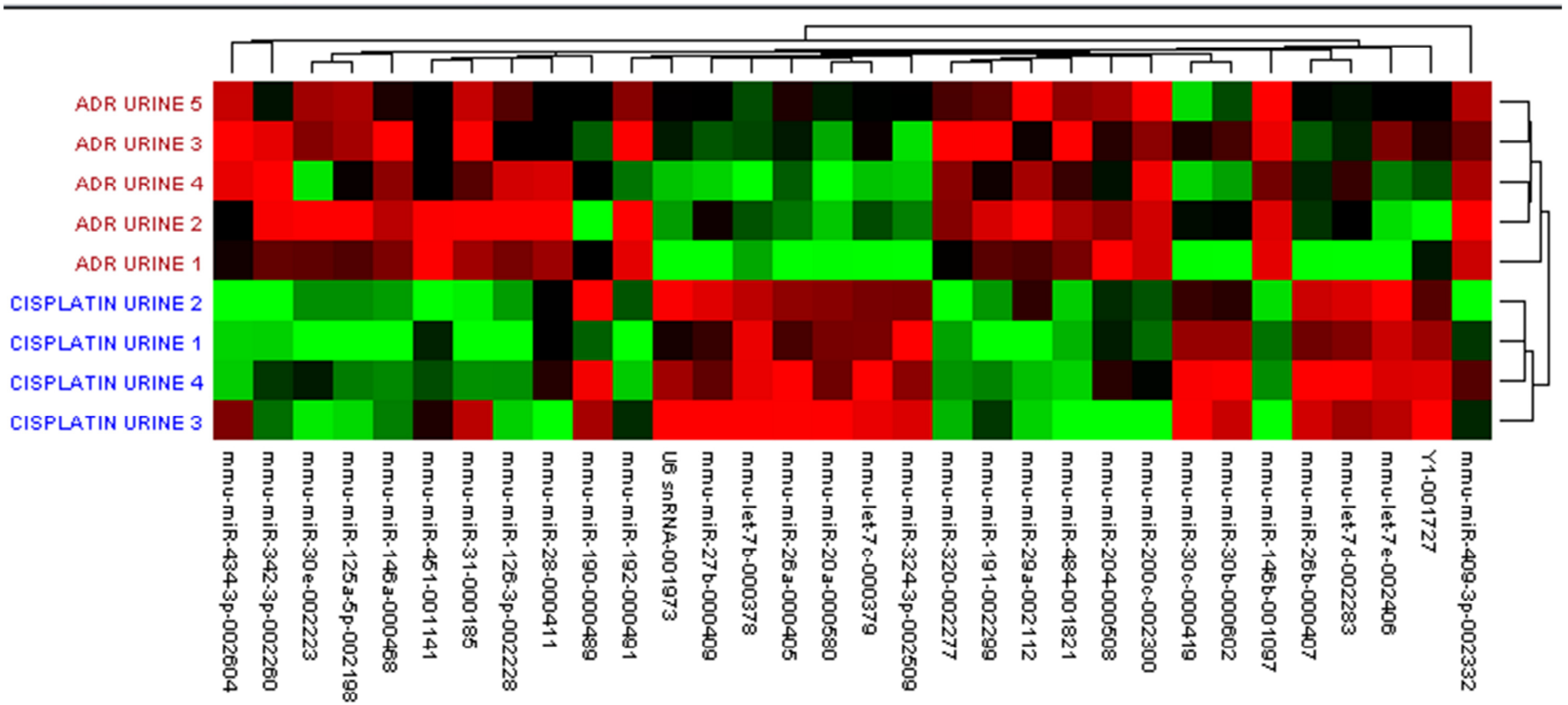

Figure 28: Hierarchical clustering analysis of all miRNAs which were found to be significantly changed between the urine of Cisplatin (20 mg/kg) dosed CD1 mice and Adriamycin (6.5 mg/kg) dosed BALB SCID mice. Expression Suite software was used to identify the significantly changed miRNA species ($P < 0.05$). The figure shows the relative miRNA concentration reported as $2^{-\Delta Ct}$. The progressively brighter shades of red indicate higher miRNA levels and the green indicates lower levels. The miRNA identities are listed on the bottom and the samples on the left

4 - Discussion

4.1 - Induction of nephrotoxicity with Cisplatin in vivo

Cisplatin was used to induce nephrotoxicity in CD1 male mice to serve as a more subtle and location specific model of kidney injury than ischemic reperfusion. CD1 male mice were administered with either saline (0.9 %) or Cisplatin (20 mg/kg) intraperitoneally and culled after 24, 48, 72 or 96 hours post dose. Urinary creatinine, NAG, Kim-1 and BUN were quantified in this model to confirm the presence of nephrotoxicity. Administration of Cisplatin caused no significant reduction in weight or changes in urine volume in any of the time points. Urinary creatinine (UCr) was quantified not as a marker of kidney injury but to serve as a normalising agent to correct for variations in urine volume and concentration. There were no significant variations in UCr in the Cisplatin administered animals at any time point. Urinary NAG was elevated significantly in Cisplatin dosed animals at 72 hours but not at any other time point. BUN was only significantly elevated 96 hours after Cisplatin administration whereas urinary Kim-1 was significantly elevated at both 72 and 96 hours post Cisplatin administration. This is to be expected given that Kim-1 has been shown to be a more sensitive marker of kidney injury than both BUN and NAG. NAG may show significant elevations at 72 hours but not 96 hours due to the degree of damage to the epithelial cells of the proximal tubules being so large that the biomarkers release is limited. This can be explained similarly to Hy's law. At 96 hours NAG may show lower levels than at 72 hours due to the unavailability of the enzyme due to loss of epithelial mass which contained the marker. Indeed, the histopathological analysis shows that at 96 hours there was marked epithelial cell loss and presence of variable numbers of apoptotic and necrotic cells. Together the markers quantified in this study show that there was significant kidney injury

72 and 96 hours post Cisplatin administration. BUN, NAG and Kim-1 were all also elevated at 48 hours but not significantly so and histological changes were evident suggesting the onset of nephrotoxicity at this time point. For this reason the 48 hour samples were chosen to be used in the miRNA array profiling studies so that the miRNA arrays may show significant elevations in miRNA species prior to elevations in other biomarkers of kidney injury.

4.2 – MiRNA profiles of Cisplatin dosed CD1 male mice

MiRNA profiling was carried out on CD1 male mice which had received either Cisplatin (20 mg/kg) or saline (0.9 %) for 48 hours as previously described. Samples were chosen from the 48 hour group as elevations in BUN, NAG and urinary Kim-1 were observed along with histological changes. These changes were not significant so it was hypothesised that changes in miRNA profiles in the urine, serum or kidney tissue would however be significant. The Cisplatin dosed animals were treated as a single biological group as were the saline administered animals and miRNA profiles were determined in the kidney tissue, serum and urine. Results were expressed as fold changes from the saline administered animals.

In the kidney tissue of Cisplatin dosed animals there were four miRNAs which were elevated by a change of greater than two fold. These included miR-685, 205 and 34a. MiR-685 has been removed as an official miRNA according to miRBase as it has been shown to be a fragment of RNase P RNA. For this reason miR-685 will be investigated no further in this thesis. MiR-205 has been shown to be involved in epithelial to mesenchymal cell transition and tumour invasion; its targets include VEGF and it has hypothesised that it may play a role in determining stem cell fate (Greene *et al.*, 2010; Majid *et al.*, 2010). MiR-34a is known to be involved in the apoptotic response of cells and plays a role in a cells susceptibility to apoptosis so the fact that this is significantly elevated in the kidneys of mice which had

received Cisplatin, a known apoptotic agent, is noteworthy and may warrant further investigation (Cannell *et al.*, 2010a; Cannell *et al.*, 2010b; Yamakuchi *et al.*, 2008). Mir-34a is expressed at high levels in the podocytes and collecting ducts. Five miRNA species were found to be significantly reduced in the kidney tissue of the Cisplatin dosed mice relative to the saline administered mice however none of which were reduced by changes greater than two fold. It may be expected that certain miRNA species would be reduced in the kidney tissue if they were lost from dying cells to the urine or serum. The fact that none were reduced by greater than twofold could possibly be explained by the sheer abundance of miRNAs in any tissue and that the loss observed after this degree of kidney injury was not sufficient to produce significant changes. It is interesting to note that one of the few miRNA species that were reduced in the kidney tissue of the Cisplatin administered mice was miR-30b which was elevated in both the urine of the IR model described in the previous chapter and in this model.

In the serum of the Cisplatin dosed animals there were a total five expressed at levels greater than two fold larger than in the saline administered animals. These were miR-429, 200a, 182, 221 and 141. MiR-429, 200a and 141 are actually all part of the same families of miRNAs (which also include miR-205 mentioned above). It seems that this family of miRNAs are expressed at greater levels in the serum and kidney tissue post Cisplatin injury (Cannell *et al.*, 2010a; Cannell *et al.*, 2010b; Yamakuchi *et al.*, 2008). This may be linked to their role in mesenchymal to epithelial cell transition. Perhaps surprisingly, two of the significantly altered miRNA species were actually found at reduced levels in the serum of Cisplatin dosed mice with fold decreases greater than two, miR652 and 340-5p (0.432 and 0.345 respectively). It is not a huge surprise that only five miRNA species were significantly

elevated by over two fold in the serum of the Cisplatin dosed animals given the proximal tubule localisation of the damage.

In the urine of the Cisplatin dosed animals there was a total of 18 miRNAs elevated by a fold change greater than two. The largest increases were found in miR-15a, 744, 365, let-7e and 15b. The miR-15 family have been shown to target Bcl-2 which is involved in the apoptotic pathway (Aqeilan *et al.*, 2010; Bonci *et al.*, 2008). Let-7e is expressed highly in the collecting ducts of the kidney so is likely to be found in the urine post injury. Amongst the other significantly elevated miRNA species are miR-26b, 30b and 30c, all of which were found to be significantly elevated in the urine of the IR mouse model of kidney injury and are expressed at high levels in the kidney. It should also be noted that U6snRNA was elevated by 5.4 fold in the urine of the Cisplatin dosed animals. This further reinforces the fact that it is an inappropriate endogenous control for miRNA studies in urine at least. No miRNA species were found to be significantly reduced in the urine of the Cisplatin administered mice relative to the control animals. This is not surprising given the fact that the urine should not contain a large amount of cellular debris under physiological conditions, especially when compared to urine of mice exposed to a nephrotoxin. Of the miRNAs which were elevated in the urine, only two were found to also be significantly altered in the kidney tissue or serum also. These were miR-744 which was found to be elevated in the serum and the urine (1.8 and 21.4 fold respectively) and miR-30b which was decreased in the kidney tissue (1.4 fold) and increased in the urine (2.6 fold). MiR-744 has been shown to enhance cell proliferation so may be elevated in response to Cisplatin to repair the damage induced by the compound (Huang *et al.*, 2012). It is reasonable to assume that this loss of miR-30b in the kidney tissue

is responsible for the increase in the urine, especially given the fact that miR-30b is enriched in the kidney.

4.3 – MiRNA profiles of Adriamycin dosed BALB SCID mice

MiRNA profiling was carried out on Adriamycin (6.5 mg/kg) or saline (0.9 % saline) administered BALB with SCID. Mice received Adriamycin once and were culled two weeks post administration. Adriamycin has been shown to be nephrotoxic and causes damage to the podocytes of the glomerulus (Ayla *et al.*, 2011; Yang *et al.*, 2009b). As with the Cisplatin administered animals, the kidney tissue, serum and urine of the Adriamycin and saline administered BALB SCID mice were profiled for miRNA species. In the kidney of the Adriamycin dosed mice there were a total of three significantly decreased and 14 elevated miRNAs. None of the significantly decreased miRNA species were by a fold change greater than two which again may be a result of the sheer abundance of miRNAs expressed in tissues. Eight of the elevated miRNA species were elevated by greater than twofold, these included miR-380-5p, 376a and 34a. There is not much literature regarding the majority of these miRNA species although miR-370 and miR-409-3p are expressed highly in the embryonic kidney. Much like the kidney tissue of the Cisplatin dosed animals, miR-34a is also significantly elevated by over two fold in the Adriamycin dosed animals possibly suggesting an important role for this miRNA in nephrotoxicity. It is also worth noting that another member of the miR-34 family, miR-34c, is also elevated in the kidney tissue of Adriamycin dosed mice.

In the serum of Adriamycin dosed mice there were a total of 31 miRNA species which were significantly altered, 28 were elevated and three were reduced. None of the significantly decreased miRNA species were decreased by over two fold which is not surprising as the

majority of circulating miRNAs would be a result of release from cells so a reduction would not be expected. Of the 28 elevated miRNAs, 20 were elevated by a fold change of greater than two. The largest fold changes were found in miR-129-3p, 544, 224, 495 and 539. Again, there is not a lot of literature which links these elevated miRNA species to the kidney or cell death or response in injury, however a number of the miRNAs, like in the kidney tissue, are expressed greatly in embryonic kidneys. It should be noted that miR-205 (part of the 200 family) is also elevated in the serum and tissue of the Cisplatin dosed animals. Another notable elevated miRNA miR-146b which is involved in inflammation and the innate immune response. Cisplatin dosed animals showed only five serum elevated miRNAs with a fold change greater than two where Adriamycin showed 20. This is surprising given that both compounds are nephrotoxic although could possibly be explained by the localisation of the kidney injury. Cisplatin causes proximal tubule damage whereas Adriamycin causes damage to the podocytes which surround the capillaries of the glomerulus. Damage to the podocytes could therefore cause miRNAs to be released preferentially into the blood rather than the urine which would be much more prevalent after proximal tubule injury. However it is also known that Adriamycin is cardiotoxic so this increase may be a result of release from damaged cardiomycocytes rather than the glomerulus. Two of the miRNA species which were significantly elevated in the kidney tissue were also elevated in the serum; miR-376a (3.5 and 3.8 fold change respectively) and miR-127 (1.8 and 3.6 fold change respectively).

In the urine of the Adriamycin dosed animals one miRNA was significantly elevated by over twofold, miR-152 (2.4 fold) while three were significantly reduced. MiR-152 is expressed highly in podocytes so its presence in the urine after Adriamycin administration may be

expected. MiR-9, 10a and 20a were reduced by over twofold. MiR-9 has been implicated in clear renal carcinomas. MiR-10a has been shown to be highly expressed in the kidney tissue of mice and regulate a number of HOX genes which regulate embryonic development (Lund, 2010). The fact that a greater amount of miRNA were reduced in the urine of the Adriamycin dosed mice relative to the saline dosed ones is unexpected given the nephrotoxic effect of the compound. It does however further support the notion suggested previously that many of the miRNAs may be lost into the serum rather than the urine. It is also possible that the fact that this Adriamycin model is a chronic rather than acute like Cisplatin influences the release of miRNAs into the urine.

4.4 – MiRNA profiles of Cisplatin and Adriamycin dosed mice

The miRNA profiles of the 48 hour Cisplatin dosed (20 mg/kg) CD1 male mice were compared to those of the two week Adriamycin (6.5 mg/kg) dosed BALB SCID mice. This was to determine whether there are any differences between the miRNA profiles in the kidney tissue, serum or urine of the mice in response to two nephrotoxins with different kidney specific sites of toxicity. Due to the fact that the mice used in two studies were not the same strain and also that the BALB mice had SCID then it is difficult to determine whether any changes observed between the Cisplatin and Adriamycin dosed mice were a result of the nephrotoxic agents themselves or a result of strain or immunological differences. For the purposes of this study any changes between the Cisplatin and Adriamycin dosed mice which were also observed between the saline (0.9 %) administered mice of both strains were ignored due to the inability to say confidently that the changes were a result of nephrotoxin induced changes rather than strain differences. In the kidney tissue two miRNAs were elevated (miR-685, and 196b) and eight were decreased (miR- 302b, 216b and 217 were

most changed) by greater than twofold in the Cisplatin dosed mice relative to the Adriamycin mice. Again, miR-685 will not be taken into account as it has been removed from the miRBase database.

In the serum of the Cisplatin dosed mice relative to the Adriamycin dosed mice six miRNAs were elevated by over two fold; miR-667, 429, 150, 134, 200a and 141 (21.8, 6, 4.3, 3.4, 2.5 and 2 fold respectively). 30 were reduced by greater than two fold, the largest reductions were in miR-216a, 216b, 872, 217 and 338-3p (0.02, 0.03, 0.04, 0.06 and 0.06 fold change respectively). The number of miRNA species which are reduced in the Cisplatin dosed mice relative to the Adriamycin dosed mice is largely due to the numbers which are elevated by over two fold in the Adriamycin dosed model.

In the urine of the Cisplatin dosed mice relative to the Adriamycin dosed mice there were 13 elevated and 15 reduced miRNA species. Let-7e, 324-3p, let-7d and 26b (32.1, 16.9, 9, 8.6 fold respectively) were elevated the largest reductions were in miR-343-3p, 146, 484, 434-3p and 146a (0.01, 0.02, 0.05, 0.06 and 0.07 respectively).

It is possible that the data gathered in these array studies could provide potential miRNA biomarkers which may be specific for either podocyte or proximal tubular injury. In order to determine these targets the miRNA species which were significantly altered between the saline administered CD1 mice and the BALB SCID mice were removed from the analysis to account for possible strain differences as previously described. This was followed by the removal of miRNA species from the analyses which are significantly altered between the Cisplatin and the saline administered mice to remove any changes in miRNAs which were specific to Cisplatin. The Adriamycin specific miRNA species were then also removed from the analysis in the same way. This leaves a group of miRNA species which were only

significantly altered when the Cisplatin dosed mice are compared to the Adriamycin dosed mice but not in the control mice or as a result of Cisplatin or Adriamycin alone. All changes are treating Adriamycin as the reference group so increases refer to increases in the Cisplatin dosed group for example. This leaves 32 significantly changed miRNAs in the kidney, 11 of which are elevated and 21 which are reduced. Of these 11 only one was elevated by greater than two fold; miR-196b (2.1 fold) and eight are reduced by greater than two fold; these include miR-302b, 216b, 217 and 345-3p (0.03, 0.06, 0.2, 0.4 and 0.4 fold respectively). MiR-196b is known to be highly expressed in the podocytes and glomeruli of kidneys. Many of the significantly reduced miRNA species are highly expressed in the embryonic kidney.

In the serum three miRNAs were elevated and 27 reduced. All three elevated species had a fold change greater than two; miR-667, 150 and 134 (21.7, 4.3 and 3.4 respectively). MiR-150 and 134 are both expressed highly in the kidneys. Of the 27 reduced species, 25 were by a fold change greater than two fold. The greatest reductions were in miR-216a, 216b, 872, 217 and 338-3p (0.02, 0.03, 0.04, 0.05, 0.06 fold respectively). The greater number of reduced miRNA species in the serum further add weight to the notion that podocyte damage may allow the release of miRNAs from dying cells into the circulation rather than the urine. In the urine there were four elevated and 13 reduced miRNA species. All of the four were elevated by greater than two fold change; miR-324-3p, Y1, let-7d and 190 (16.9, 10.6, 9.1, 2.9 fold respectively). Of the reduced species 12 were reduced by greater than two fold; these included miR-342-3p, 146b, 484, 434-3p and 146a (0.01, 0.02, 0.05, 0.06, 0.07 fold respectively). The fact that a greater number of miRNAs are reduced in the urine of Cisplatin dosed mice (elevated in Adriamycin dosed mice) is perhaps initially surprising given

the small amount of significantly altered miRNAs in the Adriamycin model compared to the Cisplatin model. However the majority of elevated miRNAs in the urine of Cisplatin model were also elevated significantly when compared to the Adriamycin model so have not been included in this analyses. A general rule seems to be when comparing the Cisplatin and the Adriamycin dosed models that the miRNA species are elevated in the Cisplatin model are expressed highly in the kidney whereas the ones expressed in the Adriamycin model seem to be expressed in the embryonic kidney.

The work in this chapter has shown that in a Cisplatin induced model of DIKI a number of urinary miRNAs are significantly elevated earlier than Kim-1, NAG or BUN suggesting they are more sensitive than other markers of kidney injury. This study has also highlighted a number of miRNAs which are significantly elevated in the kidney tissue of both an Adriamycin and a Cisplatin model of DIKI. MiR-34a was elevated in both models and the miR-34 family of miRNAs have been shown to be involved in cell cycle progression and apoptosis. The following chapter will investigate miR-34a and the role it may play in susceptibility to toxicity.

Chapter 5

Investigating the Functional Intracellular Role of MiRNAs as Regulators of Susceptibility to DIKI

Contents

1 - **Introduction**

Cell death is an important event in organ toxicity and nephrotoxicity is no exception. The type of cell death which occurs during toxicity can also play a role in the severity and response of the body to the toxic event. The two most important forms of cell death are apoptosis and necrosis. Apoptosis is a "programmed" form of cell death (Majno *et al.*, 1995) which is also known as cell suicide whereas necrosis is an "accidental" or "unplanned" form of cell death. One of the main differences which can be shown experimentally between apoptosis and necrosis is that the apoptotic process can be interfered with through modulation of intracellular signalling whereas necrosis cannot (Broker *et al.*, 2005).

Apoptosis occurs in response to many factors and plays an important role in events such as growth and embryonic development as well as a defensive mechanism. These factors include control by genetic cues, exogenous factors such as hormones or cytokines as well as in response to toxic insult or viral agents (Majno *et al.*, 1995). Upon the induction of apoptosis the cell becomes denser and shrinks, the chromatin condenses, and membrane blebbing occurs. The cell continues to shrink and condense and becomes a number of apoptotic bodies (Broker *et al.*, 2005). The organelles in the cell do not swell unlike necrosis. Nuclear DNA is cleaved by specific endonucleases which cleave the DNA into fragments which are multiples of 185 base pairs. Once these apoptotic bodies have been formed they can be cleared by macrophages by phagocytosis or lyse and release their content into the extracellular environment by a process called secondary necrosis (Majno *et al.*, 1995). There are two main mechanisms of apoptosis, the extrinsic (death receptor) and the intrinsic (mitochondrial) pathways, both of which however result in caspase 3 activation (Elmore, 2007; Morley *et al.*, 2000). The intrinsic pathway involves the outer membrane of the

mitochondria becoming permeable allowing the release of toxic proteins. Once this occurs the cell has passed “the point of no return” for apoptotic cell death. This is regulated by members of the Bcl-2 family which include Bax and Bak which allow the release of cytochrome C which triggers the caspase cascade through the cleavage of caspase 9 (Broker *et al.*, 2005). The extrinsic pathway involves binding of a ligand such as tumour necrosis factor α (TNF-α) to a receptor such as TNFR1 which in turn leads to the activation of the death inducing signalling complex (DISC) followed by activation of caspase 8 which cleaves caspase 3 leading to activation of executioner proteins (Welch *et al.*, 2007).

Necrosis on the other hand is an unprogrammed form of cell death which is a result of extracellular stimuli rather than internal cues. Necrosis is an energy independent process whereas apoptosis requires energy. Unlike apoptosis, necrosis leads to disruption of the cellular membrane, which results in leakage of the cellular contents into the extracellular environment. This is preceded by swelling of the cytoplasm and oedema of the cell. Once the integrity of the plasma membrane is lost and the contents of the cell are released an inflammatory response occurs through chemotactic signalling which can exacerbate damage to a tissue (Broker *et al.*, 2005; Vakkila *et al.*, 2004).

In recent years certain miRNA species have been implicated in cell survival and apoptosis and around 30 different miRNA species had been implicated in apoptosis in 2009 (Yang *et al.*, 2009a). This has largely been investigated in the setting of cancer research where certain miRNAs have been shown to be differentially regulated in tumours and affect the survival phenotype of the tumour. The miR-34 family have been a particular focus of this research. This family contains miR-34a, b and c and were all originally identified as targets of p53 and there are many cancers where the expression of this family are altered. The miR-34 family

are actually directly induced by p53 in response to DNA damage or stress (He *et al.*, 2007). Each of these miRNAs have antiproliferative effects but miR-34a in particular promotes apoptosis in addition to this (Cannell *et al.*, 2010a; Cannell *et al.*, 2010b; He *et al.*, 2009). In approximately 30 % of neuroblastomas miR-34a is deleted which may contribute towards the tumours survival. MiR-34a has been shown experimentally to promote apoptosis and also to inhibit proliferation in vitro in various cancer cell lines (Yang *et al.*, 2009a). Targets of miR-34a which have been experimentally validated include CDK4, CDK6, cyclin E2 and Bcl-2 (He *et al.*, 2007; Kong *et al.*, 2012; Yamakuchi *et al.*, 2008).

The hypothesis of this chapter is that modulation of specific miRNA species can influence susceptibility to various toxins in vitro. In particular miR-34a was chosen due to the role the miR-34 family play in cell survival and apoptosis coupled with the fact it was found to be significantly elevated after both Cisplatin and Adriamycin exposure in vivo. The aims of this study were:

- To validate the toxic response of HEK293 cells to Cisplatin and Staurosporine in terms of apoptosis and necrosis.
- To modulate miR-34a activity in HEK293 cells and investigate if this modulation of miRNA activity influenced susceptibility of HEK293 cells to Cisplatin or Staurosporine in terms of cell death and apoptosis.

2 - **Materials and Methods**RNA oligonucleotides (synthetic miRNAs) were purchased from integrated DNA technologies (IDT). RNA isolation and purification kits were purchased from QIAGEN (miRNeasy mini kit, RNeasy minElute cleanup kit). Reverse transcription specific and qPCR specific miRNA primers (TaqMan® MicroRNA Assays) were obtained from Applied Biosystems. Reagents for reverse transcription and for qPCR were also obtained from Applied Biosystems (TaqMan® MicroRNA Reverse Transcription Kit and TaqMan® Universal PCR Master Mix). Dulbecco's modified eagle medium (DMEM) supplemented with glucose (4.5 g/L) was obtained from Lonza, Switzerland. Cytotoxicity was detected through either the MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS)which was obtained from Promega or the Lactate Dehydrogenase (LDH) assay(Aqueous Non-Radioactive Cell Proliferation Assay) which was obtained from Roche Applied Science. Apoptosis was detected through either the Annexin V apoptosis detection kit which was obtained from BD Pharminogen or through detection of caspase 3 via western blot (AbCam). MiRNA mimic, inhibitor and other reagents were obtained from QIAGEN. Lipofectamine RNAiMAX was obtained from Invitrogen. HEK293 cell line was obtained from the European Collection of Cell Cultures (ECACC). All other reagents were purchased from Sigma (Poole, UK) unless otherwise stated.

2.1 - Cell culture and experimental set up

HEK-293 cells stably expressing either a PcDNA vector or Kim-1 were maintained in DMEM supplemented with foetal bovine serum (10 % v/v), L-glutamine (1 % v/v) and Penicillin/Streptomycin solution (1 % v/v). Cells were incubated in 75 cm^2 culture flasks under humidified air containing 5 % CO_2 at 37 °C. Upon reaching confluency, cells were dislodged from the flasks using trypsin solution (4 ml), washed with serum free media (6 ml)

and counted with a haemocytometer and a light microscope and trypan blue then either cultured in 75 cm^2 flasks or plated in flat bottomed 24 well plates in preparation for experimental analysis. Cells were allowed to reach ~90 % confluency before being exposed to Cisplatin (50 µM – 500 µM) in serum free media (0.1 % DMSO) for up to 24 hours. Control cells were exposed to serum free media (0.1 % DMSO) alone.

2.2 - MTS assay

The MTS assay is a colourimetric assay for cell viability based on the ability of cells to reduce a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) via intracellular dehydrogenase enzymes to a soluble formazan compound. The quantity of the formazan product formed by the cells is directly proportional to the number of viable cells. Cells were plated in flat bottomed 24 well plates (2 x 10^5 cells/well) in triplicate and allowed to reach ~ 95 % confluency before being exposed to Cisplatin (50 – 600 µM) for 1 – 24 hours for characterisation of the cell line experiments. Cells were plated in 24 well plates (1.5 x 10^5 cells/well) and transfected with a miRNA mimic or inhibitor for 6 – 72 hours followed by exposure to Cisplatin. Following incubation, media was removed and discarded and serum free media (120 µl) was added to each well followed by MTS solution (20 µl). Plates were returned to the incubator (1 hour) before media (100 µl) was transferred to a flat bottomed 96 well plate and the absorbance read at a wavelength of 490 nm. Results were blank corrected and expressed as a percentage of vehicle only cells.

2.3 - LDH Assay

The LDH assay is a colourimetric assay for the quantification of cell death through the measurement of lactate dehydrogenase activity in the media of *in vitro* cell systems which has been released from the cytosol of dead or dying cells. Cells were plated in flat bottomed 24 well plates (2 x 10^5 cells/well) in triplicate and allowed to reach ~ 95 % confluency before being exposed to Cisplatin (50 – 600 µM) for 1 – 24 hours for characterisation of the cell line experiments. Cells were plated in 24 well plates (1.5 x 10^5 cells/well) and transfected with a miRNA mimic or inhibitor for 6 – 72 hours followed by exposure to Cisplatin. Following incubation media was removed (50 µl) from each well and transferred to a flat bottomed 96 well plate. The remaining media was discarded and 2 % Triton X solution (100 µl) and serum free media (100 µl) was added to cells. Once cells were lysed media was removed (5 µl) and added to separate wells of a flat bottomed 96 well plate and diluted 1 in 10. Freshly made up reaction mixture (according to manufacturer's instructions) was added (50 µl) to each well of the 96 well plates and incubated in the dark for 30 minutes (room temperature). Absorbance was measured using a plate reader at a wavelength of 490 nm. Results were expressed as LDH leakage as a fraction of total LDH.

2.4 - Transfection of cells with miRNA mimics or inhibitors

Cells were transfected with either miR-34a mimic or inhibitor. 20 µM MiRNA mimic (0.3 µl) or inhibitor (3 µl) was combined with lipofectamine (3 µl) and serum free media (50 µl) per 24 well to give a final mimic and inhibitor concentration of 5 nM and 50 nM respectively. Mixture was incubated at room temperature (10 minutes) while cells were prepared to allow formation of transfection complexes. Cells were plated in 24 well plates as previously described in serum containing media (500 µl). Transfection mixture was added drop wise

(50 µl) to appropriate wells of the 24 well plate and plate was incubated at 37 °C for an appropriate amount of time. Transfection experiments also involved transfection of a miScript inhibitor negative control and a mock transfection which were prepared in the same way as the mimic or inhibitor.

2.5 - Western blot analysis of caspase 3, CDK6 and Bcl-2

Cell lysates were prepared after treatment by removal of media and washed with hanks balanced salt solution. Cells were then lysed with RIPA buffer (20 µl) and lysates transferred to an Eppendorf tube. Cell lysate protein concentration was determined through the methods of Bradford *et al* (Bradford, 1976) and 20 µg of protein was loaded onto an acrylamide gel (10 % for caspase 3 and Bcl-2 and 8 % for CDK6) after combination with reducing agent and LDS buffer (30 µl and 70 µl respectively) and heating for five minutes (80 °C). Power was supplied to the gel for ten minutes (80 V) to level the protein followed by an hour (170 V). The proteins were transferred to a nitrocellulose membrane through the application of a current (230 A) for one hour. Ponceau red was added to membranes to ensure even loading and sufficient transfer before membranes were blocked with non-fat milk (10 % w/v) for one hour and then primary antibody added at the appropriate concentration in non-fat milk (2 % w/v) and for the appropriate period of time; β-actin, 1/50,000 for one hour, caspase-3, 1/10,000 overnight, BCL2, 1/500 overnight, CDK6 1/2000 overnight. Membranes were then washed with TBST (0.1 % TWEEN/ 1 % TBS buffer) four times (five minutes). Appropriate secondary antibodies were added to the membranes (1/10000) for one hour and then washed again. Membranes were then incubated with ECL-plus (one minute) lighting solution (according to manufacturer's instructions) before exposure to hyperfilm (10 seconds – three minutes).

2.6 - Cell death analysis of by annexin V/ PI FACS analysis

Apoptotic and necrotic cells were determined using a flow cytometer (BD FACScanto II). Unlabelled cells were used to set the parameters for the assay and cell debris was excluded by altering the voltage threshold. Cells labelled with only PI or only Annexin V were also used to set parameters for the assay to avoid the two dyes "bleeding" into each other. Cells (>500,000) were washed twice with ice cold PBS then resuspended in binding buffer and incubated at room temperature with Annexin V (5 µl) and propidium iodide (PI) (5 µl) for 15 minutes. Dyed cells were than analysed using the set parameters. The fractions of viable, necrotic, early apoptotic and apoptotic cells were determined using CyFlogic software.

2.7 - Detection of Bcl-2 and CDK6 mRNA using qPCR

Bcl-2 and CDK6 mRNA levels were quantified in cell lysates by qPCR. Primers to CDK6, Bcl-2 and GAPDH mRNA were designed and purchased Eurofins, UK. RNA was isolated from cell lysates using the RNeasy mini kit as previously described for miRNAs. RNA was then reverse transcribed to cDNA with the SuperScript III Reverse Transcriptase kit (Invitrogen) according to manufacturer's instructions and quantified using the SYBR green Jumpstart Taq Ready Mix (Sigma, UK) and qPCR as previously described for miRNAs. Bcl-2 and CDK6 were normalised to the endogenous GAPDH using the ΔΔCt method.

2.9 - Statistical Analysis

Each experiment involved duplicate qPCR repeats of the reverse transcription cDNA product. Each independent experiment was repeated to generate at least an n of three. Error bars represent standard error of the means (SEM) of the three independent experiments. Shapiro Wilk test was used to test all data for normality followed by the Kruskal Wallis or two way Anova test to assess statistical significance.

3 - **Results**

3.1 - Characterisation of the response of HEK293 cells to Cisplatin and Staurosporine

The response of HEK-293 cells to Cisplatin and Staurosporine were characterised using a number of cell death assays. The MTS assay was used to quantify the fraction of viable cells remaining through the colourimetric detection of the production of a formazan compound. The LDH assay was used to quantify LDH leakage in the surrounding media which is a reflection of the loss of membrane integrity.

Dose responses after 24 hours of exposure to Cisplatin were carried out using HEK293 cells stably expressing an empty PcDNA vector and those expressing Kim-1. Figure 1 shows the fraction of viable cells remaining as determined by the MTS assay in both the Kim-1 and PcDNA expressing cell lines. Cisplatin caused significant reductions in the HEK-293 Kim-1 cells at concentrations of 50 µM (mean 79.8 %) and continued to reduce viability in a dose dependent manner until 200 µM (mean 52.5 %). Between 200 µM and 500 µM the cell viability remained between 50 and 60 % followed by a drop to 31.2 % at 600 µM. PcDNA expressing HEK-293 cell viability was significantly decreased at doses of 200 µM Cisplatin (Mean 52.3 %). Cell viability remained at approximately 50 % at 300 and 400 µM Cisplatin before further reduction to 28.2 %. LDH activity was found to be significantly elevated in the media surrounding the Kim-1 expressing HEK-293 cells (figure 2A) at concentrations of 100, 300 and 600 µM Cisplatin (mean 6.5 %, 7.5 % and 6.5 % of total LDH in media respectively).

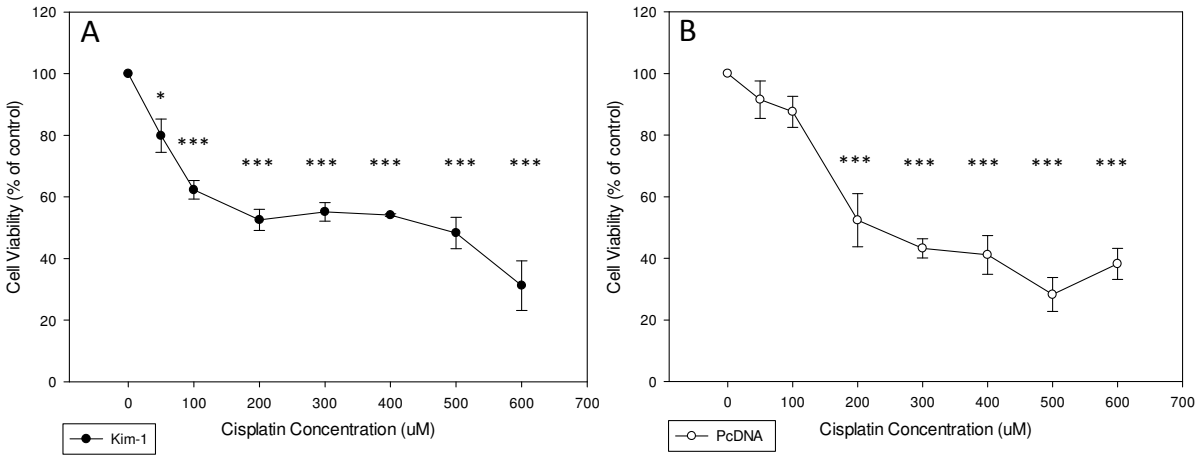

Figure 1: - Cell viability determined by MTS assay in HEK293 cells expressed as a fraction of untreated cells in HEK-293 cells expressing Kim-1 (A) or PcDNA (B) in response to various concentrations of Cisplatin for 24 hours. Error bars represent SEM of three independent experiments. *P<0.05, ***P<0.005.

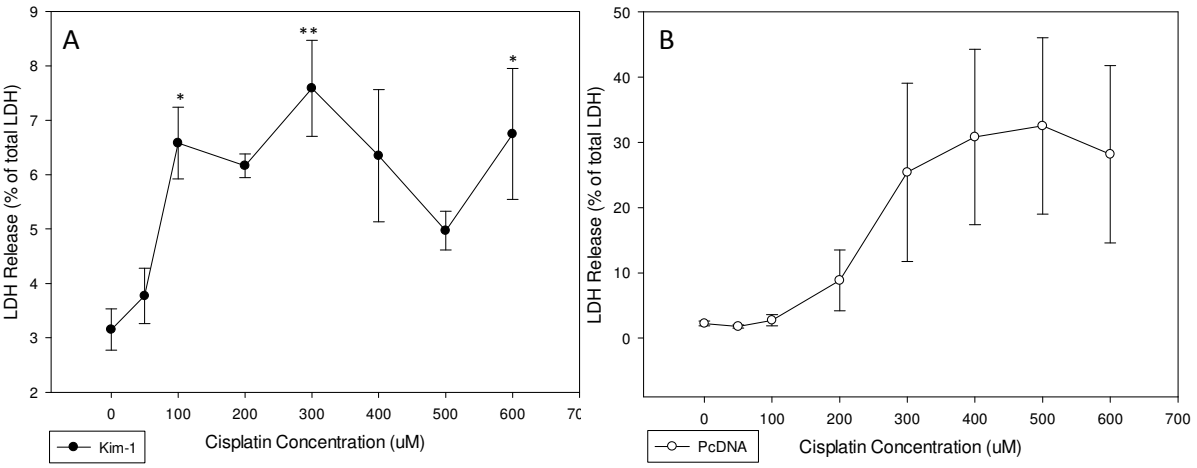

Figure 2: - LDH activity in the media surrounding HEK-293 cells expressing Kim-1 (A) and PcDNA (B) in response to various concentrations of Cisplatin for 24 hours. Error bars represent SEM of three independent experiments. *P<0.05, **P<0.01.

In the PcDNA expressing cells there were no significant elevations in the fraction of LDH in the media surrounding the cells (figure 2B) although there was over 25 % of LDH found in the media at concentrations greater than 300 µM Cisplatin.

The effect of Cisplatin on the two cell types was also examined over various periods of time using both the MTS and LDH assays. The Kim-1 expressing HEK293 cells (figure 3) showed no significant decreases in cell viability at a Cisplatin concentration of below 500 µM until 24 hours (figure 3F) of exposure to Cisplatin where significant decreases were observed also at 100 µM Cisplatin (mean 77 (100 µM) and 68 % (500 µM) respectively) . A 16 hour exposure to Cisplatin (figure 3E) caused a significant decrease in cell viability only at 500 µM Cisplatin (mean 77 %). One hour exposure (figure 3B) to Cisplatin caused a significant decrease in cell viability at 500 µM Cisplatin (mean 88 %) in the Kim-1 expressing HEK293 cells. No other significant reductions in cell viability were observed. No significant elevations in the fraction of LDH found in the media of the Kim-1 expressing HEK293 cells (figure 4) were observed at any time point other than 24 hour exposure to Cisplatin where a concentration of 500 µM (mean 6.4 %) caused a significant increase (figure 4A). The PcDNA expressing HEK293 cells showed no significant decreases in cell viability from in the MTS assay at any time point prior to 16 hours of Cisplatin exposure (figure 5). After 16 (mean 79, 80 and 75 % respectively) and 24 hours (mean 71, 62, 63 % respectively) of exposure to Cisplatin significant decreases in cell viability were observed at 50 µM, 100 µM and 500 µM Cisplatin (figure 5E, F). There was no significant increase in the fraction of LDH found in the media surrounding the PcDNA expressing HEK293 cells until 24 hours of Cisplatin exposure at 500 µM which caused 50 % of the total LDH to be found in the media.

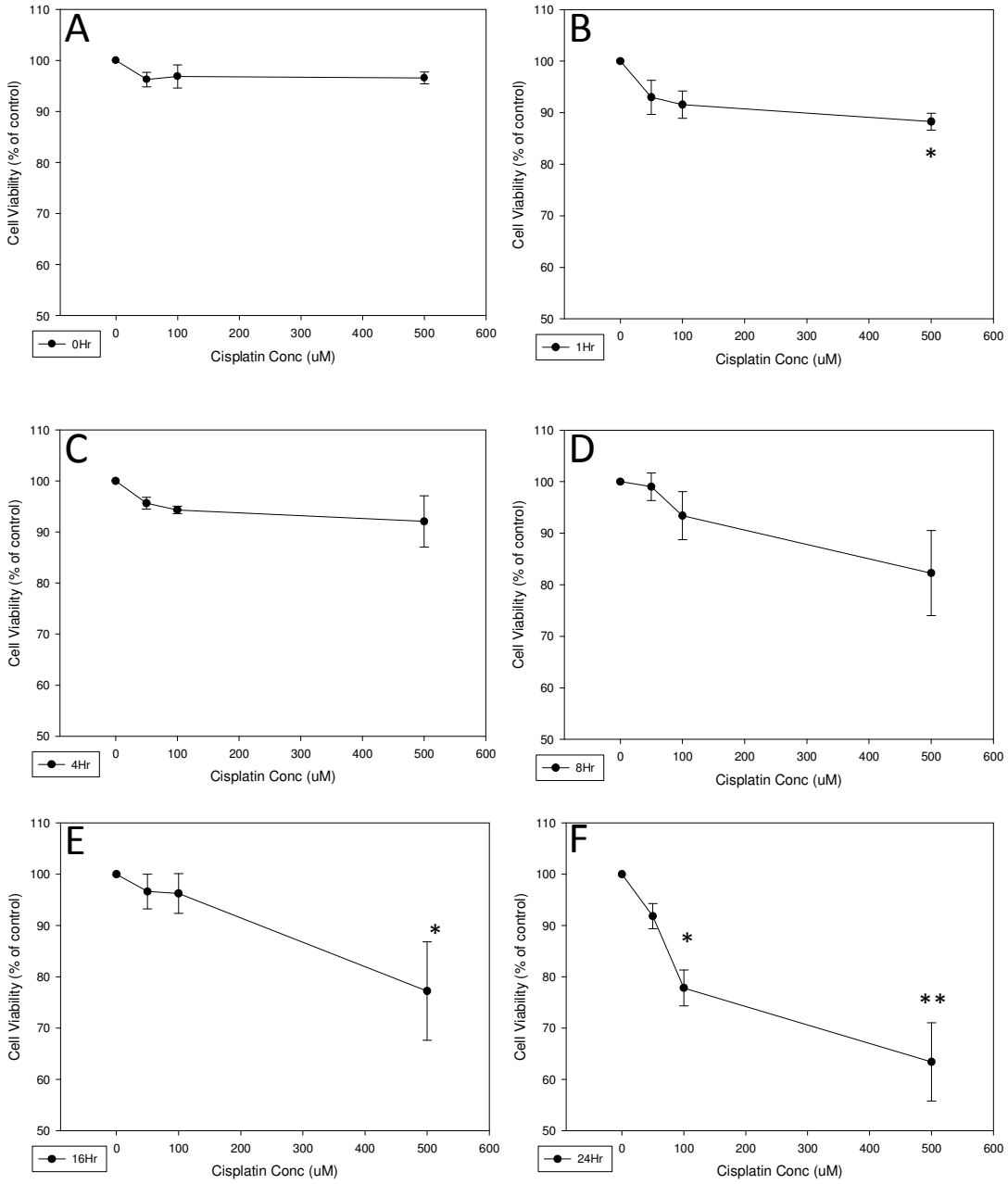

Figure 3:- Cell viability determined by MTS assay in Kim-1 expressing HEK293 cells expressed as a fraction of untreated cells over various periods of time. Zero hours (A), one hours (B), four hours (C), eight hours (D), 16 hours (E) and 24 hours (F) were evaluated in response to various concentrations of Cisplatin. Error bars represent SEM of three independent experiments. *P<0.05, **P<0.01.

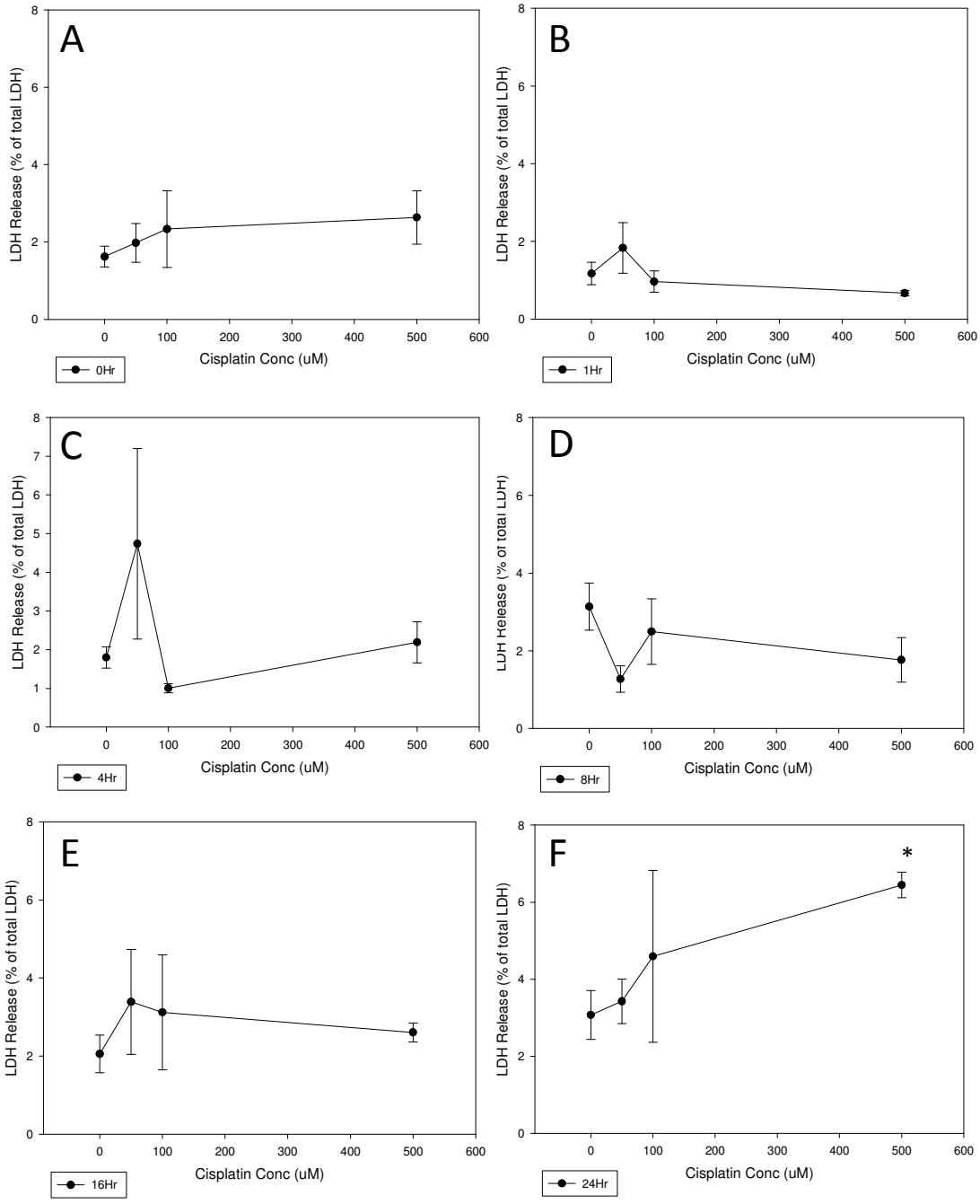

Figure 4:- LDH activity in media surrounding Kim-1 expressing HEK293 cells over various periods of time. Zero hours (A), one hours (B), four hours (C), eight hours (D), 16 hours (E) and 24 hours (F) were evaluated in response to various concentrations of Cisplatin. Error bars represent SEM of three independent experiments. *P<0.05.

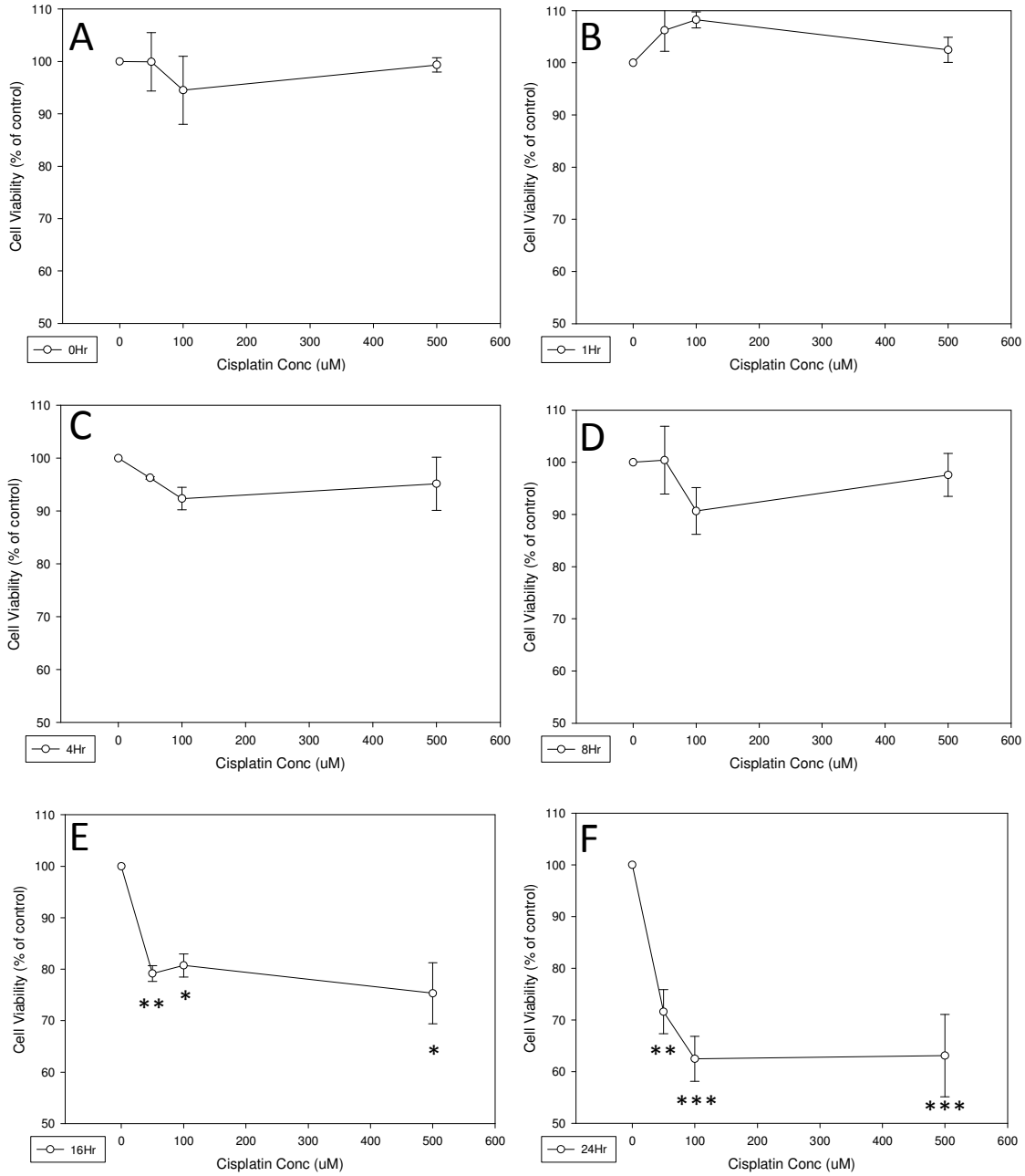

Figure 5:- Cell viability determined by MTS assay in PcDNA expressing HEK293 cells expressed as a fraction of untreated cells over various periods of time. Zero hours (A), one hours (B), four hours (C), eight hours (D), 16 hours (E) and 24 hours (F) were evaluated in response to various concentrations of Cisplatin. Error bars represent SEM of three independent experiments. *P<0.05, **P<0.01, ***P<0.005.

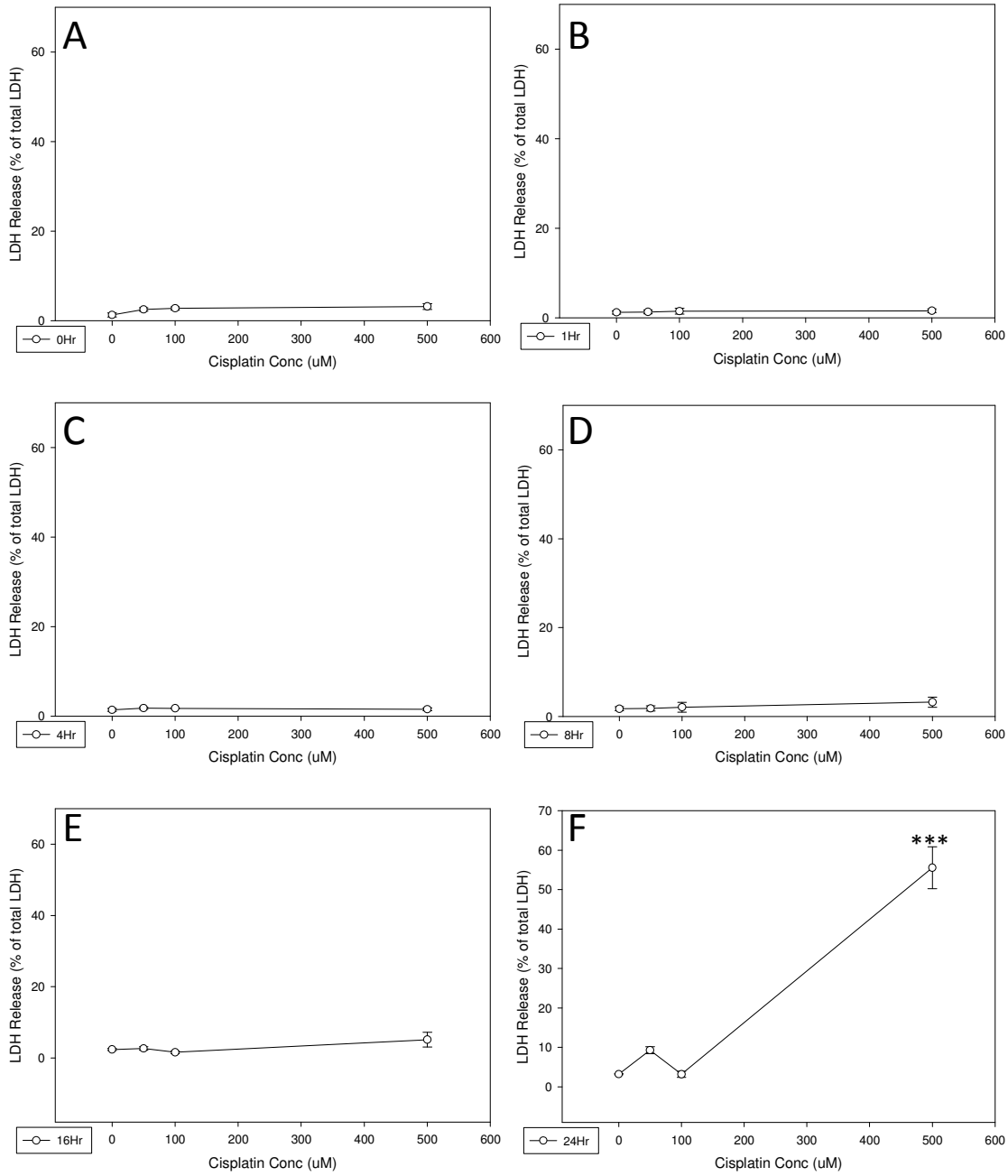

Figure 6:- LDH activity in the media surrounding PcDNA expressing HEK293 cells over various periods of time. Zero hours (A), one hours (B), four hours (C), eight hours (D), 16 hours (E) and 24 hours (F) were evaluated in response to various concentrations of Cisplatin. Error bars represent SEM of three independent experiments. ***P<0.005.

The data for longer time periods with the PcDNA expressing HEK293 cells however are misleading as the PcDNA cells were extremely difficult to work with and would stop adhering to the flasks readily over timepoints of eight hours. This makes the latter timepoints for MTS and LDH assays misleading. For this reason further use of the PcDNA expressing cells was discontinued in favour of the Kim-1 expressing cells.

Staurosporine was also used as a second apoptosis inducing agent in the Kim-1 expressing HEK293 cells. Their response of the cells was also assessed after exposure to varying concentrations of Staurosporine for 24 hours (figure 7). Significant decreases in cell viability quantified by the MTS assay were observed only after exposure to 1.5 µM and 2 µM (mean 51 and 55 % respectively) Staurosporine (figure 7A). The same was observed for the fraction of LDH released into the media surrounding the cells as assessed by LDH activity. No significant increases in LDH found in the media were observed prior to concentrations of 1.5 µM and 2 µM (mean 22 and 24 % respectively) (figure 7B).

3.2 - Assessment of Apoptotic response to Cisplatin and Staurosporine in Kim-1 expressing HEK293 cells

The response of Kim-1 expressing HEK293 cells to Cisplatin and Staurosporine was also assessed in terms of apoptosis. This was achieved though quantification of cleaved caspase 3 by western blotting and through FACS analysis of Annexin V and PI stained cells. Cisplatin caused significant elevations in levels of cleaved caspase 3 at 100 µM only (mean 1.14) however a band is also clearly visible at 250 µM Cisplatin although the change was not significant (figure 8 A, C). Staurosporine caused a dose dependent increase in caspase 3 expression which was found to be significant only at concentrations of 1.5 and 2 µM (mean 1.2, 1.3 respectively).

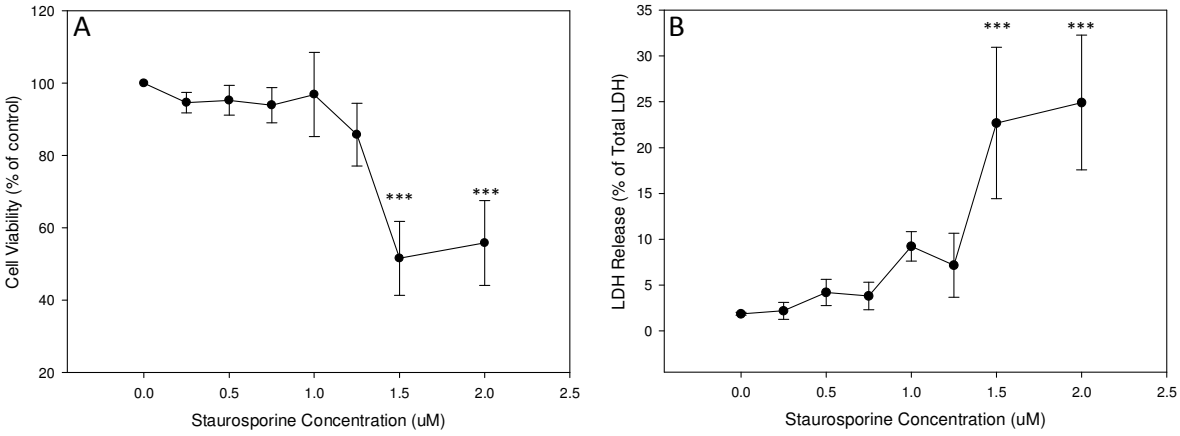

Figure 7:- Response of Kim-1 expressing HEK293 cells to increasing concentrations of Staurosporine measured as cell viability by MTS assay (A) and LDH release by LDH assay (B). . Error bars represent SEM of three independent experiments. ***P<0.005.

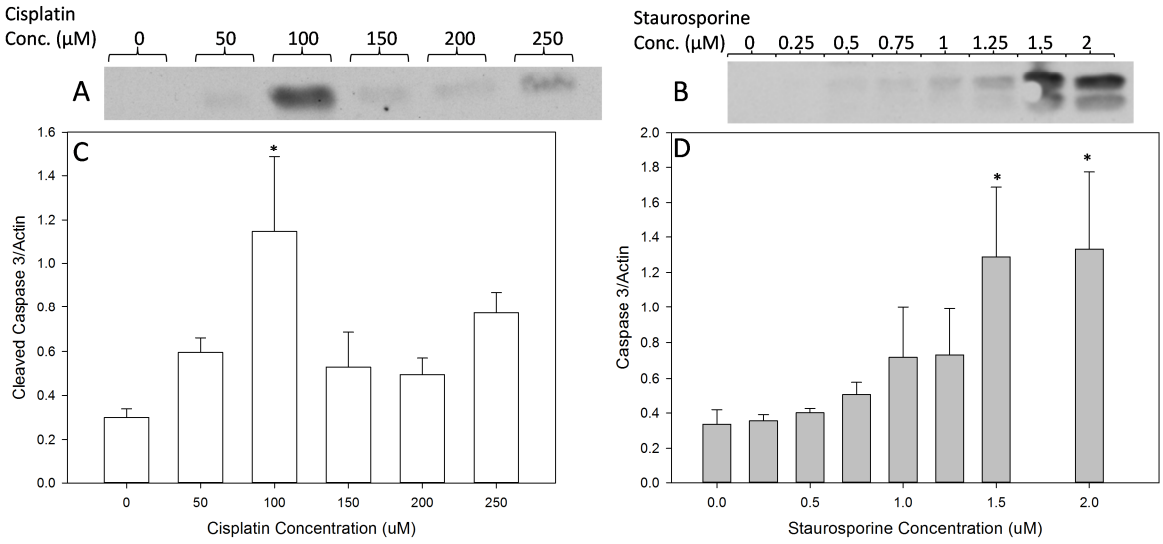

Figure 8:- Expression of cleaved caspase 3 in lysates of Kim-1 expressing HEK293 cells in response to increasing concentrations of Cisplatin (A,C)and Staurosporine (B,D). Western blots are shown for both Cisplatin treated (A) and Staurosporine treated (B) cells and are expressed graphically from densitometry data of cleaved caspase 3 normalised to β-actin. Error bars represent SEM of three independent experiments. *P<0.05.

Kim-1 expressing HEK293 cells were also incubated the concentration of Cisplatin or Staurosporine which elicited a significant increase in cleaved caspase 3 and also with the pan caspase inhibitor Z-VAD-FMK. Z-VAD-FMK was also incubated with the cells alone for 24 hours (figure 9). Cisplatin was found to elicit significant increases in cleaved caspase 3 when normalised to β-actin when incubated at concentrations of 100 and 250 μM (Mean 0.18 and 0.28 respectively) (Figure 9C). ZVAD-FMK elicited no significant response when incubated with the cells alone (Figure 9C, D). 1 μM Staurosporine caused a significant increase (Mean 0.78) in the amount of cleaved caspase 3 in the cells (Figure 9D). ZVAD-FMK did not cause significant increases in cleaved caspase 3 in the cell line when incubated with Cisplatin or Staurosporine.

FACS analysis of Annexin V and PI stained cells was also carried out to confirm the apoptotic response to Cisplatin and Staurosporine. Cisplatin caused significant increases in the fraction of early apoptotic cells at concentrations of 50 and 100 μM (Mean 25 and 32 % respectively) and significant increases in late apoptotic cells at 150, 200 and 250 μM (Mean 8, 12 and 9 % respectively). No significant changes in the fraction of alive or necrotic cells were observed (Figure 10). When incubated with ZVAD-FMK and Cisplatin as described for the caspase 3 analysis above, the significant changes in early apoptosis were abolished (Figure 11). No significant changes were observed in response to Staurosporine concentrations of 0.25, 0.5, 0.75, 1, 1.5 and 2 μM (data not shown). When incubated with Z-VAD-FMK, Staurosporine also elicited no significant changes in fraction of cells positive for Annexin V/PI staining (Figure 12).

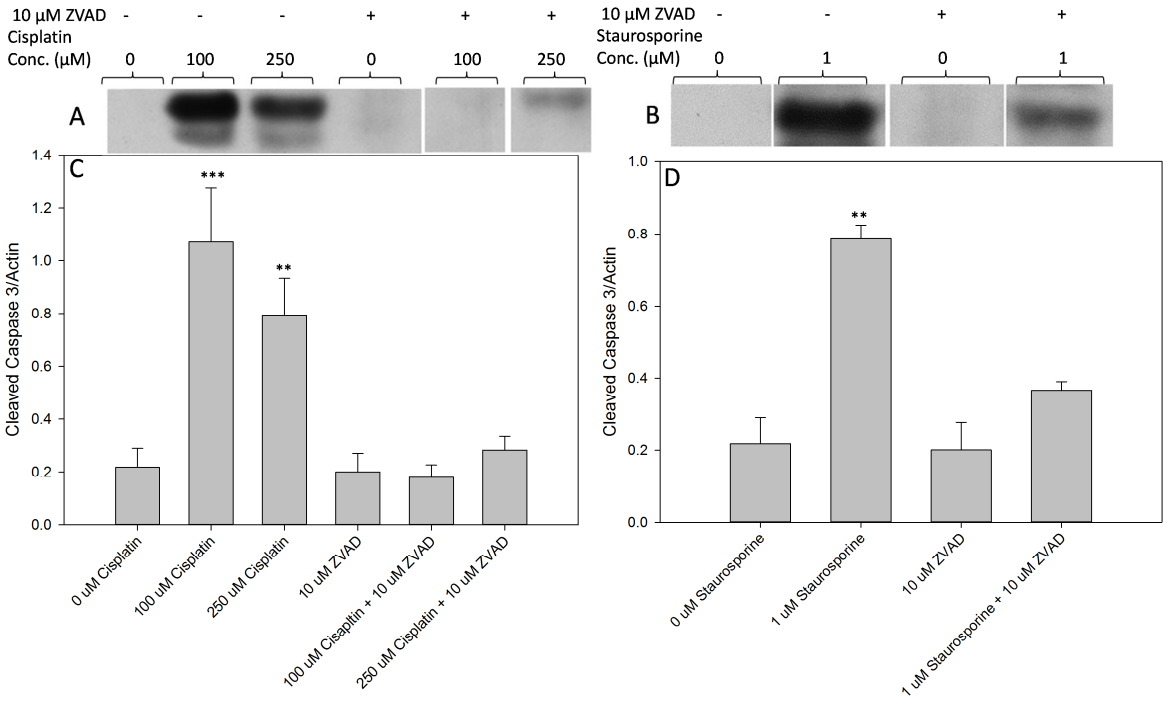

Figure 9:- Expression of cleaved caspase 3 in lysates of Kim-1 expressing HEK293 cells in response to Cisplatin (A,C) and Staurosporine (B,D) with and without incubation with ZVAD-FMK. Western blots are shown for both Cisplatin treated (A) and Staurosporine treated (B) cells and are expressed graphically from densitometry data of cleaved caspase 3 normalised to β-actin (C,D). Error bars represent SEM of three independent experiments. **P<0.01, ***P<0.005.

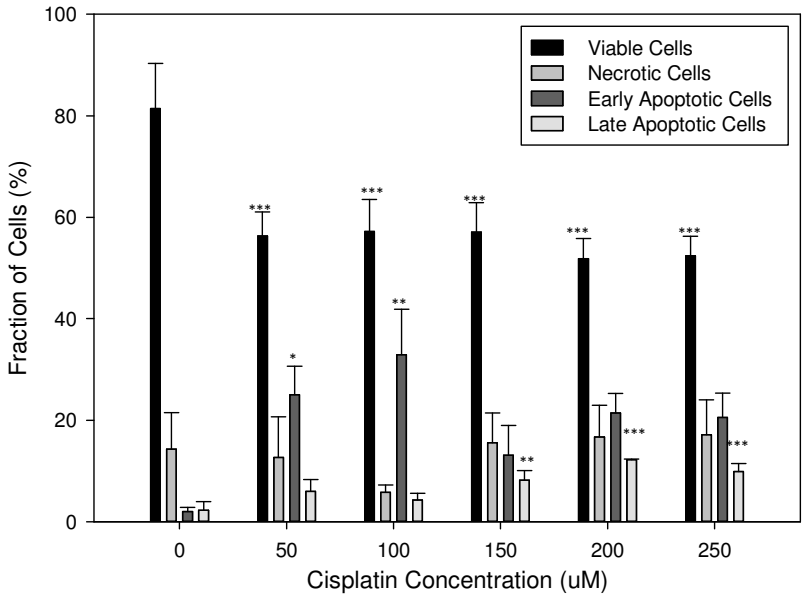

Figure 10:- Fraction of alive, necrotic, early and late apoptotic cells in Kim-1 expressing HEK293 cells in response to Cisplatin as determined by Annexin V/PI staining and FACS analysis. Error bars represent SEM of three independent experiments. *P<0.05, **P<0.01, ***P<0.005.

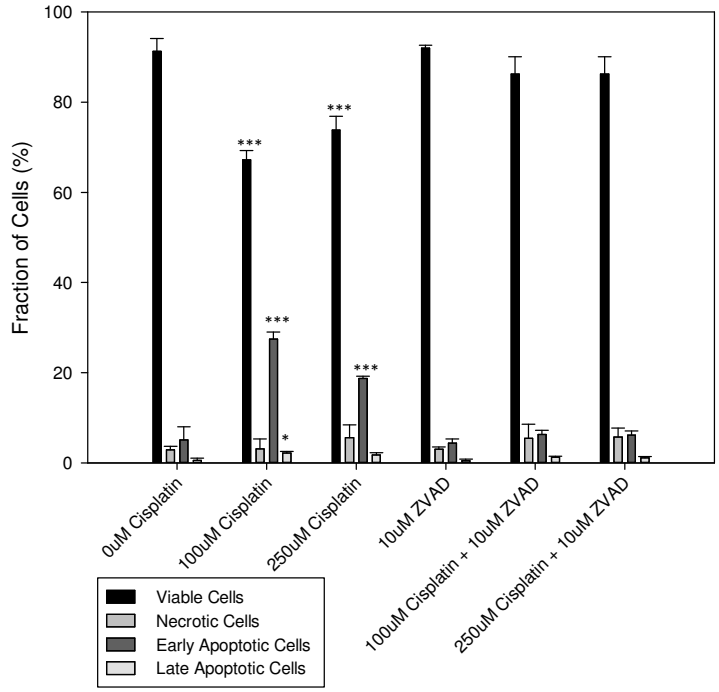

Figure 11:- Fraction of alive, necrotic, early and late apoptotic cells in Kim-1 expressing HEK293 cells in response to Cisplatin and/or ZVAD-FMK (10 µM) as determined by Annexin V/PI staining and FACS analysis. Error bars represent SEM of three independent experiments. *P<0.05, ***P<0.005.

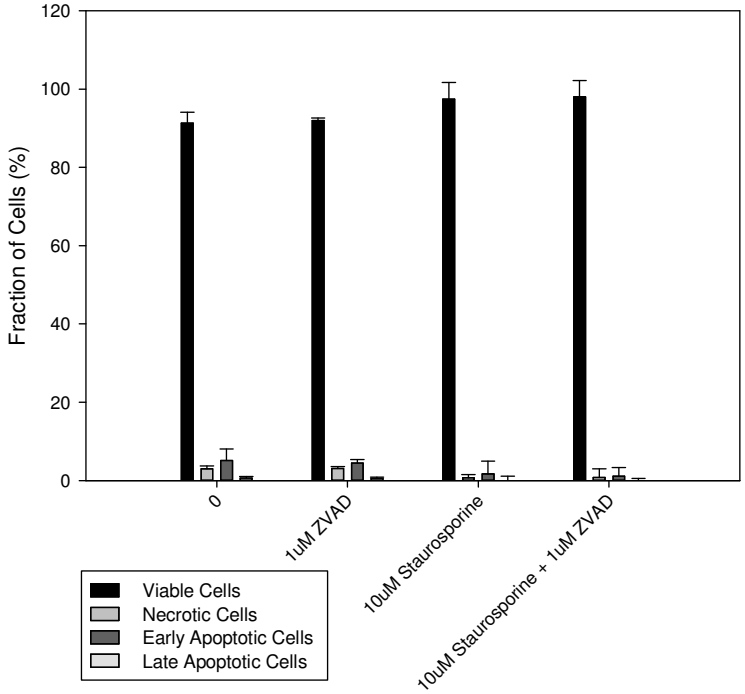

Figure 12:- Fraction of alive, necrotic, early and late apoptotic cells in Kim-1 expressing HEK293 cells in response to Staurosporine and/or ZVAD-FMK (10 µM) as determined by Annexin V/PI staining and FACS analysis. Error bars represent SEM of three independent experiments.

3.3 - Baseline miRNA analysis in Kim-1 expressing HEK293 cells and THP1 cells

A number of miRNAs were quantified in the Kim-1 expressing HEK293 cells in order to provide information on the relative expression of the kidney enriched miRNAs which had been previously examined in chapter three plus the muscle specific miR-1 and miR-34a. Results are presented as mean Ct (Figure 13A) or mean Ct per ng per µl RNA (figure 13B). miR-1 was found to be the least abundant miRNA species in the HEK293 cells and the miR-30 family were found to be the most abundant miRNA species. The same pattern was also observed in the THP-1 cell line.

3.4 - Transfection of Kim-1 expressing HEK293 cells with a miR-34a inhibitor

Kim-1 expressing HEK293 cells were transfected with an inhibitor of miR-34a in an effort to modulate susceptibility to toxicity. Cells were transfected with a miR-34a inhibitor for various periods of time. A mock transfection was also carried out where the inhibitor was added to the media then removed after 10 minutes to make sure the miR-34a being quantified was a true reading and not a result of the inhibitor itself. MiR-34a was normalised by the ΔΔCt method to miR-192 (Figure 15A) and to both miR-192 and total RNA (Figure 15B). When normalised to miR-192 alone the level of miR-34a in the cells was found to be significantly elevated after a 72 hour transfection only (Mean ΔΔCt 17.3). When normalised to miR-192 and to total RNA there were no significant changes in miR-34a.

Kim-1 expressing HEK293 cells were transfected with a miR-34a inhibitor for 72 hours and then exposed to Cisplatin for 24 hours. Cell viability was then assessed via the MTS assay (Figure 16A), cell death via the LDH assay (Figure 16B) and apoptosis assessed via quantification of cleaved caspase 3 via western blot (Figure 16C and D).

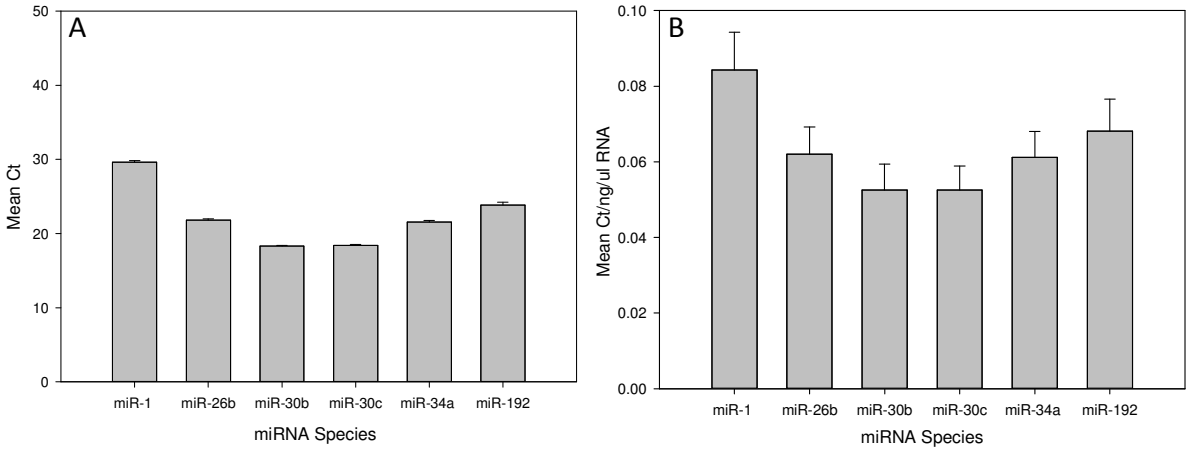

Figure 13:- miR-1, miR-26b, miR-30b, miR-30c, miR-34a and miR-192 quantified in Kim-1 expressing HEK293 cells under normal conditions. All miRNA species quantified by qPCR and expressed either as mean Ct (A) or mean Ct normalised to total RNA in the sample. Error bars represent SEM of three independent experiments.

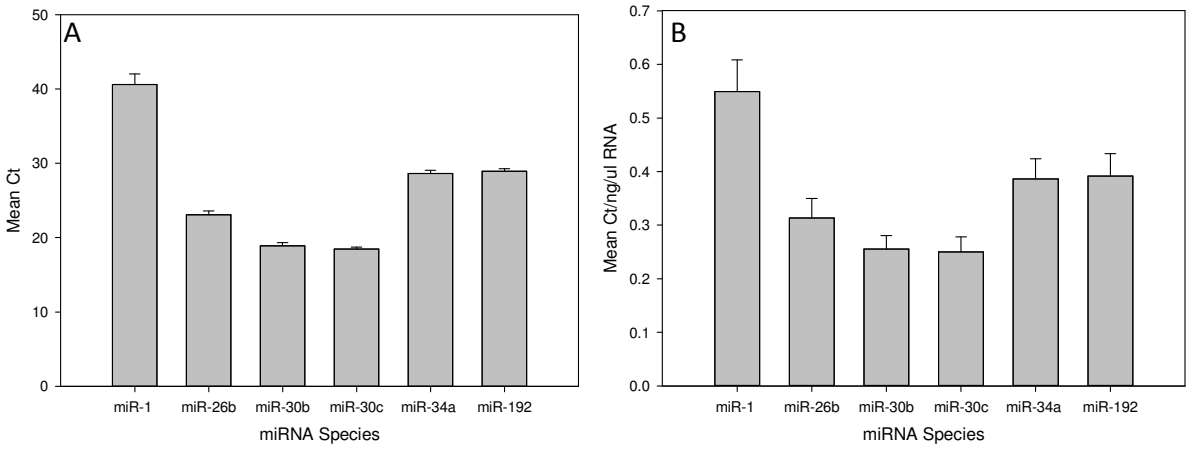

Figure 14:- miR-1, miR-26b, miR-30b, miR-30c, miR-34a and miR-192 quantified in THP-1 cells under normal conditions. All miRNA species quantified by qPCR and expressed either as mean Ct (A) or mean Ct normalised to total RNA in the sample. Error bars represent SEM of three independent experiments.

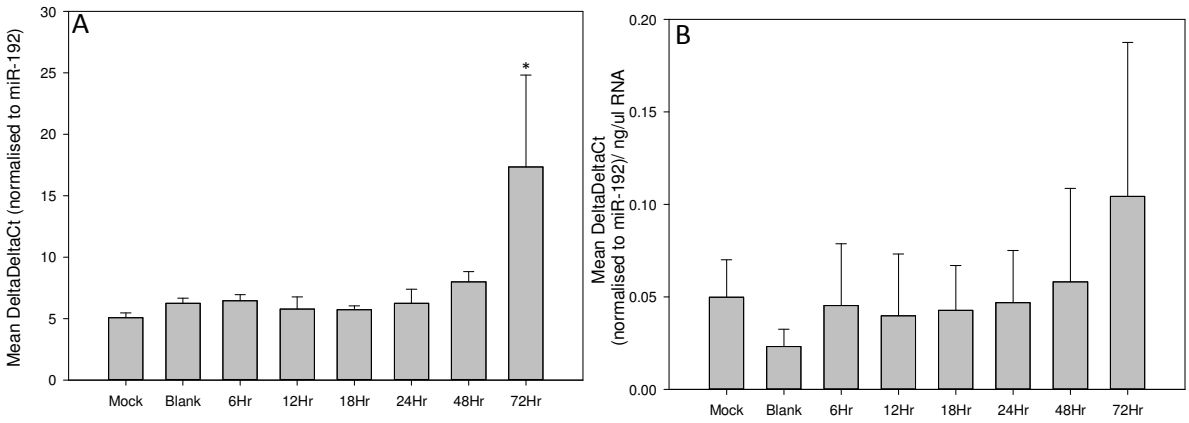

Figure 15:-miR-34a quantified in Kim-1 expressing HEK293 cells transfected with a miR-34a inhibitor for varying periods of time. A mock transfection involved the addition of the miR-34a inhibitor to the media for 10 minutes followed by its removal.MiR-192 was also quantified in the cells and was used to normalise miR-34a to. All miRNA species quantified by qPCR and expressed either as mean ΔΔCt (A) or mean ΔΔCt normalised to total RNA in the sample. Error bars represent SEM of three independent experiments (B).

Cell viability was significantly decreased after exposure to 100 µM Cisplatin in both the control cells (Mean 77 %) and the miR-34a inhibitor transfected cells (Mean 62 %). There was however no significant differences between the control and inhibitor transfected cells (Figure 16A). LDH was found to be significantly elevated in the media after exposure to 100 µM Cisplatin (Mean 8.4 %) but not from any other treatment (Figure 16B). Cleaved caspase 3 was found to be significantly elevated after exposure to 100 µM Cisplatin in both the control and miR-34a inhibitor transfected cells (Mean 1.1 and 1.4 respectively) (Figure 16C and D).

3.5 - Transfection of Kim-1 expressing HEK293 cells with miR-34a mimic

As there were no significant changes with the miR-34a inhibitor and it could not be confirmed with any certainty that the transfection was affecting the cell line it was decided to also investigate the effects of a miR-34a mimic on the same cell line. Kim-1 expressing HEK293 cells were transfected with a mimic of miR-34a in an effort to modulate susceptibility to toxicity. Cells were transfected with a miR-34a mimic (10 nM) for various periods of time. A mock transfection was also carried out where the mimic was added to the media then removed after 10 minutes to make sure the miR-34a being quantified was a true reading and not a result of the mimic itself. MiR-34a was normalised by the ΔΔCt method to miR-192 (Figure 17A) and to both miR-192 and total RNA (Figure 17B). MiR-34a was found to be significantly elevated in the Kim-1 expressing cells at six, 12, 18 and 72 hours post miR-34a mimic transfection (Mean 107, 113, 132 and 267 respectively). The only significant elevation in miR-34a normalised to miR-192 by the ΔΔCt method and also to total RNA in the sample was found to be after 72 hour exposure to the mimic (Mean 2.5).

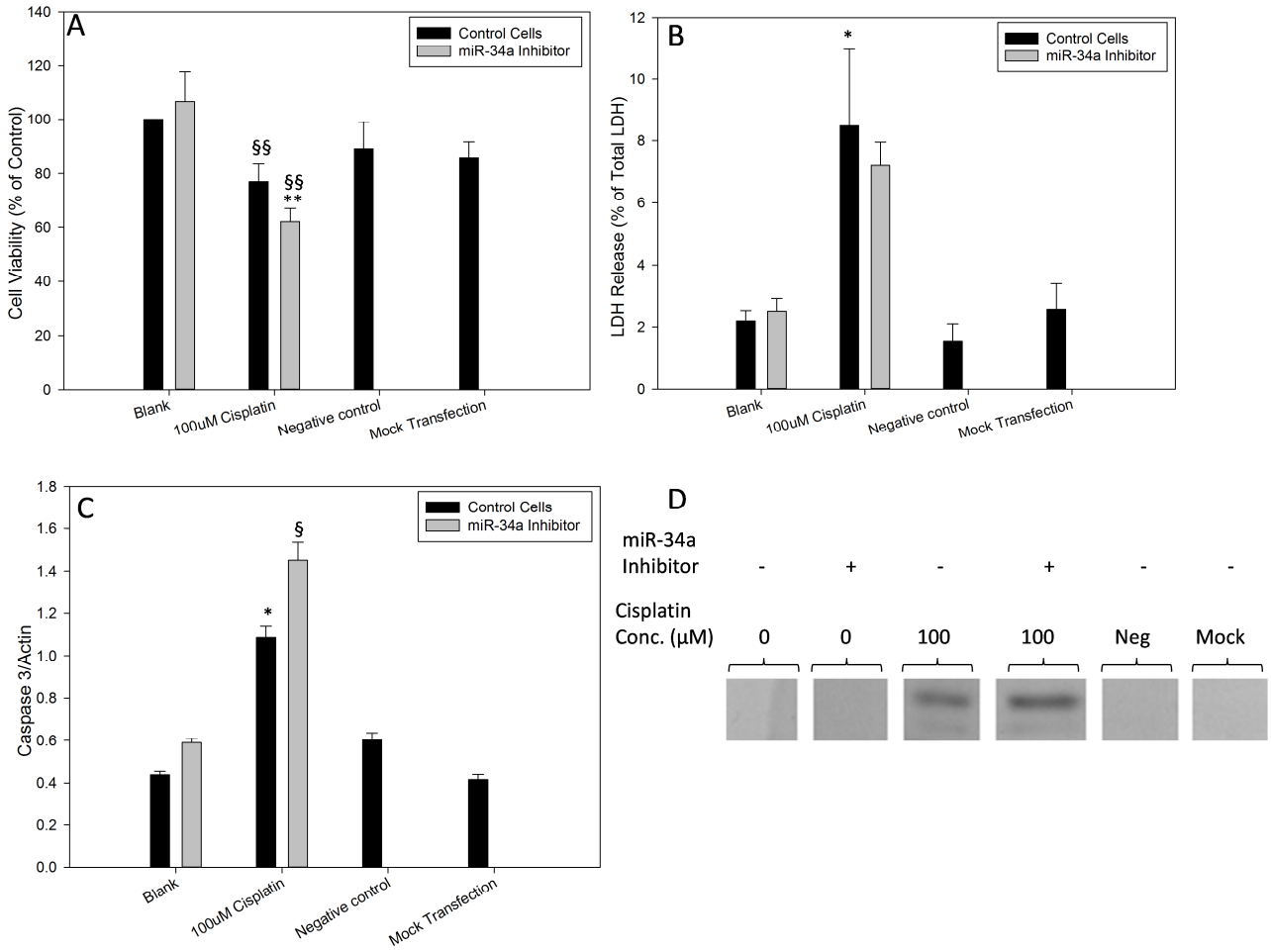

Figure 16:- Kim-1 expressing HEK293 cells transfected with a miR-34a inhibitor for 72 hours followed by exposure to Cisplatin (100 µM) for 24 hours. Cell viability was assessed via MTS assay (A), Cell death was assessed by the LDH assay (B) and quantification of cleaved caspase 3 via western blotting was used to assess apoptosis (C and D). Error bars represent SEM of three independent experiments. * represent significant changes to control cells and § represent significant changes to miR-34a inhibitor transfected cells. */§ P<0.05, **/§§ P<0.01.

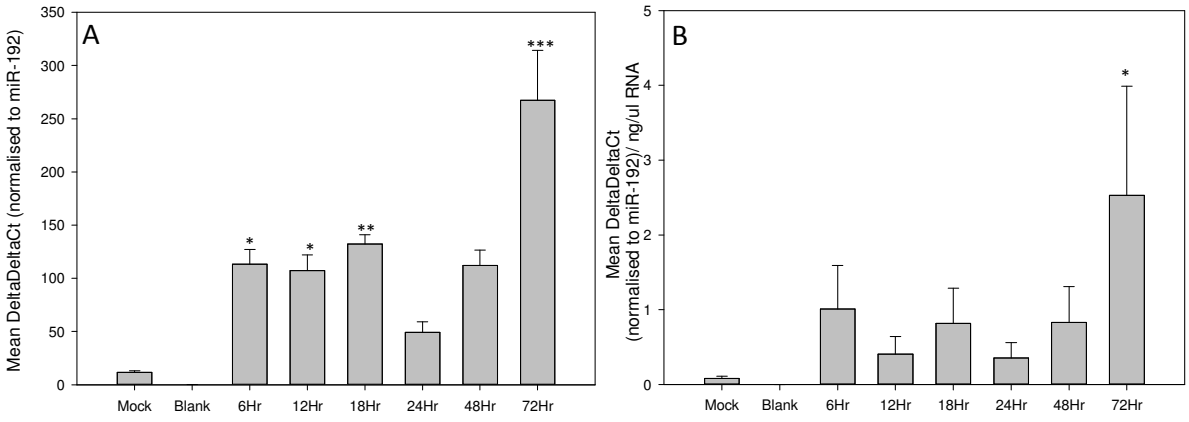

Figure 17:- miR-34a quantified in Kim-1 expressing HEK293 cells transfected with a miR-34a mimic for varying periods of time. A mock transfection involved the addition of the miR-34a mimic to the media for 10 minutes followed by its removal. MiR-192 was also quantified in the cells and was used to normalise miR-34a to. All miRNA species quantified by qPCR and expressed either as mean ΔΔCt (A) or mean ΔΔCt normalised to total RNA in the sample (B). Error bars represent SEM of three independent experiments. *P<0.05, **P<0.01, ***P<0.005.

To investigate the most appropriate time for miR-34a mimic transfection and the most appropriate concentration of the mimic to be used Kim-1 expressing cells were transfected with increasing concentrations of miR-34a mimic for various periods of time. The effect of the mimic on apoptosis of the cells was assessed via quantification of cleaved caspase 3 via western blot. No significant increases in cleaved caspase 3 were observed after six or 12 hour exposure to any concentration of miR-34a mimic examined. After 18 hours, concentrations of 5, 10 and 20 nM miR-34a mimic all caused significant elevations in cleaved caspase 3 normalised to β-actin (mean 0.48, 0.48, 0.44 respectively) when compared to untransfected cells (figure 18C). Exposures of 24 and 48 hours to miR-34a mimic also produced significant increases in cleaved caspase 3 normalised to β-actin when compared to control at 5, 10 and 20 nM miR-34a mimic (figure 18D and E). 72 hour transfection with miR-34a mimic caused a significant elevation in cleaved caspase 3 normalised to β-actin at only 20 nM mimic (mean 1.76) when compared to control (figure 18F). Cell viability was also assessed via the MTS assay using the same experimental conditions as for the assessment of caspase 3 (figure 19). Significant reductions in cell viability were observed as early as six hours after transfection (figure 19A) with a miR-34a mimic at concentrations of 5, 10 and 20 nM (mean 84, 76, 84 % respectively). 12 hour transfection (figure 19B) also yielded significant decreases in cell viability at 5, 10 and 20 nM (mean 87, 63, 87 % respectively). The 24 hour transfection (Figure 19D) only yielded a significant reduction in cell viability at 10 nM mimic (57 %) whereas a 48 hour transfection (figure 19E) caused significant decreases in cell viability at 5, 10 and 20 nM mimic (mean 64, 43, 51 % respectively).

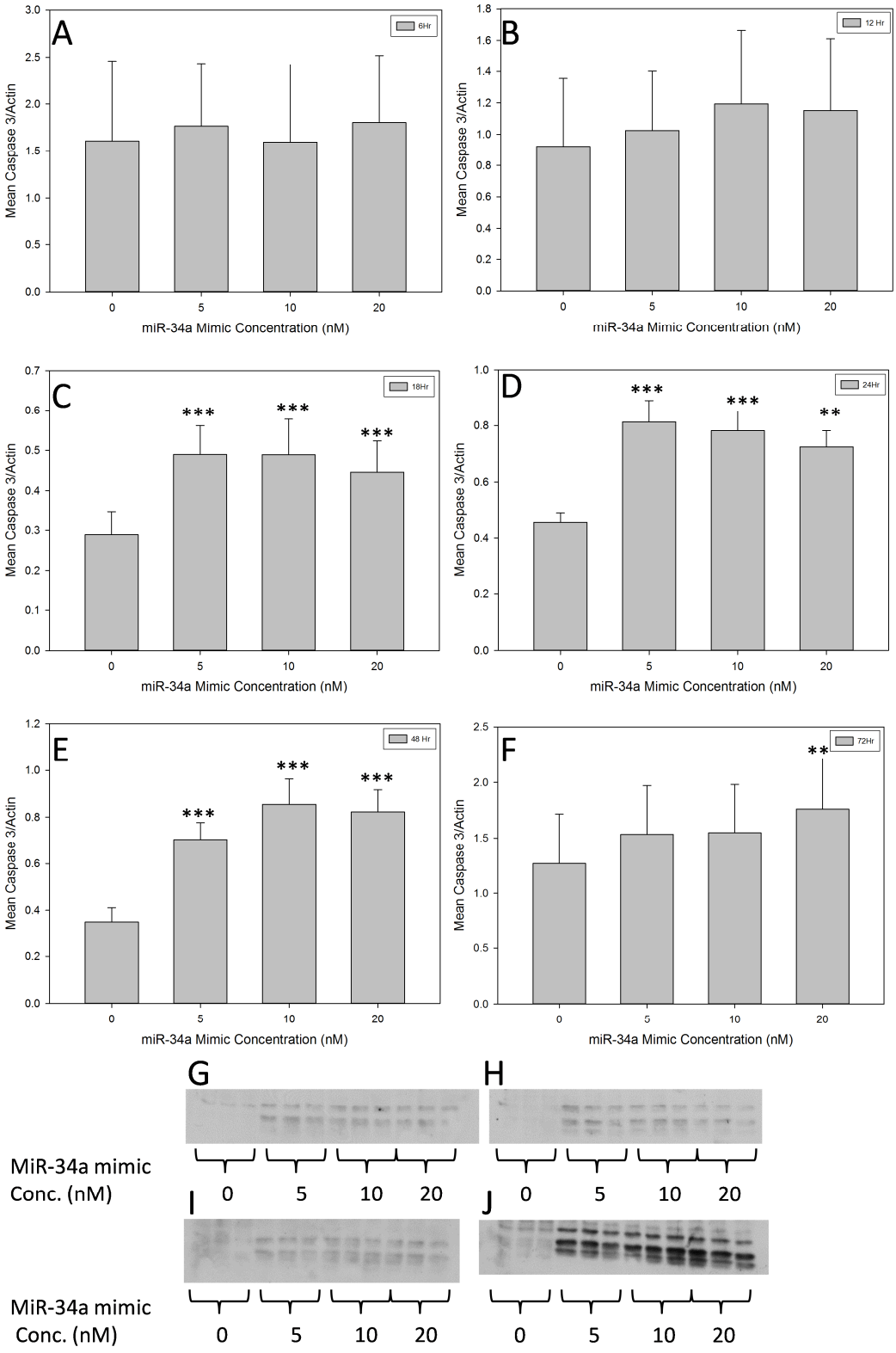

Figure 18:- Cleaved caspase 3 normalised to β-actin in Kim-1 expressing HEK293 cells transfected with increasing concentrations of miR-34a mimic for six, 12, 18, 24, 48 and 72 hours (A, B, C, D, E, F respectively). Western blots show expression of cleaved caspase 3 after 18, 24, 48 and 72 hours (G, H, I, J respectively). Error bars represent SEM of three independent experiments. **P<0.01, ***P<0.005.

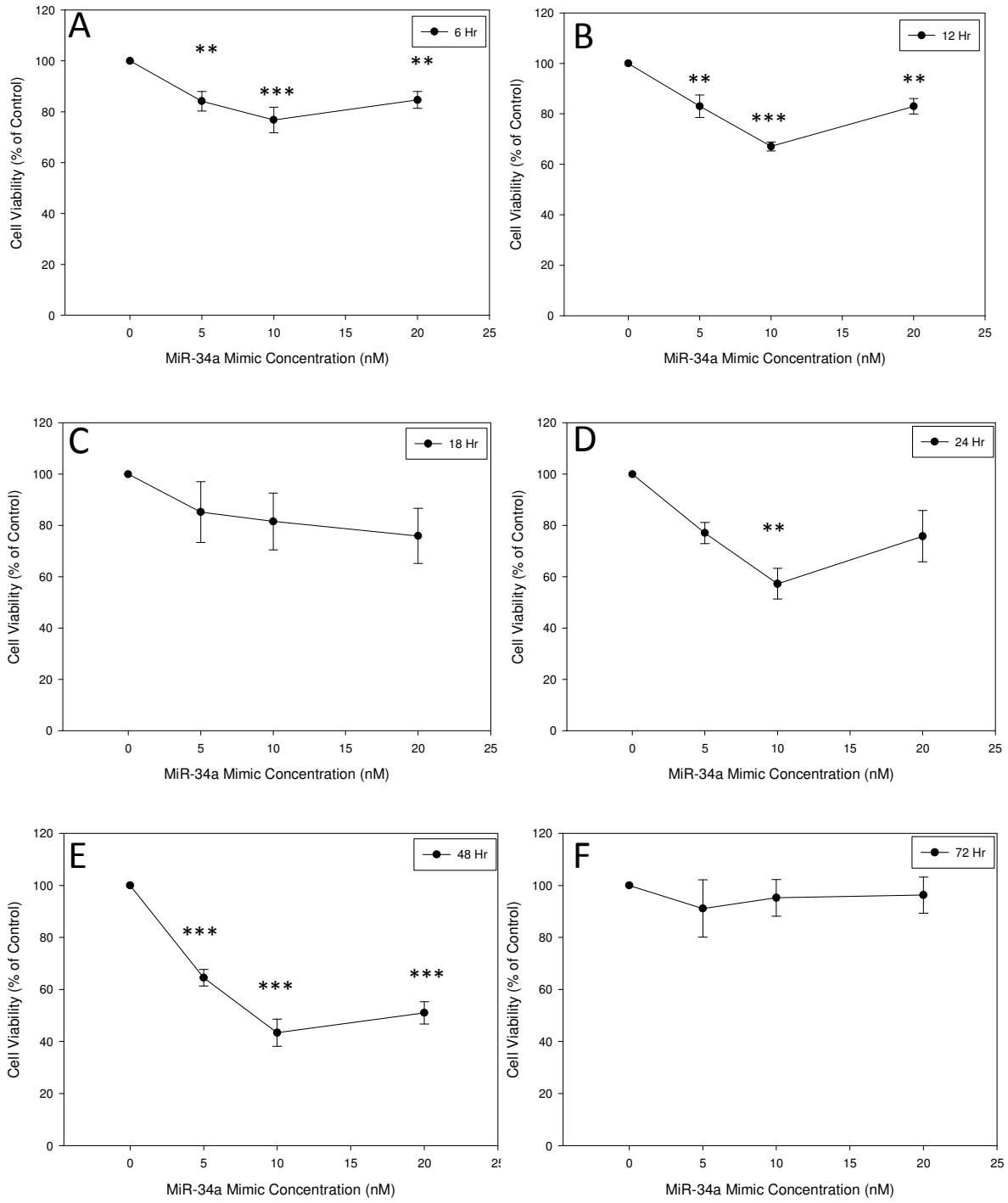

Figure 19:- Cell viability determined via MTS assay in Kim-1 expressing HEK293 cells transfected with increasing concentrations of miR-34a mimic for six, 12, 18, 24, 48 and 72 hours (A, B, C, D, E, F respectively). Error bars represent SEM of three independent experiments. **P<0.01, ***P<0.005.

To further assess whether the changes in cell viability and cleaved caspase 3 were a result of the transfection of the miR-34a mimic, two targets were quantified at the protein level via western blot after a transfection of 48 hours (figure 20). Both Bcl-2 (figure 20A) and CDK6 (figure 20B) were expressed at significantly lowered levels in the miR-34a mimic transfected cells (mean 0.56 and 0.83 respectively). The mRNA of Bcl-2 and CDK6 were also quantified via qPCR in 48 hour miR-34a mimic transfected cells and normalised to GAPDH (figure 21). Bcl-22 mRNA was found to be significantly elevated from control in miR-34a mimic transfected cells (mean 2.3) however CDK6 mRNA was found to be slightly reduced in the mimic transfected cells although not significantly so.

3.6 - Transfection of Kim-1 expressing HEK293 cells with miR-34a mimic for 48 hours followed by incubation with Cisplatin

Kim-1 expressing HEK293 cells were transfected with miR-34a mimic for 48 hours which was decided as the optimal time for transfection from previous experiments. Media was then replaced with Cisplatin containing media (0, 50 or 100 μM) for 24 hours. Control cells which were not transfected as well as a negative transfection control which contained a random sequence and a mock transfection which had only lipofectamine were also assessed alongside the transfected cells. Cell viability was assessed via MTS (figure 22), LDH in media via LDH assay (figure 23), apoptosis by caspase 3 western (figure 24) and cell fate via annexin V/PI FACS analysis (figure 25).

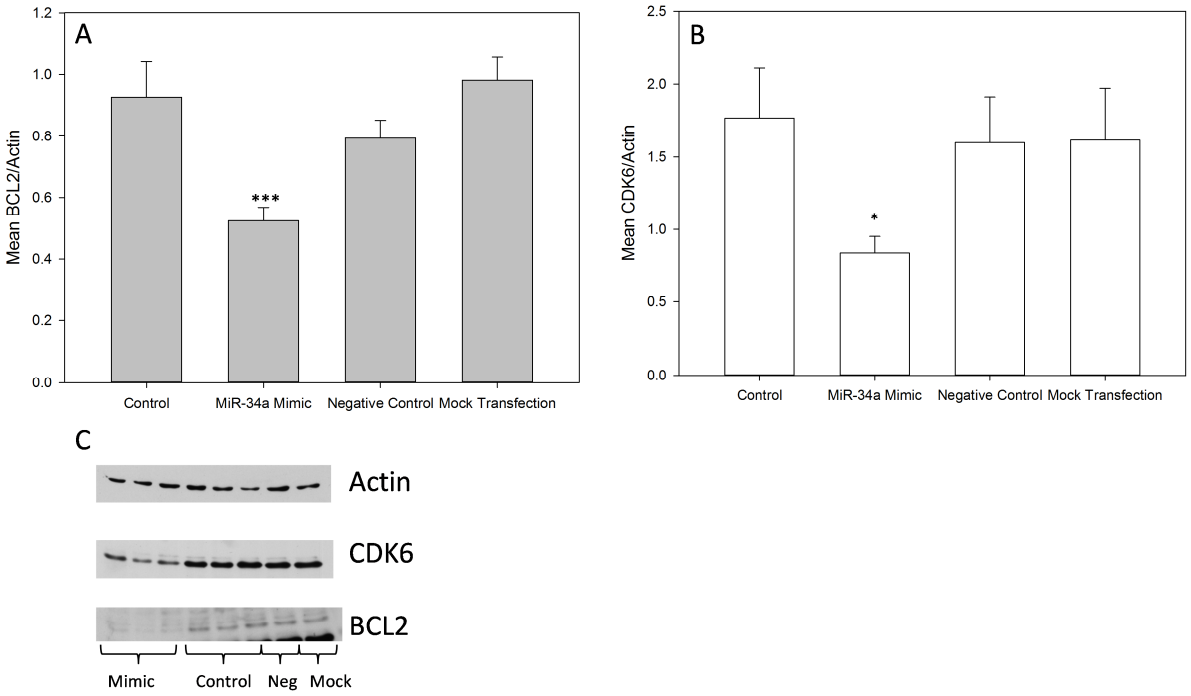

Figure 20:- Quantification of BCL2 and CDK6 protein in Kim-1 expressing HEK293 cell lysates via western blot. Western blots to BCL2 (A) and CDK6 (B) were assessed via densitometry and normalised to β-actin (C). Error bars represent SEM of three independent experiments. *P<0.05, ***P<0.005.

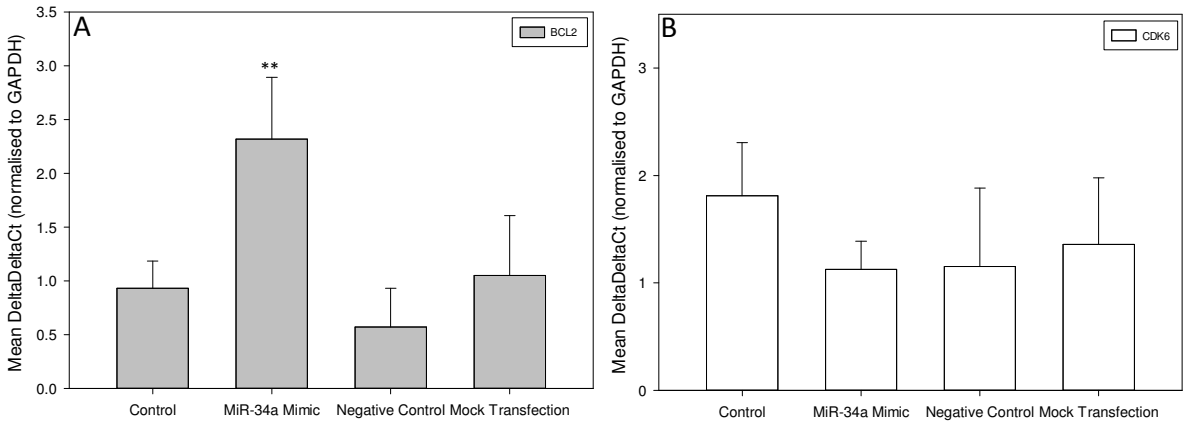

Figure 21:- Quantification of BCL2 and CDK6 mRNA in Kim-1 expressing HEK293 cell lysates via qPCR. BCL2 (A) and CDK6 (B) mRNA levels were normalised by the ΔΔCt method to GAPDH. Error bars represent SEM of three independent experiments. **P<0.01.

Transfection with miR-34a mimic caused a significant reduction in cell viability (Figure 22A) when incubated without Cisplatin when compared to untrasfected control cells (Mean 53 %). Incubation with 50 μM and 100 μM Cisplatin in the mimic transfected cells caused a significant reduction from both untransfected and transfected controls (mean 8.7 and 4.6 % respectively). MiR-34a mimic transfected cells dosed with 50 or 100 μM Cisplatin also had significantly different cell viabilities than untransfected cells treated with 50 or 100 μM Cisplatin (mean 8.7 and 78.1 % for 50 μM respectively and 4.6 and 53 % for 100 μM respectively). Results were also expressed as a fold change from their respective controls (figure 22B) to reduce the effect of the difference in cell viabilities from the untransfected and transfected control cells. Again, 50 and 100 μM Cisplatin caused significant reductions in cell viability in the mimic transfected cells (mean 0.12 and 0.02 respectively) and in the 100 μM Cisplatin dosed untransfected cells (mean 0.52). There were significant differences between the transfected and untransfected cells exposed to 50 and 100 μM Cisplatin (mean 0.12 and 0.76 for 50 μM respectively and 0.52 and 0.02 for 100 μM respectively).

The fraction of LDH released into the media of cells transfected with miR-34a mimic and exposed to 50 and 100 μM Cisplatin (figure 23A) were significantly elevated from both transfected and untransfected control cells (mean 51 and 57 % respectively). Untransfected cells were found to have a significantly larger fraction of LDH in the media of 100 μM Cisplatin dosed cells compared to untransfected controls (mean 20 %). There was a significantly greater fraction of LDH in the media of mimic transfected cells exposed to 50 μM Cisplatin compared to the untransfected cells (mean 51 and 14 % respectively). The same was observed for the transfected cells and the untransfected cells exposed to 100 μM Cisplatin (mean 57 and 20 % respectively).

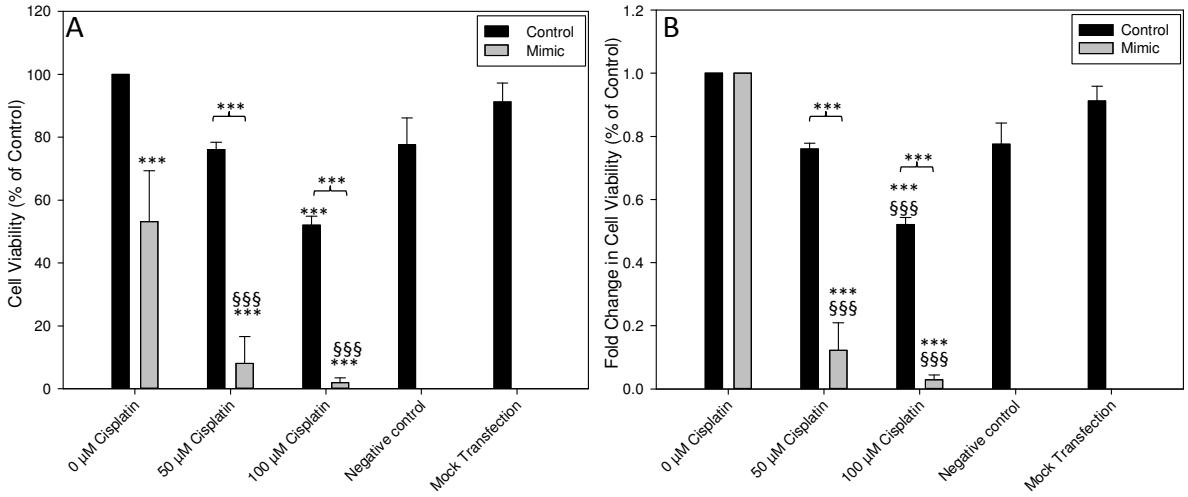

Figure 22:- Cell viability determined via MTS assay in Kim-1 expressing HEK293 cells. Cells were either transfected with miR-34a mimic (grey bars) or not (black bars) for 48 hours followed by incubation with 50 µM Cisplatin or 100 µM for 24 hours. Negative control and mock transfection samples were transfected with a random sequence which should have no effect on the cells and lipofectamine alone respectively. Results are expressed as fraction of untransfected cells (A) or as fold change of their own control (B). * represent significant changes to control cells and § represent significant changes to miR-34a mimic transfected cells. ***/§§§ P<0.005.

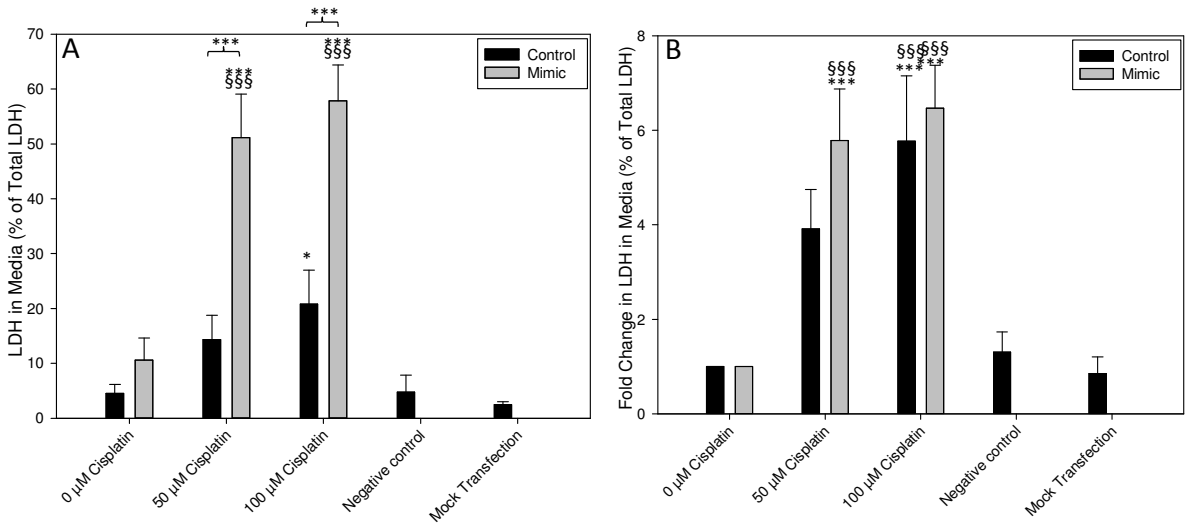

Figure 23:- Fraction of LDH leakage into media surrounding Kim-1 expressing HEK293 cells. Cells were either transfected with miR-34a mimic (grey bars) or not (black bars) for 48 hours followed by incubation with 50 µM or 100 µM Cisplatin for 24 hours. Negative control and mock transfection samples were transfected with a random sequence which should have no effect on the cells and lipofectamine alone respectively. Results are expressed as fraction of untransfected cells (A) or as fold change of their own control (B). * represent significant changes to control cells and § represent significant changes to miR-34a mimic transfected cells. */§ P<0.05, ***/§§§ P<0.005.

Results were also expressed as fold changes of respective controls as for the MTS assays (Figure 23B). The mimic transfected cells exposed to 50 and 100 μM Cisplatin were both significantly elevated from the mimic transfected control (mean fold change 5.7 and 6.4 respectively). The untransfected cells exposed to 100 μM Cisplatin were also significantly elevated from the controls (Mean 5.7). There were no significant differences between the transfected and untransfected cells exposed to the same concentrations of Cisplatin.

Apoptosis was also assessed in the miR-34a mimic transfected cells via the quantification of cleaved caspase 3 via western blot (figure 24) and also via Annexin V/PI cell staining followed by FACS analysis(figure 25). Exposure to 50 μM Cisplatin caused significant elevations in cleaved caspase 3 normalised to β-actin in cells transfected with the miR-34a mimic (mean 2.3) and also in the transfected cells exposed to 100 μM Cisplatin (mean 1.9). The untransfected cells were found to have significantly greater levels of cleaved caspase 3 after exposure to 100 μM Cisplatin (mean 1.1) but not 50 μM Cisplatin. Exposure to 50 μM Cisplatin caused exposure of significantly greater amounts of cleaved caspase 3 normalised to β-actin in mimic transfected cells compared to untransfected cells.

The FACS analysis showed significant increases in the fraction of early apoptotic cells (figure 25C) when the mimic transfected cells were exposed to both 50 and 100 μM Cisplatin (mean 20.7 and 20.4 % respectively). The fraction of late apoptotic cells were found to be significantly increased in response to the mimic alone compared to untrasfected cells, the mimic exposed to 50 μM Cisplatin and the mimic exposed to 100 μM Cisplatin (mean 12.1, 21.8 and 21.6 % respectively). Exposure of untrasfected cells to 100 μM Cisplatin caused a significant elevation in late apoptosis (mean 8.5 %).

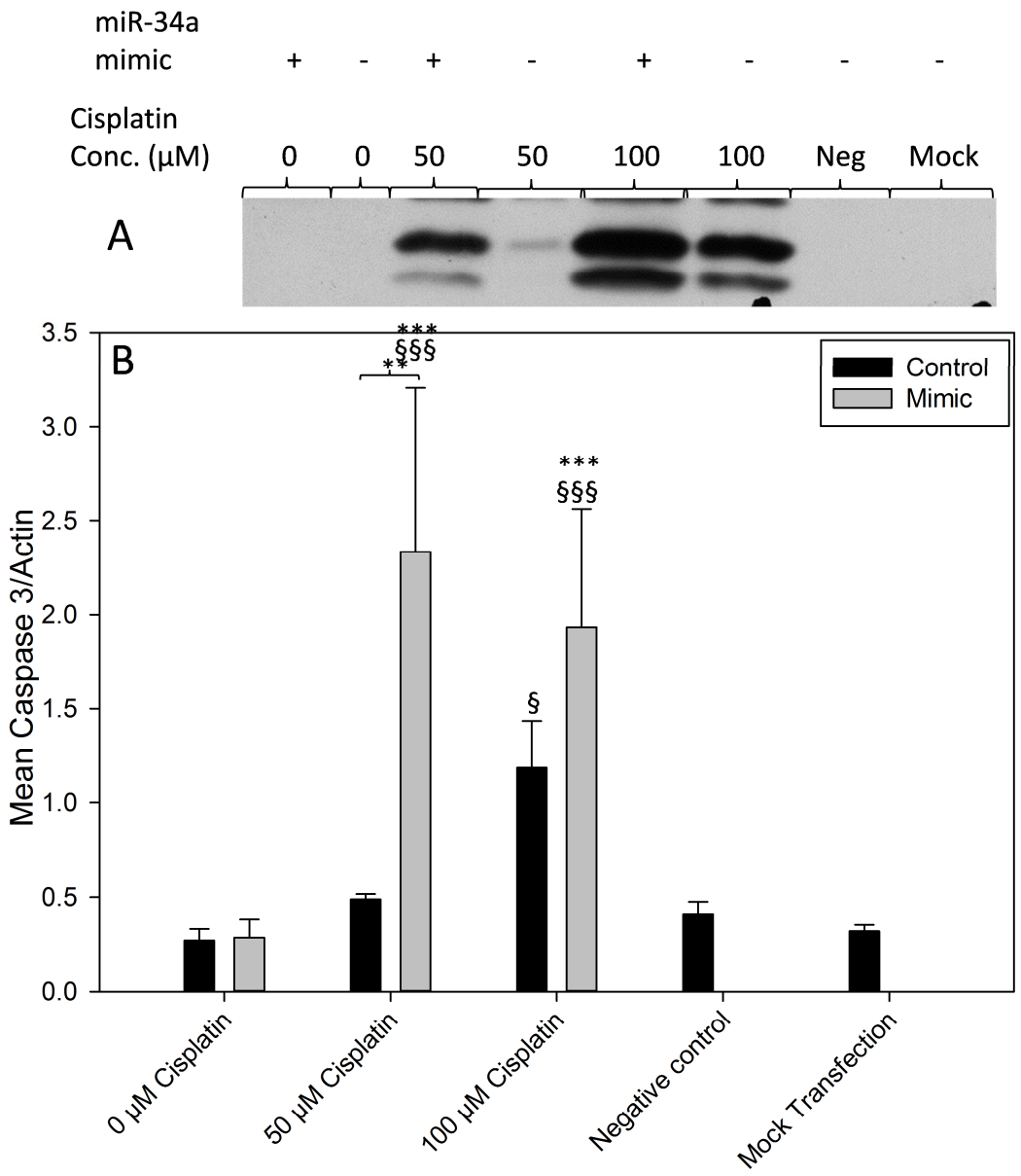

Figure 24:- Mean cleaved caspase 3 in Kim-1 expressing HEK293 cell lysates. Cells were either transfected with miR-34a mimic (grey bars) or not (black bars) for 48 hours followed by incubation with 50 µM or 100 µM Cisplatin for 24 hours. Negative control and mock transfection samples were transfected with a random sequence which should have no effect on the cells and lipofectamine alone respectively. Results are expressed as cleaved caspase 3 normalised to β-actin (B). Cleaved caspase 3 western bands are shown (A). * represent significant changes to control cells and § represent significant changes to miR-34a mimic transfected cells. */§ $P<0.05$, **/§§ $P<0.01$, ***/§§§ $P<0.005$.

The fraction of late apoptotic cells was found to be significantly elevated after exposure to both 50 and 100 µM Cisplatin in the transfected cells compared to the untransfected cells (mean 21.8 and 4.4 % respectively for 50µM Cisplatin and 21.6 and 8.5 % respectively for 100 µM Cisplatin). Results from the FACS analysis were also expressed as fold changes of the untransfected and transfected cells exposed to Cisplatin as a fold change to their respective controls (figure 26). Exposure to 100 µM Cisplatin caused a significant increase in early apoptotic cells (figure 26C) in untransfected cells (mean fold change of 2.6) and also causing significant elevations in late apoptotic cells (mean fold change of 3.9) which were significantly greater than the fold changes in the mimic transfected cells (figure 26D).

3.7 - Transfection of Kim-1 expressing HEK293 cells with miR-34a mimic for 48 hours followed by incubation with Staurosporine

Transfections of Kim-1 expressing HEK293 cells with miR-34a mimic for 48 hours were also repeated but followed by exposure to Staurosporine for 24 hours rather than Cisplatin. Transfection with miR-34a mimic caused significant reductions in cell viability as determined by the MTS assay (figure 27A) in cells exposed to 1 or 1.5 µM Staurosporine compared to untransfected control cells (mean 33.1 and 39.3 % respectively). Transfected cells exposed to 1 or 1.5 µM Staurosporine had significantly reduced cell viability when compared to untransfected cells exposed to 1 or 1.5 µM Staurosporine (mean 33.1 and 90.4 % for 1 µM respectively and 39.3 and 82.7 % for 1.5 µM respectively). Results were also expressed as fold change in cell viability of the transfected or untransfected cells' respective controls (figure 27B). Again 1 and 1.5 µM Staurosporine caused significant reductions in fold change cell viability in mimic transfected cells when compared to either control (mean fold change 0.36 and 0.43 respectively).

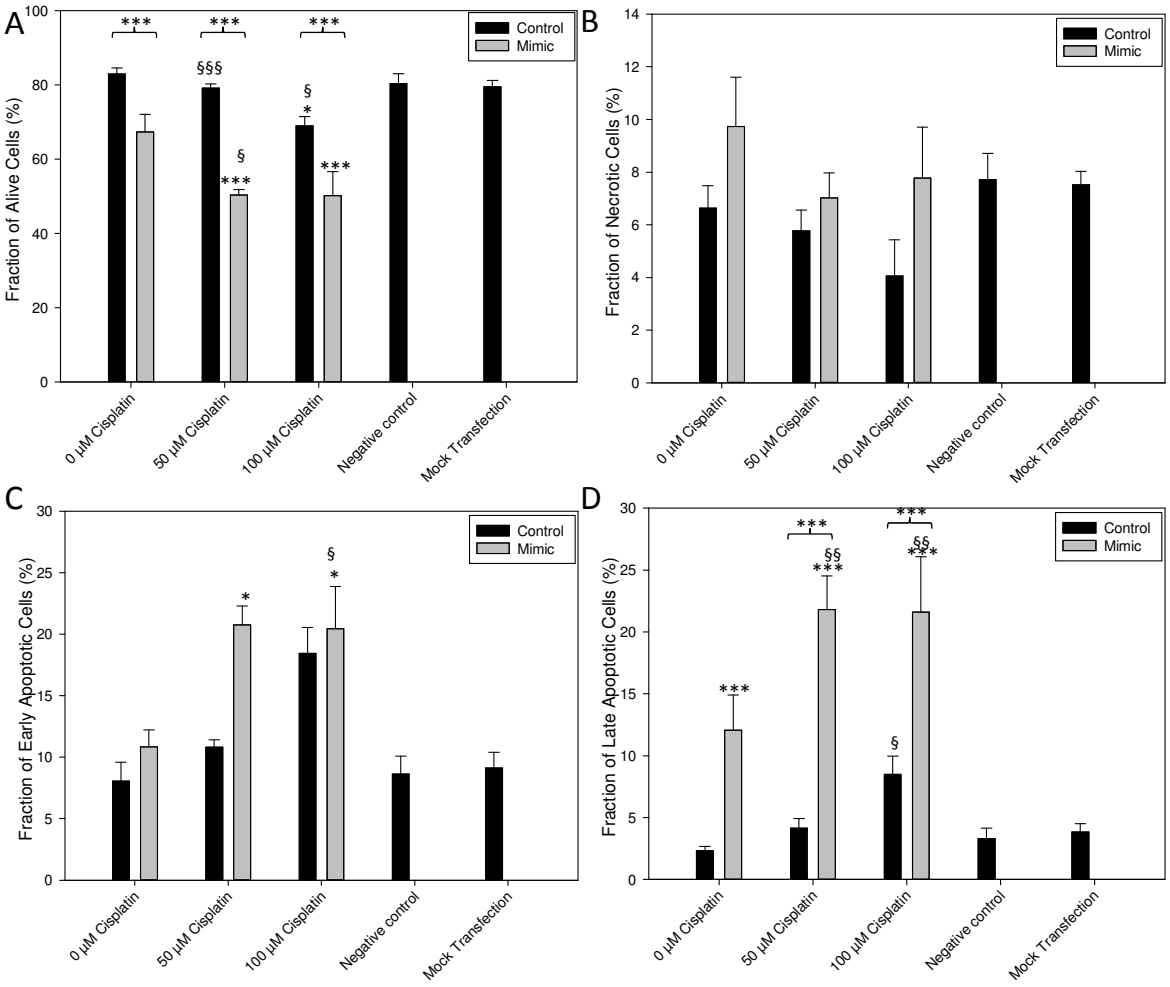

Figure 25:- Fate of Kim-1 expressing HEK293 cells as determined via Annexin V/PI staining and FACS analysis. Cells were either transfected with miR-34a mimic (grey bars) or not (black bars) for 48 hours followed by incubation with 50 µM or 100 µM Cisplatin for 24 hours. Negative control and mock transfection samples were transfected with a random sequence which should have no effect on the cells and lipofectamine alone respectively. Results are expressed as the fraction of cells which are alive (A), necrotic (B), early apoptotic (C) or late apoptotic (D). * represent significant changes to control cells and § represent significant changes to miR-34a mimic transfected cells. */§ P<0.05, ***/§§§ P<0.005.

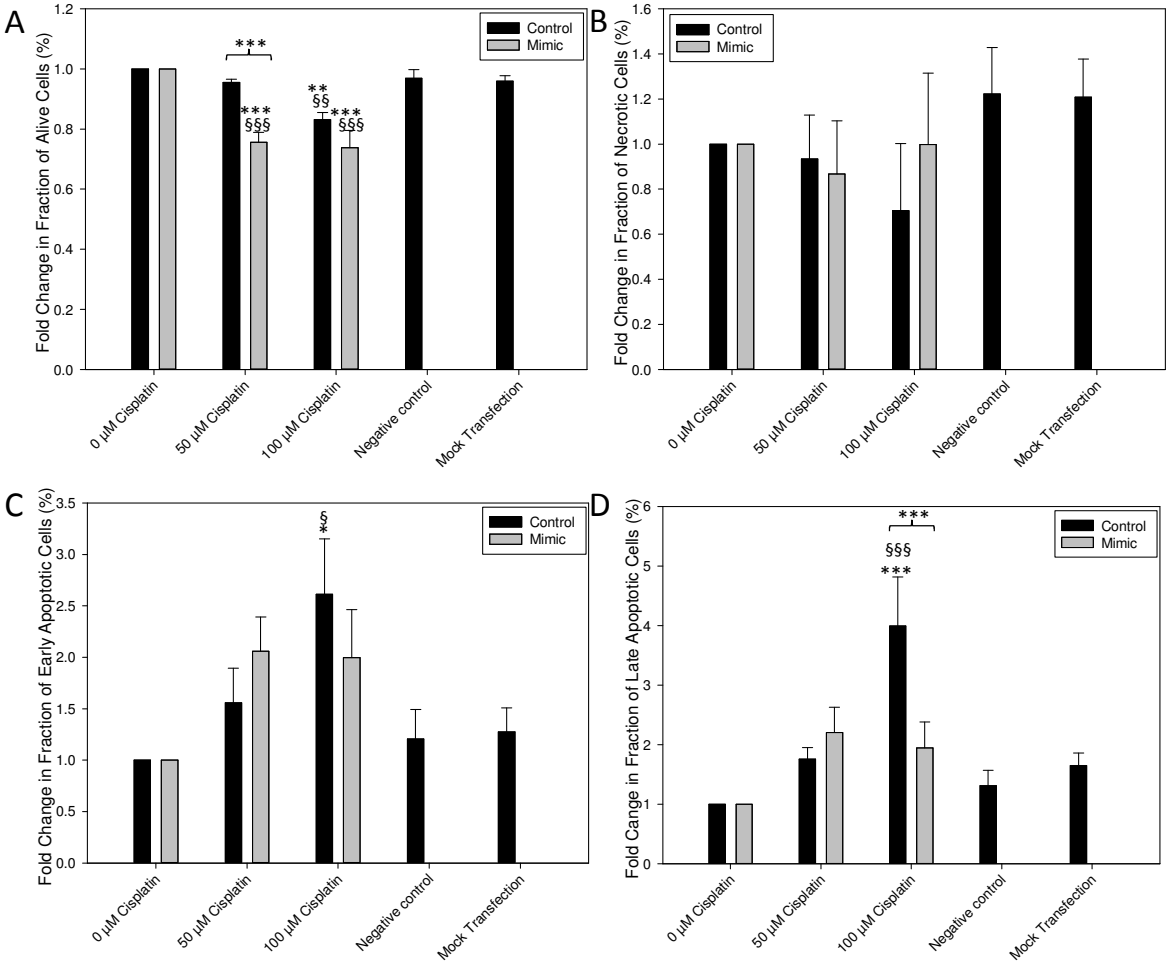

Figure 26:- Fate of Kim-1 expressing HEK293 cells expressed as fold change of respective controls as determined via Annexin V/PI staining and FACS analysis. Cells were either transfected with miR-34a mimic (grey bars) or not (black bars) for 48 hours followed by incubation with 50 µM or 100 µM Cisplatin for 24 hours. Negative control and mock transfection samples were transfected with a random sequence which should have no effect on the cells and lipofectamine alone respectively. Results are expressed as the fold change of fraction of cells which are alive (A), necrotic (B), early apoptotic (C) or late apoptotic (D). * represent significant changes to control cells and § represent significant changes to miR-34a mimic transfected cells. */§ P<0.05, **/§§ P<0.01, ***/§§§ P<0.005.

Transfected cells exposed to 1 and 1.5 μM Staurosporine also had significantly greater reduction in fold change of cell viability than untrasfected cells exposed to the same concentration of Staurosporine (mean fold change 0.36 and 0.95 for 1 μM respectively and 0.43 and 0.82 for 1.5 μM respectively).

Exposure to 1 and 1.5 μM Staurosporine caused a significant increase in the fraction of LDH in the media surrounding the mimic transfected cells (figure 28A) compared to both the transfected and untransfected controls (mean 33.2 and 33.6 % respectively). Exposure to 1.5 μM Staurosporine was found to significantly increase the LDH in the media of the untransfected cells compared to control untransfected cells (mean 20.2). The mimic transfected cells had a significantly greater amount of LDH in the media after exposure to 1 or 1.5 μM Staurosporine when compared the untransfected cells (mean 33.2 and 17.6 % for 1 μM respectively and 32.6 and 20.2 % for 1.5 μM respectively). When expressed as fold change (figure 29B) of their respective controls both the transfected and untransfected cells had significantly larger changes in LDH in the media for both 1 and 1.5 μM Staurosporine (4.5 and 4.4 for 1 μM respectively and 4.2 and 4.9 for 1.5 μM respectively) however they were not significantly different from each other.

Cell death via apoptosis was assessed after exposure to Staurosporine via quantification of cleaved caspase 3 in cell lysates as previously described (figure 29). Exposure of mimic transfected cells to 1 or 1.5 μM Staurosporine caused significant increases in cleaved caspase 3 when normalised to β-actin (mean 1.72 and 1.27 respectively). The expression of cleaved caspase 3 normalised to β-actin in transfected cells exposed to 1 or 1.5 μM Staurosporine was also significantly greater than in untransfected cells (mean 1.72 and 0.4 for 1 μM respectively and 1.27 and 0.55 for 1.5 μM Staurosporine respectively).

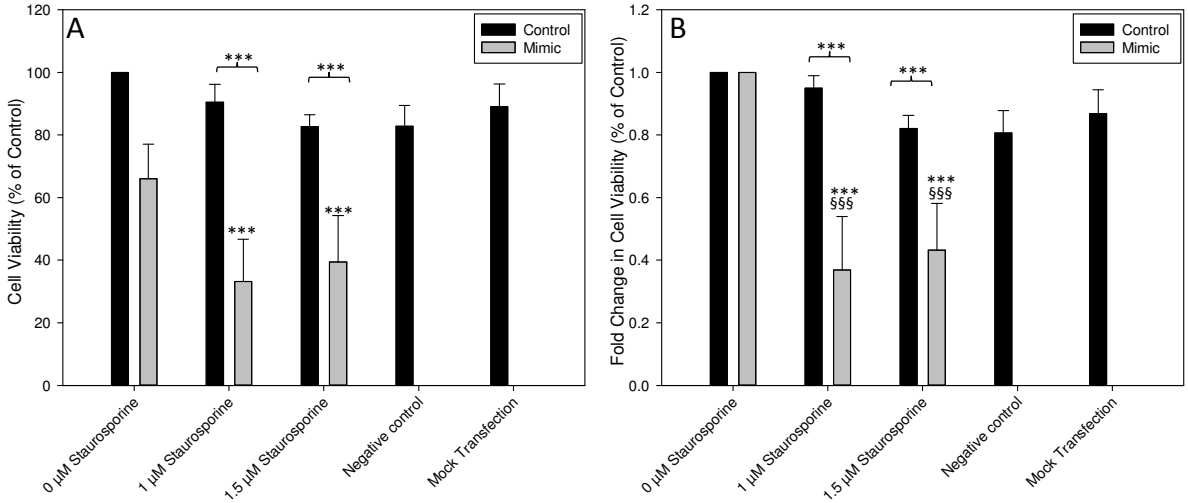

Figure 27:- Cell viability determined via MTS assay in Kim-1 expressing HEK293 cells. Cells were either transfected with miR-34a mimic (grey bars) or not (black bars) for 48 hours followed by incubation with 1 µM or 1.5 µM Staurosporine for 24 hours. Negative control and mock transfection samples were transfected with a random sequence which should have no effect on the cells and lipofectamine alone respectively. Results are expressed as fraction of untransfected cells (A) or as fold change of their own control (B). * represent significant changes to control cells and § represent significant changes to miR-34a mimic transfected cells. ***/§§§ P<0.005.

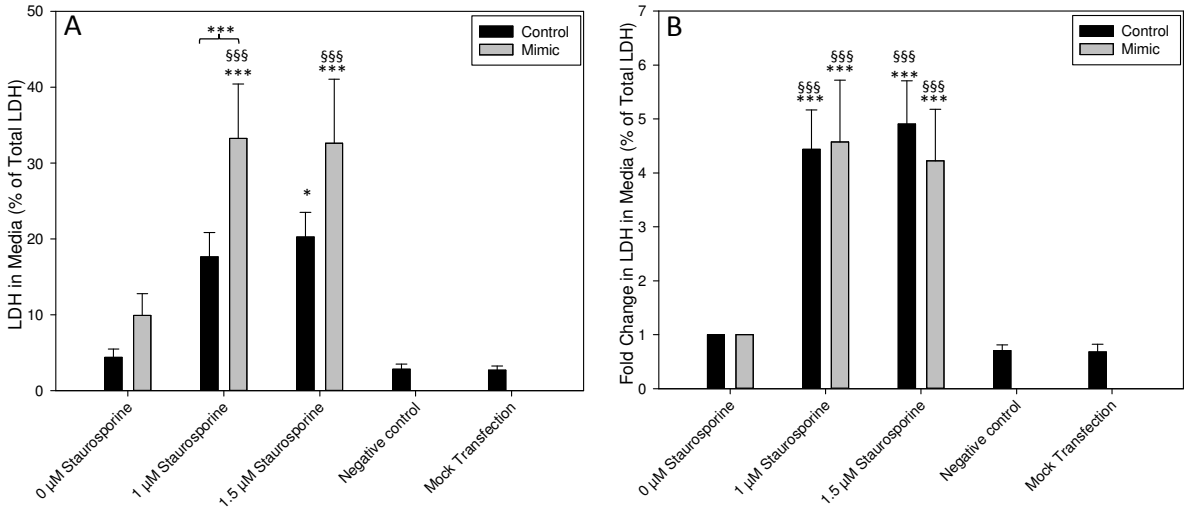

Figure 28:- Fraction of LDH leakage into media surrounding Kim-1 expressing HEK293 cells. Cells were either transfected with miR-34a mimic (grey bars) or not (black bars) for 48 hours followed by incubation with 1 µM or 1.5 µM Staurosporine for 24 hours. Negative control and mock transfection samples were transfected with a random sequence which should have no effect on the cells and lipofectamine alone respectively. Results are expressed as fraction of untransfected cells (A) or as fold change of their own control (B). * represent significant changes to control cells and § represent significant changes to miR-34a mimic transfected cells. */§ P<0.05, ***/§§§ P<0.005.

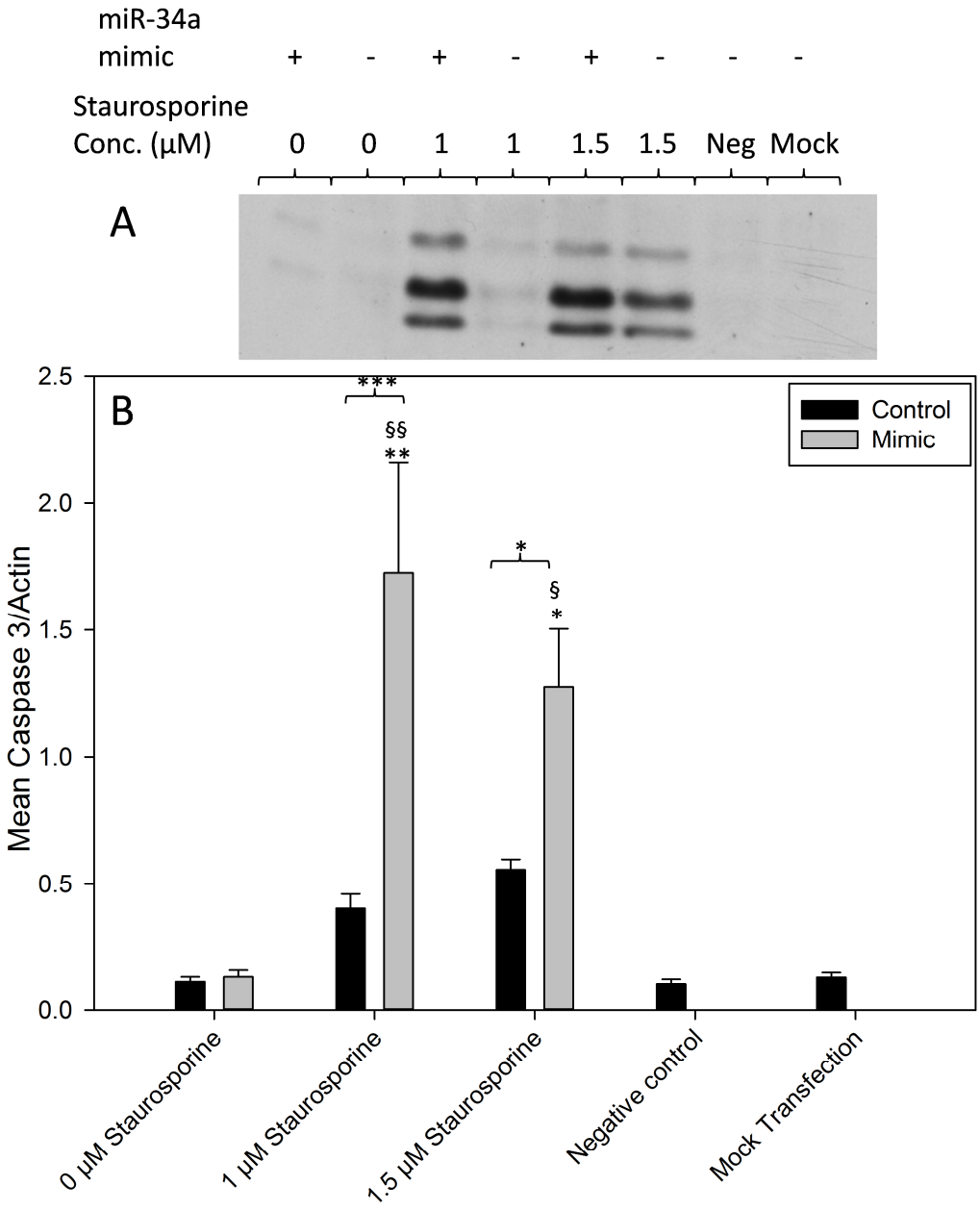

Figure 29:- Mean cleaved caspase 3 in Kim-1 expressing HEK293 cell lysates. Cells were either transfected with miR-34a mimic (grey bars) or not (black bars) for 48 hours followed by incubation with 1 µM or 1.5 µM Staurosporine for 24 hours. Negative control and mock transfection samples were transfected with a random sequence which should have no effect on the cells and lipofectamine alone respectively. Results are expressed as cleaved caspase 3 normalised to β-actin (B). Cleaved caspase 3 western bands are shown (A). * represent significant changes to control cells and § represent significant changes to miR-34a mimic transfected cells. */§ P<0.05, **/§§ P<0.01, ***/§§§ P<0.005.

There were no significant changes in the fraction of early or late apoptotic cells when exposed to Staurosporine were observed in mimic transfected or untransfected cells (data not shown).

3.8 - Transfection of Kim-1 expressing HEK293 cells with miR-34a mimic for 12 hours followed by incubation with Cisplatin

In order to try and negate the changes in cell viability, LDH leakage into media and fraction of apoptotic cells which were observed between transfected and untrasfected control cells the transfection time was reduced to 12 hours. The Kim-1 expressing HEK293 cells were then exposed to either 50 or 100 µM Cisplatin for 24 hours. MiR-34a mimic transfected cells' viability (figure 30A) was significantly reduced from the untransfected controls when exposed to 50 or 100 µM Cisplatin (mean 28.8 and 11.5 % respectively). Untransfected cell viability was significantly reduced when exposed to 100 µM Cisplatin only (mean 32.2 %). When cell viability was expressed as fold change (figure 30B) from the cells respective controls the transfected cells were found to have a significant reduction in fold change of cell viability when exposed to both 50 and 100 µM Cisplatin (mean fold change 0.3 and 0.14 respectively). Untransfected cells exposed to 100 µM Cisplatin were also found to have significantly reduced cell viability (mean fold change 0.32). Transfected cells exposed to both 50 and 100 µM Cisplatin were found to have significantly different fold changes in cell viability to untransfected cells (Mean fold change 0.3 and 0.63 for 50 µM respectively and 0.14 and 0.32 for 100 µM respectively). Exposure to 50 and 100 µM Cisplatin caused increases in the fraction of LDH in the media in both the transfected and untransfected cells however these changes were not found to be significant (figure 31A).

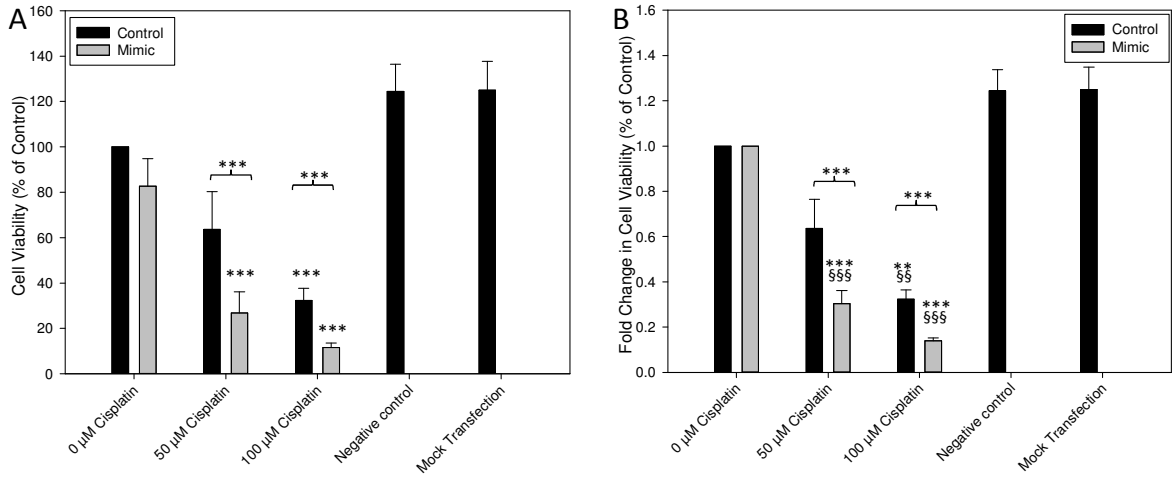

Figure 30:- Cell viability determined via MTS assay in Kim-1 expressing HEK293 cells. Cells were either transfected with miR-34a mimic (grey bars) or not (black bars) for 12 hours followed by incubation with 50 µM or 100 µM Cisplatin for 24 hours. Negative control and mock transfection samples were transfected with a random sequence which should have no effect on the cells and lipofectamine alone respectively. Results are expressed as fraction of untransfected cells (A) or as fold change of their own control (B). * represent significant changes to control cells and § represent significant changes to miR-34a mimic transfected cells. **/§§ P<0.01, ***/§§§ P<0.005.

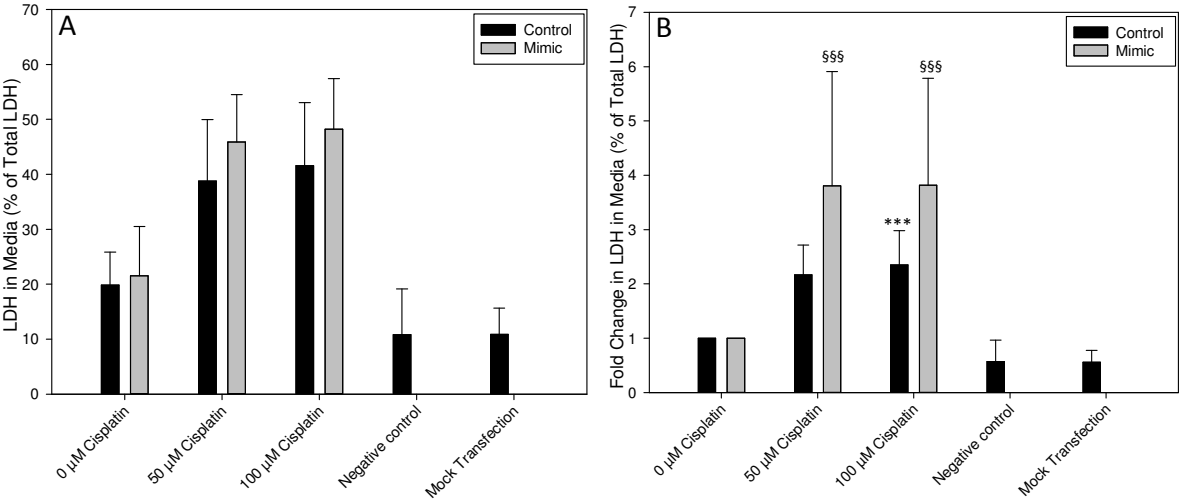

Figure 31:- Fraction of LDH leakage into media surrounding Kim-1 expressing HEK293 cells. Cells were either transfected with miR-34a mimic (grey bars) or not (black bars) for 12 hours followed by incubation with 50 µM or 100 µM Cisplatin for 24 hours. Negative control and mock transfection samples were transfected with a random sequence which should have no effect on the cells and lipofectamine alone respectively. Results are expressed as fraction of untransfected cells (A) or as fold change of their own control (B). * represent significant changes to control cells and § represent significant changes to miR-34a mimic transfected cells. ***/§§§ P<0.005.

When expressed as fold changes of their respective controls (Figure 31B) 50 and 100 μM Cisplatin caused significant increases in fold changes of LDH in the media in transfected cells (mean fold change 3.8 and 3.81 respectively). Exposure to 100 μM Cisplatin in the untransfected cells also caused a significant elevation in the fold change of LDH in media (mean 2.3) although there were no significant differences between transfected or untransfected cells after exposure to 50 or 100 μM Cisplatin.

Apoptosis was assessed in after transfection for 12 hours and exposure to Cisplatin for 24 hours by caspase 3 western and through Annexin V/PI FACS analysis. Transfected cells expressed a significantly greater amount of cleaved caspase 3 (figure 32) normalised to β-actin when exposed to 50 or 100 μM Cisplatin compared to control transfected cells (mean 1.1 and 1.8 respectively). Untransfected cells also expressed a significantly greater amount of cleaved caspase 3 than control cells after exposure to 100 μM Cisplatin (mean 1.1). There was a significantly greater expression of cleaved caspase 3 after exposure to 100 μM Cisplatin in the transfected cells than in the untransfected cells (mean 1.8 and 1.1 respectively). When assessed via FACS analysis there were no significant changes in the fraction of early or late apoptotic cells although there were elevations in both sets of cells in response to 50 or 100 μM Cisplatin (data not shown). When results were expressed as fold change of fraction of cells relative to their respective controls (figure 33B) there was a significant increase in the fraction of late apoptotic cells in the transfected cells after exposure to 100 μM Cisplatin (mean fold change 3.5) but no other significant changes were observed.

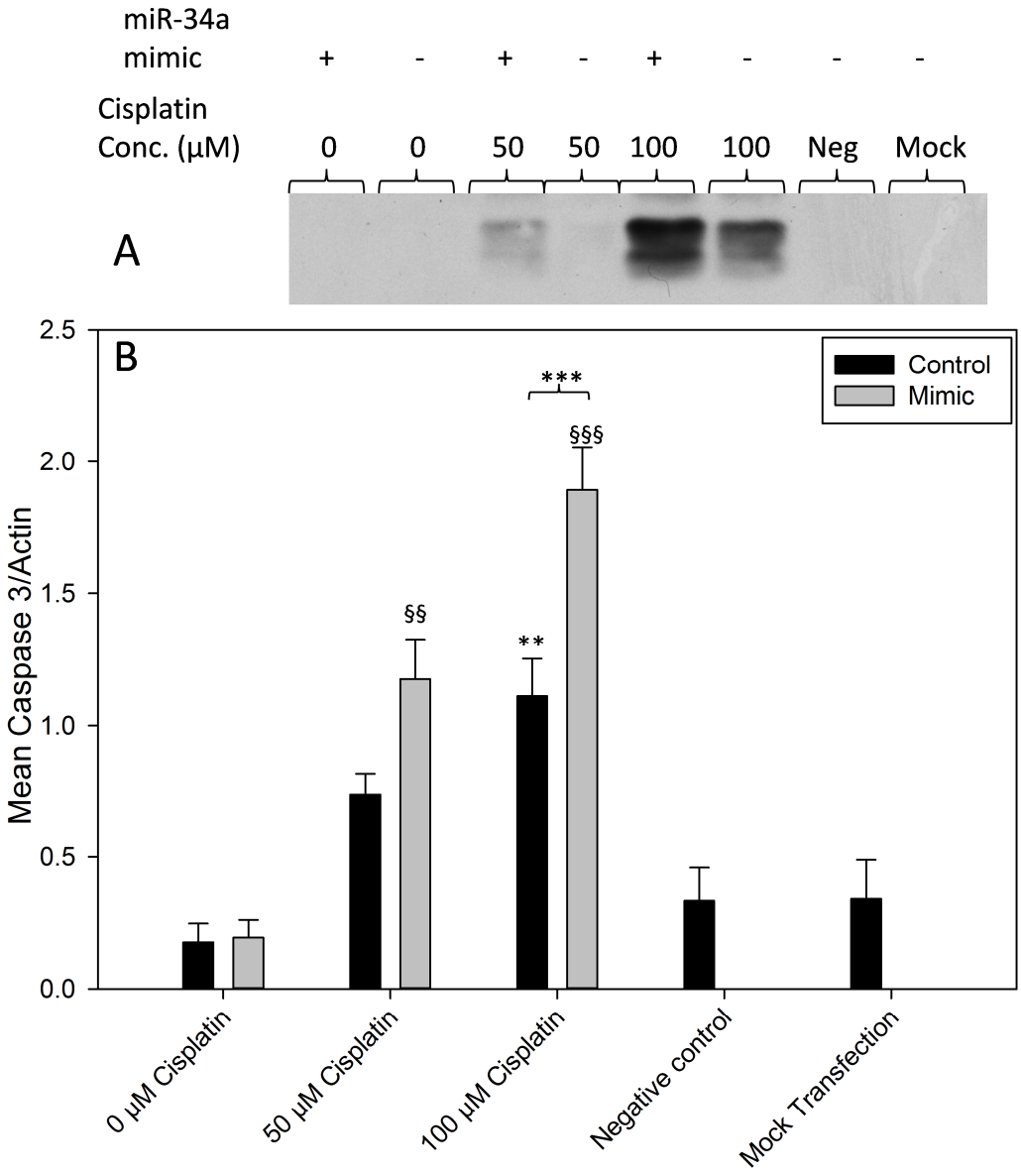

Figure 32:- Mean cleaved caspase 3 in Kim-1 expressing HEK293 cell lysates. Cells were either transfected with miR-34a mimic (grey bars) or not (black bars) for 12 hours followed by incubation with 50 µM or 100 µM Cisplatin for 24 hours. Negative control and mock transfection samples were transfected with a random sequence which should have no effect on the cells and lipofectamine alone respectively. Results are expressed as cleaved caspase 3 normalised to β-actin (B). Cleaved caspase 3 western bands are shown (A). * represent significant changes to control cells and § represent significant changes to miR-34a mimic transfected cells. **/§§ P<0.01, ***/§§§ P<0.005.

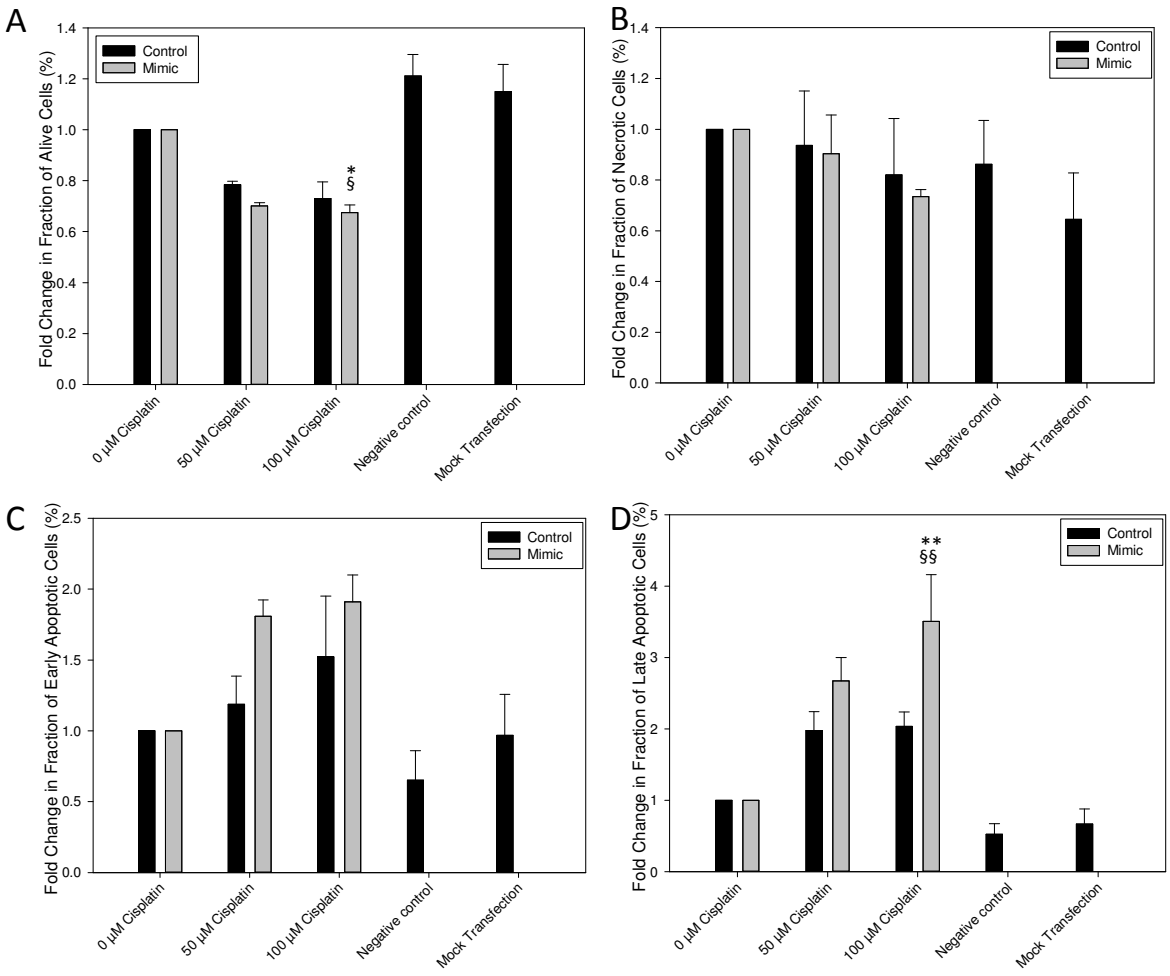

Figure 33:- Fate of Kim-1 expressing HEK293 cells expressed as fold change of respective controls as determined via Annexin V/PI staining and FACS analysis. Cells were either transfected with miR-34a mimic (grey bars) or not (black bars) for 12 hours followed by incubation with 50 µM or 100 µM Cisplatin for 24 hours. Negative control and mock transfection samples were transfected with a random sequence which should have no effect on the cells and lipofectamine alone respectively. Results are expressed as the fold change of fraction of cells which are alive (A), necrotic (B), early apoptotic (C) or late apoptotic (D). * represent significant changes to control cells and § represent significant changes to miR-34a mimic transfected cells. */§ P<0.05, **/§§ P<0.01.

3.9 - Transfection of Kim-1 expressing HEK293 cells with miR-34a mimic for 12 hours followed by incubation with Staurosporine

Kim-1 expressing HEK293 cells exposed to Staurosporine were also transfected for 12 hours with the miR-34a mimic to attempt to negate the differences in the control untransfected cells and the transfected cells as with the Cisplatin dosed cells. Cell viability was significantly reduced (Figure 34A) in the mimic transfected cells after exposure to both 1 and 1.5 µM Staurosporine (Mean 22.9 and 9.2 % respectively). The untransfected cells' viability was also significantly reduced after exposure to 1 and 1.5 µM Staurosporine (Mean 49.4 and 32.6 % respectively). There were no significant differences in cell viability between the transfected and untransfected cells exposed to 1 and 1.5 µM Staurosporine. When results were expressed as a fold change of the transfected or untransfected cells relative controls (Figure 34B) both the transfected and untransfected cells were significantly reduced in response to 1 and 1.5 µM Staurosporine (Mean fold change 0.24 and 0.49 for 1 µM respectively and 0.1 and 0.32 for 1.5 µM respectively). The fraction of LDH in the media (Figure 35A) was significantly greater than control in the transfected cells in response to both 1 and 1.5 µM Staurosporine (Mean 55.4 and 56.3 % respectively). The untransfected cells also had significantly larger amounts of LDH in the media compared to controls in response to 1 and 1.5 µM Staurosporine (Mean 37.5 and 38.8 % respectively). The transfected cells exposed to 1 and 1.5 µM Staurosporine had significantly greater amounts of LDH in the media than the untransfected cells exposed to the same concentrations of Staurosporine.

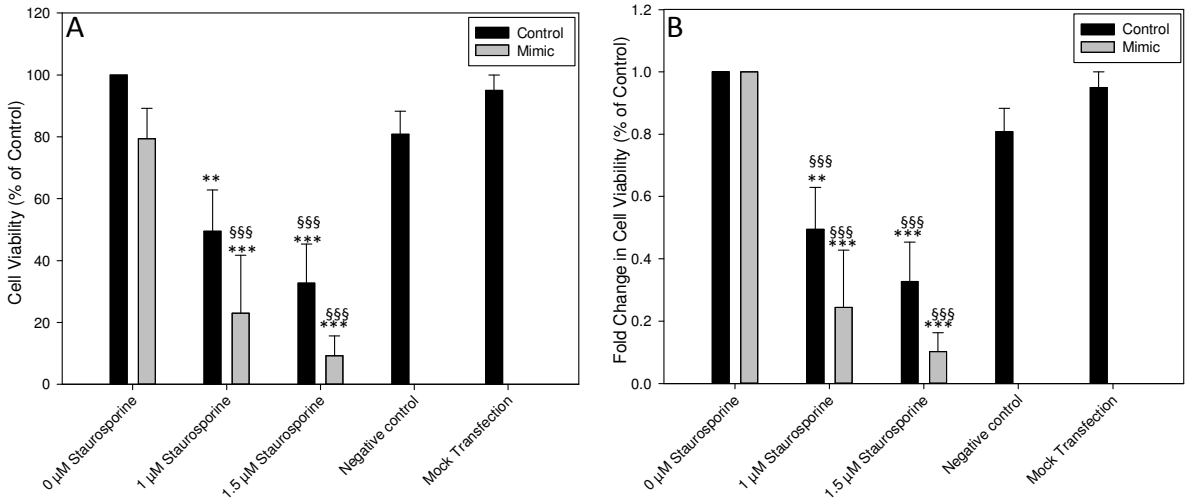

Figure 34:- Cell viability determined via MTS assay in Kim-1 expressing HEK293 cells. Cells were either transfected with miR-34a mimic (grey bars) or not (black bars) for 12 hours followed by incubation with 1 µM or 1.5 µM Staurosporine for 24 hours. Negative control and mock transfection samples were transfected with a random sequence which should have no effect on the cells and lipofectamine alone respectively. Results are expressed as fraction of untransfected cells (A) or as fold change of their own control (B). * represent significant changes to control cells and § represent significant changes to miR-34a mimic transfected cells. **/§§ P<0.01, ***/§§§ P<0.005.

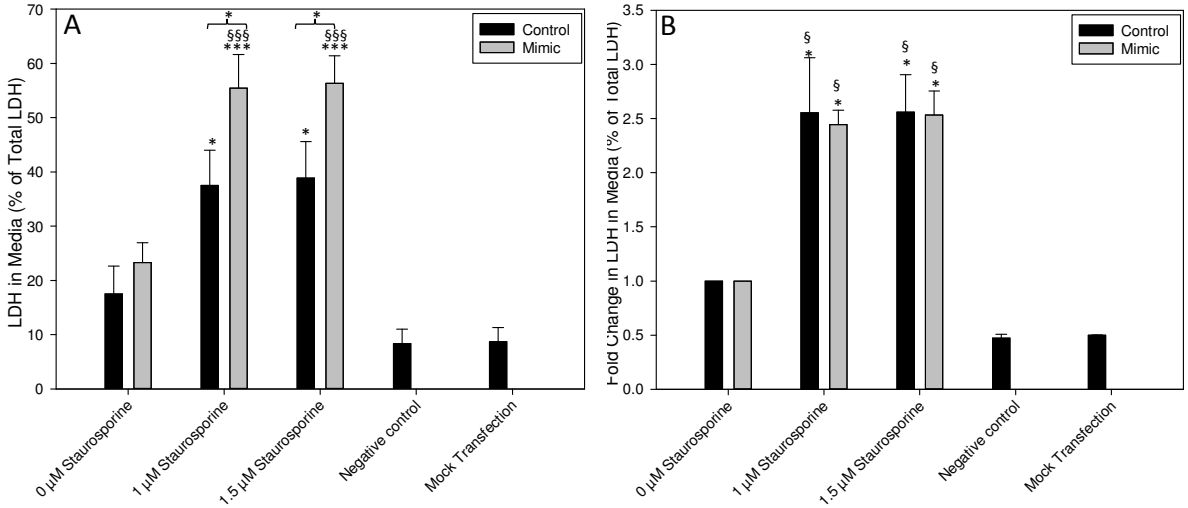

Figure 35:- Fraction of LDH leakage into media surrounding Kim-1 expressing HEK293 cells. Cells were either transfected with miR-34a mimic (grey bars) or not (black bars) for 12 hours followed by incubation with 1 µM or 1.5 µM Staurosporine for 24 hours. Negative control and mock transfection samples were transfected with a random sequence which should have no effect on the cells and lipofectamine alone respectively. Results are expressed as fraction of untransfected cells (A) or as fold change of their own control (B). * represent significant changes to control cells and § represent significant changes to miR-34a mimic transfected cells. */§ P<0.05, **/§§ P<0.01, ***/§§§ P<0.005.

When expressed as fold change in LDH release into the media relative to respective controls (Figure 35B) both the transfected and untransfected cells showed significant increases in fold change of LDH in media in response to 1 and 1.5 µM Staurosporine (Mean fold change 2.4 and 2.5 for 1 µM respectively and 2.5 and 2.5 for 1.5 µM respectively). There were no significant differences between the transfected and untransfected cell groups.

Apoptosis was assessed in the 12 hour miR-34a mimic transfected cells exposed to Staurosporine for 24 hours through quantification of cleaved caspase 3 via western blot and Annexin V/PI staining and FACS analysis. There was a significantly greater expression of cleaved caspase 3 normalised to β-actin (Figure 36) in transfected cells exposed to 1 µM Staurosporine (Mean 0.75) but not between any other groups although there was larger amounts of cleaved caspase 3 in the cells exposed to Staurosporine than the control groups. There were no significant changes in the fraction of early or late apoptotic cells as determined by FACS whether the data was expressed as a fold change of transfected or untransfected relative controls (Figure 36) or not (data not shown).

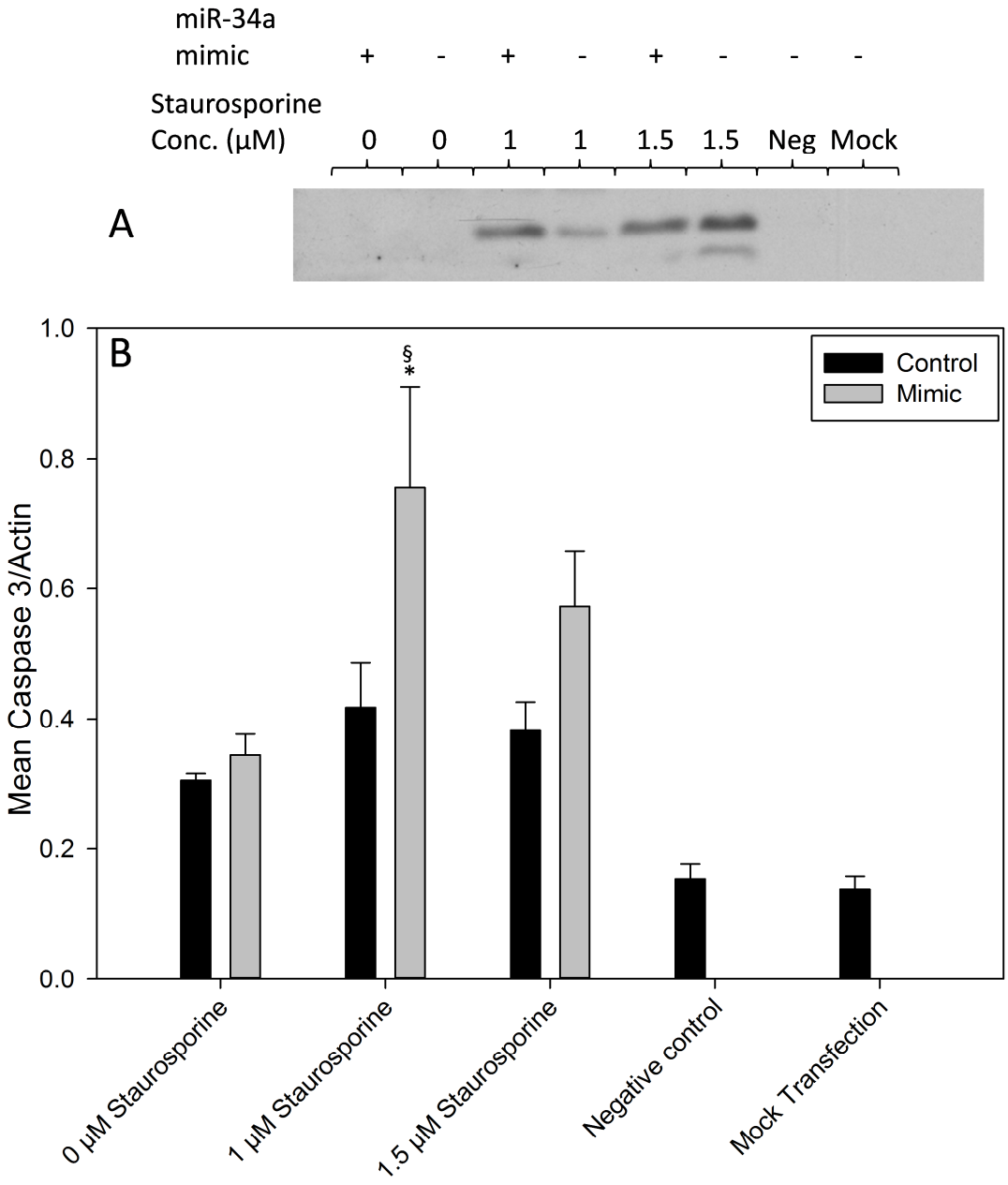

Figure 36:- Mean cleaved caspase 3 in Kim-1 expressing HEK293 cell lysates. Cells were either transfected with miR-34a mimic (grey bars) or not (black bars) for 12 hours followed by incubation with 1 µM or 1.5 µM Staurosporine for 24 hours. Negative control and mock transfection samples were transfected with a random sequence which should have no effect on the cells and lipofectamine alone respectively. Results are expressed as cleaved caspase 3 normalised to β-actin (B). Cleaved caspase 3 western bands are shown (A). * represent significant changes to control cells and § represent significant changes to miR-34a mimic transfected cells. */§ $P<0.05$.

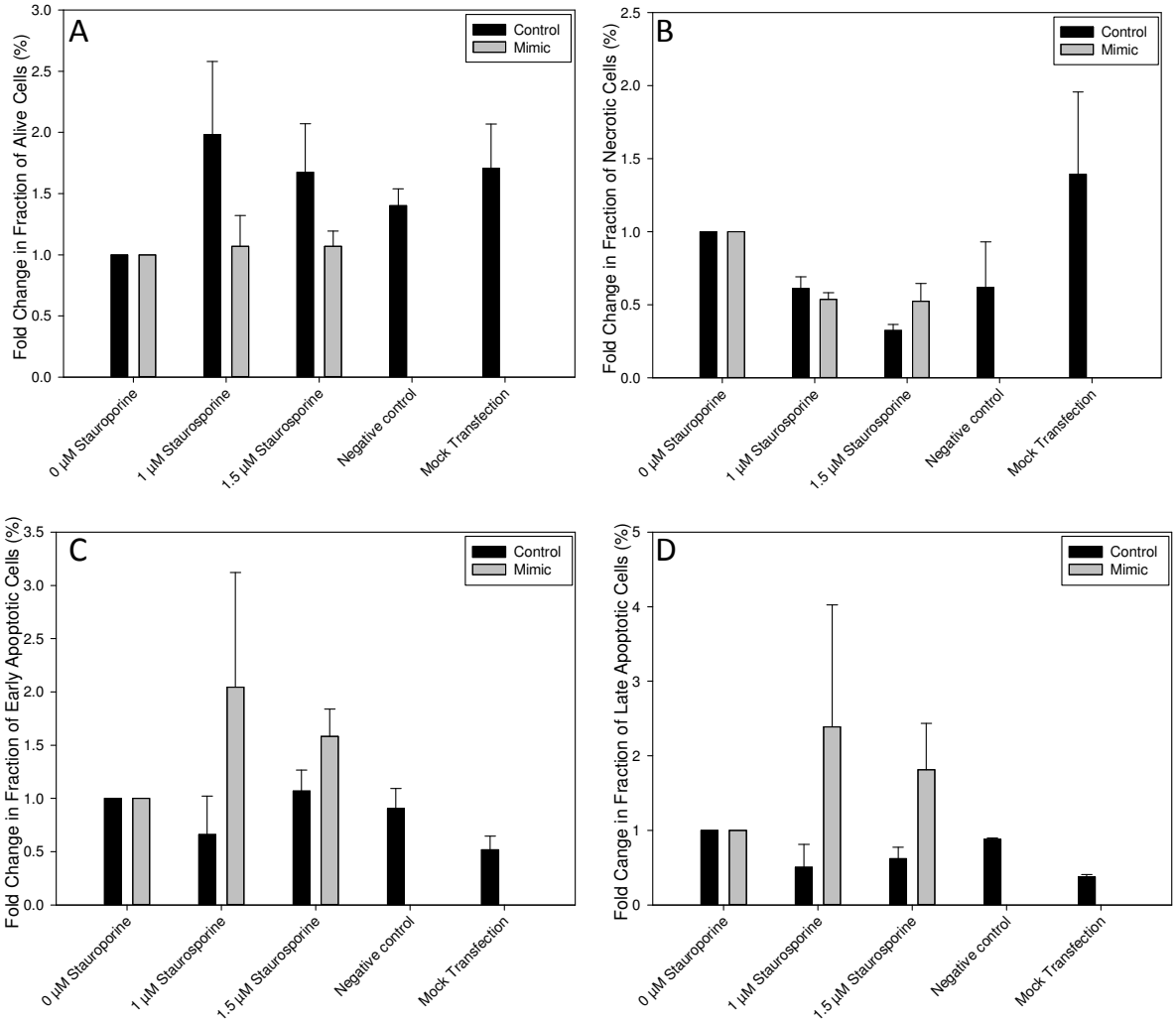

Figure 37:- Fate of Kim-1 expressing HEK293 cells expressed as fold change of respective controls as determined via Annexin V/PI staining and FACS analysis. Cells were either transfected with miR-34a mimic (grey bars) or not (black bars) for 12 hours followed by incubation with 1 µM or 1.5 µM Staurosporine for 24 hours. Negative control and mock transfection samples were transfected with a random sequence which should have no effect on the cells and lipofectamine alone respectively. Results are expressed as the fold change of fraction of cells which are alive (A), necrotic (B), early apoptotic (C) or late apoptotic (D). * represent significant changes to control cells and § represent significant changes to miR-34a mimic transfected cells.

4 - **Discussion**

Previous work in this thesis has shown that specific miRNA species are elevated in various models of kidney injury. This is in agreement with the work carried out by a number of different research groups who have also illustrated that miRNAs are elevated in many different types of organ damage as well as various disease states (Brase *et al.*, 2010; He *et al.*, 2004; Starkey Lewis *et al.*, 2011; Waldman *et al.*, 2008; Wang *et al.*, 2009). It is now widely accepted that miRNAs are released from dying cells and are present in various biological fluids such as serum or urine. It is however not known whether this process is largely due to the passive release of the contents of a cell, including miRNAs, upon loss of membrane integrity or rather that it is an active process where miRNAs are transported from cells in order to communicate situations of cellular stress to cells in their surrounding environment. It has been shown that miRNAs can be transferred from one cell to another by exocytosis of miRNA containing vesicles which can then be taken up by other cells by endocytosis. The miRNA taken up by a second cell has been shown to be functional (Valadi *et al.*, 2007). If it is the case that release of miRNAs from cells upon injury or stress has an active element then it is possible that the miRNAs released by one cell could impact the fate of surrounding cells in a tissue. Perhaps they could change expression of defensive proteins or to signal a cell to undergo apoptosis rather than succumbing to necrosis. This process can be modulated synthetically using miRNA mimics or inhibitors.

The hypothesis of this chapter was that modulation of miRNA species can represent a novel mechanism of altering a cells susceptibility to toxicity in vitro and may provide information on the function of miRNA species. The aims of this chapter were to characterise a human embryonic kidney cell line, which are readily transfectable, in terms of response to the

nephrotoxin, Cisplatin and an apoptosis inducing antibiotic, Staurosporine. Then to modulate the activity of a specific miRNA species and assess whether the susceptibility of the cell to toxicity could be altered in response to the two compounds.

4.1 - Characterisation of the toxic response of HEK293 cells to Cisplatin and Staurosporine

In order to characterise the response of the HEK293 cell line to the proximal tubular toxin, Cisplatin, the cell lines were incubated with varying concentrations of Cisplatin and for varying periods of time. HEK293 cells expressing the transmembrane protein, Kim-1 which is expressed in high levels in the proximal tubules, and those expressing an empty PcDNA vector were both used to examine the differences between the two cell lines and to determine which, if any, were more suited to this study. The toxic response was assessed through the MTS assay and the LDH assay. The MTS assay quantifies the conversion of a tetrazolium salt to a formazan product colourimetrically which is only possible in viable cells by dehydrogenase enzymes. Results of the MTS assay are expressed as viable cells as a fraction of control cells. The LDH assay is a cytotoxicity assay which quantifies colourimetrically LDH activity in the media surrounding cells. This is expressed as the fraction of total cell LDH in the media which reflects the degree of membrane damage. The MTS and LDH assay can both detect cell death but cannot distinguish between necrosis and apoptosis. The LDH assay also cannot detect early apoptosis as this does not lead to loss of membrane integrity.

After incubation with Cisplatin for 24 hours both the Kim-1 and PcDNA expressing cells had a dose dependent reduction in cell viability as determined by the MTS assay (Figure 1). There were no significant differences between the two cell lines although the Kim-1 expressing cells were found to have significant reductions in cell viability after exposure to 50 µM

Cisplatin whereas the PcDNA expressing cells did not until exposure to 200 µM. The LDH assay largely showed the same dose dependent response to Cisplatin in the Kim-1 expressing cells albeit with a higher degree of variation. The PcDNA expressing cells also responded in a dose dependent manner and a far greater fraction of LDH was released into the media than the Kim-1 expressing cells and the inter assay variation was extremely high as shown by the error bars (Figure 2B). This difference was most likely attributed to the fact that the PcDNA expressing cells do not adhere to plates as well as Kim-1 expressing cells allowing cells to be lost during washing so when expressed as a fraction of individual wells the data is skewed.

The two cell types were also incubated with increasing concentrations of Cisplatin for various periods of time and cytotoxicity assessed via MTS and LDH assays. Cisplatin reduced cell viability in a time and dose dependent manner in both cell lines with no significant reductions in cell viability occurring until incubation with Cisplatin for 16 hours and 24 hours (Figure 3, 5). The LDH assay showed that no significant increases in LDH in the media were observed until 24 hour incubation for both the Kim-1 and PcDNA expressing cells (Figure 4, 6). It is possible that the MTS assay was detecting cytotoxicity earlier than the LDH assay due to the presence of early apoptosis which is not detected by the LDH assay. Again the PcDNA expressing cells had a huge fraction of LDH in the media compared to the Kim-1 cells after 24 hours which was attributed to lack of adherence of the cells as previously described. Due to these difficulties it was decided that the PcDNA cells would be taken no further in future experiments.

Staurosporine was also incubated with the Kim-1 expressing HEK293 cells to provide a second compound which is known to induce apoptosis (Figure 7). Much like Cisplatin,

Staurosporine caused a dose dependent reduction in cell viability and increase in LDH in the media when incubated with the Kim-1 expressing cells for 24 hours. No significant cytotoxicity was evident at concentrations below 1.5 µM Staurosporine.

4.2 - Apoptotic response of Kim-1 expressing HEK293 cells to Cisplatin and Staurosporine

Apoptosis was assessed in the HEK293 cells through detection of cleaved caspase 3 via western blot and by Annexin V/PI staining of cells followed by FACS analysis. Caspase 3 is a member of the caspase family of proteins which are cysteine-dependent aspartate-directed proteases. They are also known as executioner proteins and are essential in apoptosis. Caspase 3 is synthesised as a pro-caspase which is cleaved by caspase 8 and 9 to active subunits which in turn cleave caspases 6 and 7 (Morley *et al.*, 2000). The presence of the two cleaved forms of caspase 3 indicates the activation of the caspase cascade and therefore apoptosis. Annexin V/PI staining is used to detect if cells are viable (Annexin and PI negative), necrotic (Annexin negative and PI positive), early apoptotic (Annexin positive and PI negative) or late apoptotic (Annexin and PI positive). Annexin V binds to phosphatidylserine which is present on the cell surface when a cell undergoes apoptosis. Propidium Iodide binds to DNA only when cell membrane integrity has been lost after necrosis or secondary apoptosis and is used to stain necrotic or secondary apoptotic cells.

Cisplatin caused a significant increase in cleaved caspase 3 at 100 µM but not any other concentrations indicating that cell death is due to apoptosis at lower concentrations of Cisplatin and a mixture of both necrosis and apoptosis at greater concentrations as indicated by the MTS and LDH assays (Figure 8). Unlike Cisplatin, Staurosporine caused a dose dependent increase in cleaved caspase 3 at concentrations of 1 and 1.5 µM Staurosporine which is in agreement with the MTS and LDH data which suggests that the

majority of cell death as a result of Staurosporine is a result of apoptosis. The presence of cleaved caspase 3 was confirmed through the incubation of Cisplatin and Staurosporine with the pan caspase inhibitor Z-VAD-FMK. Z-VAD-FMK irreversibly binds to the catalytic site of caspases preventing their cleavage and therefore the caspase cascade (Figure 9). FACS analysis of cells incubated with Cisplatin agreed with previous analysis in that lower concentrations of Cisplatin caused a significant increase in early apoptosis and larger concentrations caused significant increases in late (secondary) apoptosis (Figure 10). Again, this was confirmed with the addition of Z-VAD-FMK which abolished increases in early and late apoptosis (Figure 11). No significant elevations in apoptosis determined by FACS were observed after incubation with Staurosporine although the fraction of early apoptotic cells was reduced after incubation with Z-VAD-FMK (Figure 12).

4.3 - Determination of baseline miRNAs in Kim-1 expressing HEK293 cells

The levels of miR-1, miR-26b, miR-30b, miR-30c, miR-34a and miR-192 were quantified in lysates of the HEK293 cells to determine if the cell line expressed all the kidney related miRNA species which had been investigated in this thesis (Figure 13). Kim-1 expressing HEK293 cells were shown to express all miRNAs investigated in this study at various levels, miR-1 being the lowest and the miR-30 family being the most abundant. The same pattern was also observed in a THP-1 cell line (Figure 14).

4.4 - Validation of transfection of Kim-1 expressing HEK293 cells with miR-34a mimic or inhibitor

MiR-34a was chosen as the miRNA species to be investigated in this study because it had a set of well-defined target mRNAs, it had been widely investigated by other research groups and had been shown to play a role in apoptosis regulation endogenously. These properties

made miR-34a a good candidate for modulation of activity in vitro as modulation of the miRNA should alter a cells apoptotic response and change the expression of quantifiable proteins (He *et al.*, 2007; Welch *et al.*, 2007; Yamakuchi *et al.*, 2008).

Kim-1 expressing HEK293 cells were transfected with an inhibitor of miR-34a for various periods of time followed by quantification of miR-34a in the cell lysates (Figure 15). There were no significant differences in miR-34a levels when normalised to miR-192 and total RNA. This was not definite evidence that the transfection was ineffective as the inhibitor would not necessarily lower levels of intracellular miR-34a as it works by binding to the mature sequences. However analysis of cell viability and apoptotic response in control and Cisplatin dosed cells also yielded no significant differences in the transfected cells compared to control cells (Figure 16).

A similar time course study was carried out with a miR-34a mimic as it is likely that a mimic would have a greater effect than an inhibitor as the inhibitor effect would become saturated quickly whereas a mimic is the addition of more functional miR-34a molecules so is not as easily saturated. Significant increases in intracellular miR-34a were observed at six, 12, 18 and 72 hour transfection with the mimic and remained significant when normalised to total RNA (figure 17) suggesting that the mimic was successfully entering the cells. The use of the mock transfection confirms that the intracellular miR-34a was being quantified rather than miR-34a in the media. To further confirm that the transfection was having an effect and to determine the most appropriate time and concentration of the transfection the cells were transfected with increasing concentrations of mimic for increasing periods of time. The mimic produced significant elevations in cleaved caspase and decreases in cell viability at 48 hours with 10 nM mimic producing the most dramatic effects (Figure 18, 19). Transfections

of 10 nM mimic for 48 hours were used as the basis for future experiments. Quantification of Bcl-2 and CDK6 which have been shown to be targets of miR-34a (He *et al.*, 2007; Welch *et al.*, 2007; Yamakuchi *et al.*, 2008) further reinforce the success of the transfection as the expression of the proteins were significantly reduced in cells transfected with the miR-34a mimic (Figure 20). Quantification of Bcl-2 and CDK6 mRNA in transfected cells did not agree with the reduced protein levels but the mimic would not necessarily target the mRNA for degradation but rather inhibit translation of the protein so this could be expected.

4.5 - Transfection of Kim-1 expressing HEK293 cells with miR-34a mimic followed by exposure to Cisplatin or Staurosporine

Cells were transfected with 10 nM miR-34a mimic for 48 hours as previously described and then exposed to 0, 50 or 100 μM Cisplatin. This was to assess whether the miR-34a mimic was capable of altering susceptibility to toxicity or apoptosis. The mimic transfected cells had significantly reduced cell viability compared to untransfected cells when exposed to 0, 50 or 100 μM Cisplatin. This suggests that the mimic increases the cells susceptibility to Cisplatin induced cell death however the difference between the mimic transfected and untransfected cells casts uncertainty on the significance of this change. Results were expressed as fold change of respective controls to try and negate this difference (Figure 22) and significant differences between the two dose groups were still observed. A similar trend was observed in the LDH assay (Figure 23) where the mimic dosed cells had greater amounts of LDH in the media than untransfected cells although the change was not significant when expressed as fold changes. Importantly there were significantly greater amounts of cleaved caspase 3 at 50 μM Cisplatin in the transfected cells compared to the untransfected cells. FACS analysis largely agreed with the caspase 3 data in that there were significantly greater

amounts of late apoptotic cells in the transfected cells than in untransfected cells (Figure 24). There were also greater fractions of early apoptotic cells after 50 and 100 μM Cisplatin in transfected cells than untransfected cells (Figure 25, 26). Exposure to Staurosporine lead to similar results as Cisplatin in that cell viability was significantly reduced in the transfected cells compared to untransfected cells suggesting the mimic is causing cells to be more susceptible to death. This is also shown in the LDH assay however when expressed as fold changes the differences become less prominent (Figure 28). The results from the caspase 3 westerns suggest that, like with Cisplatin, that the mimic transfected cells are more susceptible to apoptosis than untransfected cells (Figure 29) as the expression of cleaved caspase 3 is significantly greater in the transfected cells. In the case of Staurosporine this was not confirmed through FACS analysis as like the Staurosporine dose responses described earlier, no significant changes were observed.

Although both Cisplatin and Staurosporine induced apoptosis at lower concentrations when the miR-34a mimic was present the fact that there were often differences between transfected and untransfected cells which were not exposed to either compound cast doubt over the significance of the changes. In an attempt to negate or reduce the differences between both controls the transfection time was reduced to 12 hours as opposed to 48 hours. Again transfected cells had significantly greater reductions in cell viability compared to the untransfected cells and no significant changes between the controls (Figure 30). LDH data largely shared the same trend with transfected cells having larger fractions of LDH in the media compared to transfected cells when exposed to Cisplatin (Figure 31). Cleaved caspase 3 expression was also greater in the transfected cells after exposure to Cisplatin, significantly so after exposure to 100 μM Cisplatin and significant exposure in cleaved

caspase 3 was observed at 50 µM Cisplatin whereas it wasn't in the untransfected cells (Figure 32). The FACS analysis agreed with the caspase 3 western data in that there were greater fractions of early and late apoptotic cells in the transfected cells exposed to Cisplatin although the differences were not significant (Figure 33). Exposure to Staurosporine largely showed the same trend as Cisplatin. The mimic transfected cells had lower cell viability in the transfected cells than the untransfected ones although not significantly so. LDH was significantly elevated in the media of transfected cells exposed to Stauroporine however this seems to be largely a result of the larger baseline of LDH in the media of the transfected cells as the significance is abolished when expressed as a fraction of their respective controls (Figure 35). Cleaved caspase 3 expression was elevated in the transfected cells compared to the untransfected cells and the FACS analysis showed elevations in early and late apoptosis in transfected cells when expressed as fraction of respective controls however not significantly so. Again, like all previous FACS analysis after exposure to Staurosporine there were no elevations in apoptotic cells in untransfected cells exposed to Staurosporine.

The results from the 48 hour and 12 hour miR-34a mimic transfections show that the overexpression of miR-34a in Kim-1 expressing HEK293 cells increases susceptibility of the cells to undergo apoptosis. The MTS assay and the LDH assay both show that there is a reduction in cell viability in response to both Cisplatin and Staurosporine which is dose dependent. This reduction is greater in the mimic transfected cells, often significantly so. The use of cleaved caspase 3 westerns and Annexin V/PI FACS analysis confirm that this reduction in cell viability is a result of apoptosis. The Caspase 3 westerns illustrate the differences the mimic makes to the expression of Caspase3 in the lower doses of Cisplatin and Staurosporine where cleaved caspase 3 often was not visible in the absence of the

mimic (Figure 24, 29, 32). As a whole these data show that a miRNA can be modulated in vitro and this modulation of a specific miRNA species can affect the susceptibility of a cell to apoptosis. No significant changes were observed when using a miRNA inhibitor but this could be a result of many different miRNA species affecting the same mRNA transcript so compensation of the inhibition may occur. If inhibition was required to achieve a desired affect a number of miRNA species may have to be inhibited simultaneously to achieve significant effects. The results from the 12 hour transfections with the miR-34a mimic did reduce differences between control transfected and untransfected although there were still differences. These differences are not only likely to be due to the induction of apoptosis as a result of the increased levels of miR-34a mimic intracellularly but also due to the fact that the miR-34 family also have antiproliferative effects (He *et al.*, 2007; Welch *et al.*, 2007; Yamakuchi *et al.*, 2008). It may be possible to negate these differences while still observing alterations when exposed to toxins through alteration of concentration of mimic and time of exposure. Now that it has been shown that modulation of miRNA activity in vitro can alter susceptibility to toxicity it would be interesting to investigate whether this affect can be observed in vivo. It may be possible that through modulation of miRNA activity the susceptibility to nephrotoxins could be reduced which may be able to improve the utility of pharmacologically efficacious nephrotoxins.

Chapter 6

Concluding Discussion

Contents

1 - Introduction

ADRs are not only a large financial burden to society and a serious issue to patients but also one of the leading reasons for the withdrawal of drugs from the market and attrition from drug development. They have been shown to be responsible for up to five % of hospital admissions, can occur in 20 % of inpatients and can increase the length of stay in hospitals. These factors all decrease the patient's quality of life and increase the financial burden of patients to society (Park *et al.*, 2005; Park *et al.*, 1998; Pirmohamed *et al.*, 2004). AKI is also a major health issue, particularly so in the western world where it is responsible for complicating up to seven % of all admissions and up to 25 % of all intensive care unit admissions. The mortality rate for AKI has been difficult to predict over the past decades due to the lack of a universally accepted definition of AKI however it is hypothesised to be between 50 and 70 % in adults and approximately 30 % in infants. Over recent years a number of initiatives have been created to define AKI allowing more accurate diagnosis, prognosis and treatment options. Drug induced AKI is widely accepted to be responsible for as many as 60 % of cases of AKI which are presented in hospitals (Chertow *et al.*, 2005a; Ferguson *et al.*, 2008; Ricci *et al.*, 2008). The risk factors for nephrotoxicity induced kidney injury include age, sex, racial and genetic differences although increasing age has the largest influence on susceptibility. The aforementioned factors make kidney injury, particularly acute injury induced by a nephrotoxic agent an extremely important event, not only clinically but also throughout drug development. In order to detect kidney injury in clinical and preclinical scenarios a number of biomarkers are required which can sensitively and specifically detect the injury.

Historically and also to a large extent to this day there are only small group of measurements which have been used as markers of kidney injury. The most widely used and "gold standards" of these markers are BUN and SCr however it is accepted that they are suboptimal biomarkers of kidney injury. Issues with SCr include the fact that changes in SCr are dependent of GFR, secretion of creatinine by tubules, its rate of synthesis and its volume of distribution. BUN also suffers similar issues due to the fact that increases in urea production and reabsorption of urea from the nephron can influence its levels in the blood. The lack of sensitivity of these markers not only affects the timely diagnosis of kidney injury but also affects the development of newer therapies and may influence the outcomes of clinical trials (Bonventre *et al.*, 2010; Ferguson *et al.*, 2008; Vaidya *et al.*, 2010; Vakkila *et al.*, 2004; Welch *et al.*, 2007). Although there are a number of newer candidate biomarkers of kidney injury which show greater sensitivity than SCr or BUN in detecting kidney injury they often have their own issues which make their use less than ideal. These include the fact that many require the use of patented antibodies making them expensive and often species specific, they are often present at very low abundances and may also suffer from high rates of degradation. There is still a great need for markers of kidney injury which are highly specific, sensitive, translatable between preclinical and clinical species and which could also ideally provide information in the location or mechanism of the injury. MiRNAs are short RNA sequences which physiologically serve to negatively regulate gene expression. In recent years there has been an increasing body of research which has suggested the potential of various miRNA species to serve as biomarkers of specific conditions. The majority of this research has focussed of miRNA expression in various cancers although a few groups have focussed on the potential of miR-122 to serve as a sensitive biomarker of liver injury. MiRNAs are promising candidate biomarkers due to the fact that they are expressed

ubiquitously throughout the body but with different tissue specific expression profiles and they are known to be highly conserved throughout evolution so the same techniques to quantify them can be used across species. This thesis set out to investigate the potential of miRNAs to be used as more sensitive biomarkers of kidney injury than current "gold standards".

2 - Validation of the techniques used to isolate, purify and quantify miRNA species from biological samples

MiRNAs are a relatively recent discovery, with the first miRNA being described just under two decades ago (Yang *et al.*, 2009a). In the immediate time after their discovery the most widely used method of detecting miRNA species was through northern blotting which provided information on the size and relative abundance of a miRNA species. Northern blotting also had its drawbacks in that it was only semi-quantitative, was low throughput and was not particularly sensitive. Since then qPCR techniques have been developed which allow the quantification of individual miRNA species with high specificity and sensitivity. This method relies on the isolation of miRNA species from various biological matrices, followed by reverse transcription of specific miRNA species to cDNA sequences and finally qPCR to quantify the miRNA. These techniques were still in their relative infancy at the beginning of this thesis and were becoming more widely used by many research groups, including groups in this department. For this reason it was appropriate to evaluate and validate the commercially available kits and techniques used to isolate, purify and quantify miRNA species from various biofluids as well as investigations into the stability of these miRNAs when exposed to conditions which they may routinely be in a laboratory. This would provide information on the limitations of the techniques and allow the application of appropriate

measures to ensure the most efficient, accurate and robust methods to isolate, purify, handle and quantify various miRNA species. This thesis showed that the precision of the qPCR procedure in terms of intra and inter-assay variation was less than 15 % which is acceptable in the FDA guidelines for biomarker validation. The qPCR technique was actually found to show coefficients of variation of less than 10 % for all concentrations greater than a fM range which suggests the qPCR technique is highly precise and "fit for purpose". It was also of the uttermost importance that that the techniques used to isolate miRNA species from various biofluids were able to robustly recover consistent amounts of miRNAs. It was shown that this was not the case and it was decided that in order to take this inconsistency into account the development of an internal standard was necessary. The internal standard was chosen to be C.elegans Lin-4 which is known not to be present in mammalian species under physiological conditions. Synthetic miRNA species were shown to be extremely stable when stored in RNase free water and exposed to multiple freeze-thaw cycles or stored refrigerated for a number of weeks. They were also quantifiable after addition to denatured serum or urine, however when a synthetic miRNA species was spiked into a undenatured serum or urine it was not quantifiable at expected levels which was attributed to rapid degradation by endonuclease activity in the biofluid. This raises some interesting questions on how endogenous miRNA species are able to be detected in the serum or urine while synthetic ones are not. Some miRNA species are known to have unprotected 3' and 5' ends which allow them to be targeted by endonucleases while others do not. This suggests that some miRNAs can be degraded in matrices with endonuclease activity while others cannot. It is likely that synthetic miRNAs have unprotected ends so are rapidly degraded (Bail et al. 2010).

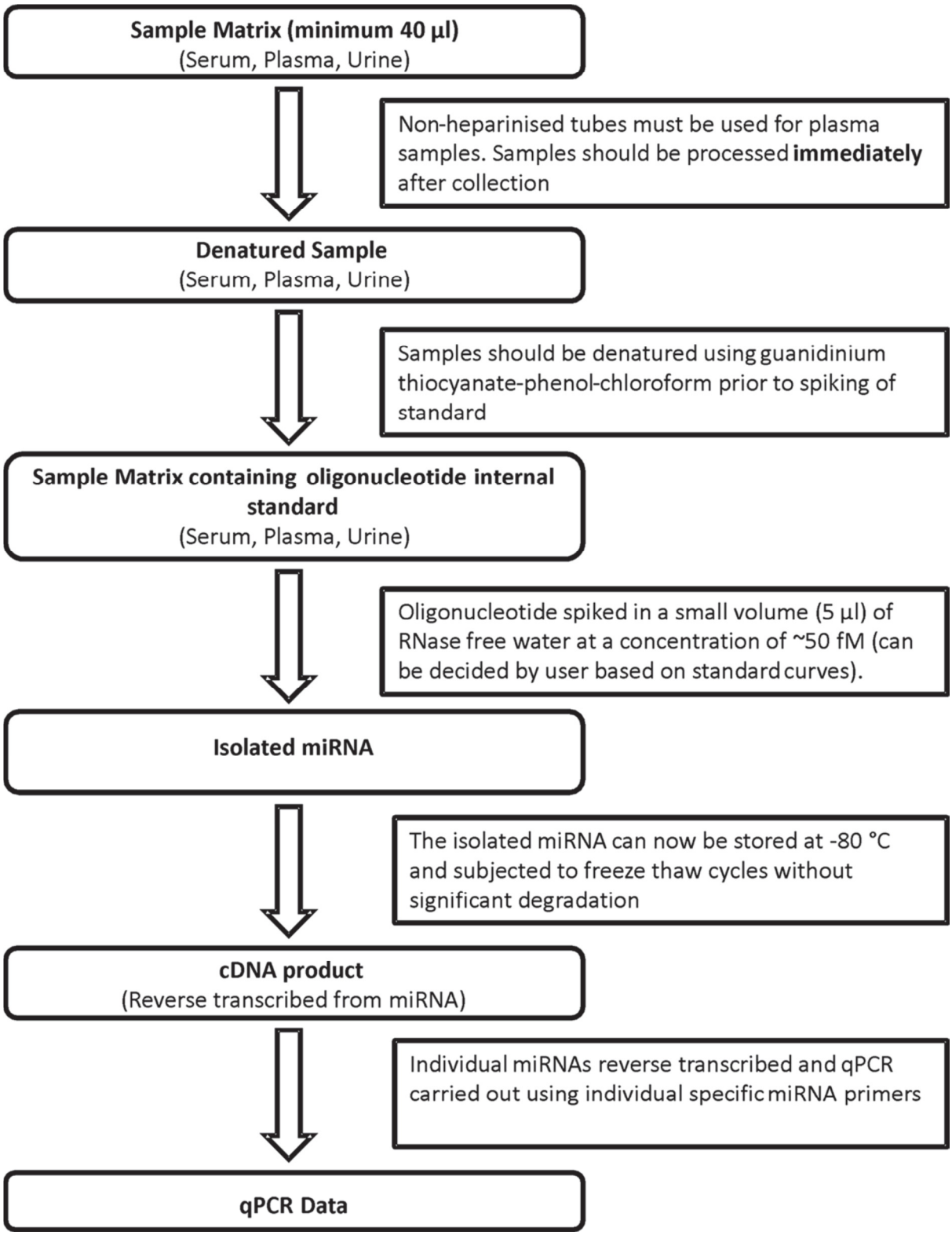

Figure 1:- Overview of validated methodology and protocol for the isolation and quantification of miRNAs from biofluids for their use as biomarkers of organ injury.

Conversely some human miRNA species have been reported to bind to proteins *in vivo* which actually increase their stability. One such miRNA species is the liver specific miR-122 which has been shown to be bound by translin *in vivo* increasing its stability. This, along with its sheer abundance may be why miR-122 represents such a promising biomarker of DILI (Yu & Hecht 2008).

Indeed it is known that miRNAs are found in exosomes but the sheer amount of miRNAs which can be detected in the serum suggests that they cannot solely be found in exosomes. Perhaps the post-translational modification is a scenario which is more likely as this would allow free circulating miRNAs to be protected from endonuclease activity after necrosis for example. Previous studies have shown that endogenous miRNAs are incredibly stable in serum. The research in this thesis is in agreement with these findings and it has also been shown that miRNAs can be detected in, and are incredibly stable in urine. Loss in stability was found after multiple freeze thaw cycles of urine prior to isolation of miRNAs but not after freeze-thaw cycles of isolated miRNAs in RNase free water. This body of work showed that translational endogenous miRNAs reflective of renal injury represent stable analytes with a large dynamic range that can be quantified easily in most laboratories by validated qPCR technologies. The data presented from this investigation go some way to help define best practice parameters for the most accurate method for the quantification of these analytes and this method was used throughout this thesis (Figure 1).

3 - MicroRNAs as potential biomarkers of kidney injury in vivo

As previously discussed the current "gold standard" biomarkers of kidney injury are suboptimal in terms of sensitivity and specificity (Ferguson *et al.*, 2008). MiRNAs have shown potential as biomarkers of DILI and in various forms of cancer however there was not a large amount of literature at the beginning of this thesis regarding miRNAs as biomarkers of kidney injury. Reproduction of a published method of APAP induced liver injury and subsequent quantification of serum miR-122 using the workflow described previously confirmed the utility of the validated methods. In order to test the hypothesis that miRNA species had the potential to be used as biomarkers of kidney injury four kidney enriched miRNA species were quantified in the urine of an in vivo model of kidney ischemic reperfusion. This method was chosen to serve as a global model of acute kidney injury. The four miRNA species quantified were chosen based on literature reports of their kidney enriched expression profiles when compared to other organs. Ideally, a kidney specific miRNA species would have been chosen, much like miR-122 in the liver, however a kidney specific miRNA does not exist to our knowledge. The utility of a kidney enriched miRNA as a biomarker of kidney injury may present problems with specificity given the fact that it would also be expressed in other tissues, similar to those observed with SCr or BUN. To go some way to answering this question of specificity the four kidney enriched miRNA species and the liver specific miR-122 were also quantified in the serum of APAP administered mice. Only the liver specific miR-122 and the highly liver enriched miR-192 were significantly elevated in the serum which suggests a degree of kidney specificity of the remaining three miRNAs.

Each of the four miRNA species quantified and urinary Kim-1 showed significant increases in the urine of the mice as early as three hours post 10 minute IR injury whereas SCr was not significantly elevated until nine hours post injury. The fact that each of the four miRNA species all showed the same trend of an initial increase in the urine post injury followed by a gradual decrease back towards baseline levels provides insight into the method in which the miRNAs are entering the urine. The fact that all four show the same trend, albeit at different amounts in the urine suggests that their release is likely to be largely a result of passive loss from necrotic/late apoptotic cells rather than active release from viable cells in response to stress. The fact that each miRNA quantified was present in the urine at varying levels is most likely a direct result of its expressional abundance in the kidney tissue itself rather than a reflection of any response to stress of toxicity for example. However this is speculation and there is no direct evidence to support this hypothesis.

Importantly the same trends were observed in the miRNA data whether it was normalised to the endogenous U6snRNA or the exogenous internal standard giving the results more confidence. Urinary Kim-1 is now accepted as a sensitive biomarker of kidney injury and it was elevated as early as three hours post IR injury. This suggests that miRNA are at least as sensitive as urinary Kim-1 in the detection of IR induced kidney injury. The data from this particular study cannot determine which the most sensitive marker is as the first measurement taken was at three hours post IR. It cannot also be determined whether changes in miRNA levels in the urine precede histological changes in the kidney for similar reasons; histological changes were observed as early as three hours post injury. Further investigation on the sensitivity of these miRNAs as urinary markers of kidney injury in relation to urinary Kim-1 and histological changes is necessary and would provide further

insight into the utility of miRNAs as biomarkers of kidney injury. The promising nature of the results from the IR model and the fact that IR injury affected the whole kidney globally prompted the development of a more subtle, location specific model of kidney injury. Cisplatin was used due to its well characterised nephrotoxic affects. BUN was significantly elevated 96 hours post Cisplatin administration whereas urinary Kim-1 and NAG were at 72 hours; further illustrating the lack of sensitivity of BUN. Excitingly, 18 miRNAs were significantly elevated by greater than two fold in the urine of the Cisplatin dosed mice 48 hours post administration. These included miR-26b, 30b and 30c which were quantified in the IR model. This not only confirms that these miRNAs are elevated in the urine after kidney injury but also suggests that urinary miRNAs are significantly elevated in the urine at an earlier time point than urinary Kim-1 and NAG in a model of Cisplatin induced kidney injury. It should also be noted that miR-30b was significantly reduced in the kidney of Cisplatin administered mice providing direct evidence that loss of a miRNA from the kidney tissue can lead to increases in the urine.

4 - MiRNA profiling of Cisplatin or Adriamycin Dosed Mice

As touched on previously there are no known kidney specific miRNA species which suggests that any miRNAs which show promise as biomarkers of kidney injury may also show elevations when damage to other tissues expressing the same miRNA occurs, influencing their specificity. In order for a non-tissue specific miRNA to have value as a biomarker of kidney injury it must show greater sensitivity than current gold standard markers or provide information which current markers cannot. Data from the IR and the Cisplatin in vivo models show that miRNAs are not only significantly elevated post kidney injury but also are found to be significantly elevated earlier than markers such as BUN and urinary Kim-1. This, in itself is

an interesting finding, but if it could be shown that certain miRNA species may be able to differentiate between specific sites of damage to the kidney then they may truly provide an exciting novel class of biomarker. In order to test this hypothesis miRNAs were profiled in a Cisplatin model of kidney injury as well as in an Adriamycin model of kidney injury. There were a total of nine significantly altered by greater than twofold kidney, 28 serum and 16 urine miRNA species between the two models of DIKI. It is important to note that these were the miRNA species which were altered as a result of the two compounds only; this does not include miRNAs which are elevated between control animal groups, between Cisplatin or between Adriamycin and their respective controls. This criteria was used to negate any strain differences and any miRNA species which were altered in response to either compound only. This study indicates that the urinary and serum miRNA expression profile after a proximal tubule toxin is different to that of a podocyte specific toxin. It highlights a number of potential target miRNA species which could be further investigated as markers of glomerular or proximal tubule injury. A number of these serum and urinary miRNA species are altered by fold changes of greater than 20 fold and present potential novel markers location specific damage. It should also be noted that it is possible that these differences do not reflect only differences in the location of injury in the kidney but may also be a result of other differences between the two models of KI. The fact that the Adriamycin model is a chronic model while the Cisplatin model is an acute model may play a role in the miRNA expression profiles. Indeed, it is interesting that many of the significantly altered miRNA species in response to Cisplatin are predicted to be involved in epithelial cell repair, the apoptotic response, angiogenesis and proliferation, many of which are known to be highly expressed in the kidney. Conversely many of the Adriamycin altered species seem to be highly expressed in the embryo or the embryonic kidney with many having unconfirmed

targets. Interestingly, only one miRNA species was commonly elevated by greater than twofold in the kidney tissue of the Cisplatin and Adriamycin administered mice; miR-34a. This provided an interesting candidate miRNA for further investigation as the miR-34 family has been shown to be involved in the apoptotic response and cell survival.

5 - The role of miRNAs as regulators of susceptibility to DIKI

The significant over expression of miR-34a in the kidney tissue of both the Cisplatin and Adriamycin dosed mice by greater than twofold lead to research into the potential intracellular roles of the miR-34 family. The miR-34 family have been relatively widely studied and it is known that they play roles in cell survival, apoptosis and have been implicated in the development of various cancers (Cannell *et al.*, 2010a; Cannell *et al.*, 2010b; He *et al.*, 2009). They are known to be directly induced in situations of cellular stress or DNA damage which would explain why they were overexpressed in the kidney tissue of mice exposed to various nephrotoxins. All members of the miR-34 family have been shown to have antiproliferative effects but miR-34a in particular has been shown to promote apoptosis in addition to its antiproliferative effects through targeting such transcripts as CDK6, Bcl-2 and cyclin E2. For the aforementioned reasons miR-34a was chosen to be investigated as a potential modulator of susceptibility to nephrotoxins and other apoptosis inducing agents with the hypothesis that modulation of miRNA species can influence a cells susceptibility to toxicity in mind. Cisplatin was shown to cause apoptosis in vitro, which agreed with much research which had been carried out previously by other groups. Cell death was largely due to apoptosis at lower concentrations of Cisplatin (50 – 100 μM) with larger proportions of the cell death a result of necrosis or secondary apoptosis at higher concentrations (> 250 μM). This study was not able to confirm any effect on the cell lines of

an inhibitor to miR-34a; at the protein level, physiologically or in response to Cisplatin. This is perhaps surprising given what is known about the physiological role of miR-34a. It may be expected that the inhibition of miR-34a would cause an increase in its target proteins, Bcl-2 or CDK6 at the protein level, however none were observed. This was decided to be either a result of inefficient transfection which seemed unlikely given the previous work which had been carried out to validate the transfection protocol, or as a result of the function of miRNAs. The latter of which is more likely the case as it is known that miRNAs can target hundreds of different mRNA transcripts so it is possible that the effect inhibition of one miRNA species had could readily be compensated for by the effects of other miRNA species which target the same mRNA transcripts. For this reason it was decided that a more effective approach was to use a mimic of miR-34a rather than an inhibitor. This would not only have more of an effect on the mRNA targeted by miR-34a due to the lower saturability of the effect of the mimic compared to the inhibitor but also may induce cell death which would be easier to quantify than cell survival. Successful transfection with the miR-34a mimic was confirmed via quantification of Bcl-2 mRNA by qPCR and by quantification of Bcl-2 and CDK6 protein via western blot. The miR-34a mimic was shown to increase apoptosis in vitro via quantification of cleaved caspase 3 and FACS analysis which support literature reports of its effect. MiRNA-34a transfection was also shown to induce significantly greater fractions of cell death in cells treated with Cisplatin than in those treated with Cisplatin without transfection with the mimic. The cell death was shown to be a result of increased apoptosis rather than necrosis through quantification of cleaved caspase 3 and Annexin V/PI FACS analysis. These data show that susceptibility to a nephrotoxic agent can be altered through modulation of the activity of a single miRNA species in vitro. In this case the susceptibility to toxicity has been increased which may not have therapeutic implications in

itself however it has shown that miRNAs influence susceptibility to toxicity. It would be interesting to see whether this effect could be replicated in vivo however it may be that the complexity of signals from tissues and surrounding cells in an in vivo model may prevent such an approach being successful. If successful however, the potential for therapeutic approaches based of modulation of miRNA expression could be vast. In this case it is plausible that modulation of miR-34a could protect against nephrotoxicity and the cell death and resultant immune response could be reduced by modulation of miRNA expression/activity.

6 - Final Comments

In this thesis the following aims and hypotheses have been addressed:

The commercially available techniques to isolate purify and quantify miRNA species from various biological matrices are sensitive, have high precision and miRNAs are stable once isolated.

- The Taqman based qPCR techniques used to quantify miRNA species were shown to be highly precise in terms of intra and inter-assay variation. Fractional recovery from miRNA isolation kits was shown to be variable so a method for the addition of an internal standard was developed. MiRNA species were shown to be stable in RNase free water over a range of conditions after isolation from urine.

MiRNAs which are enriched in the kidney have utility as urinary biomarkers of kidney injury in vivo.

- MiR-26b, 30b, 30c and 192 were all signficantly elevated in the urine of mice exposed to 10 minutes of kidney ischemia followed by reperfusion. This occurred three hours post IR whereas SCr was not significantly elevated until nine hours post IR. MiR-192 was found to be significanrtly elevated in the serum of an in vivo model of APAP hepatotoxicity while miR-26b, 30b and 30c were not indicating a degree of kidney specificity.

Profiling miRNA expression in the urine, serum and kidney tissue of a Cisplatin model of nephrotoxicity provide a number of miRNAs which have potential as biomarkers of DIKI.

- BUN was significantly elevated in vivo 96 hours while urinary NAG and Kim-1 were elevated 72 hours post Cisplatin (20 mg/kg) administration. 18 miRNA species were found to be significantly elevated by greater than twofold in the urine of Cisplatin (20 mg/kg) administered mice at 48 hours. These included miR-26b, 30b and 30c.

Comparing miRNA expression profiles of Cisplatin or Adriamycin dosed mice yields a number of miRNAs which provide information on the location of kidney injury.

- Nine, 28 and 16 miRNA species were expressed at signifcantly different quantities in the kidney, serum and urine respectively of Cisplatin (20 mg/kg) or Adriamycin (6.5 mg/kg) administered mice. Both the Cisplatin and Adriamycin Administered mice had significantly elevated levels of miR-34a in the kidney tissue compared to their respective controls.

Modulation of miRNA activity can influence susceptibility to toxicity in vitro.

- Moudulation of miR-34a activity using a mimic for miR-34a significantly increased the apoptotic response to Cisplatin when compared to cells with normal miR-34a activity. This was confirmed with western blot analysis of cleaved caspase 3 and Annexin V/PI FACS analysis.

Bibliography

Amacher DE (2002). A toxicologist's guide to biomarkers of hepatic response. *Hum Exp Toxicol* **21**(5)**:** 253-262.

Antoine DJ, Srivastava A, Pirmohamed M, Park BK (2010). Statins inhibit aminoglycoside accumulation and cytotoxicity to renal proximal tubule cells. *Biochem Pharmacol* **79**(4)**:** 647-654.

Antoine DJ, Williams DP, Park BK (2008). Understanding the role of reactive metabolites in drug-induced hepatotoxicity: state of the science. *Expert Opin Drug Metab Toxicol* **4**(11)**:** 1415-1427.

Aqeilan RI, Calin GA, Croce CM (2010). miR-15a and miR-16-1 in cancer: discovery, function and future perspectives. *Cell death and differentiation* **17**(2)**:** 215-220.

Ayla S, Seckin I, Tanriverdi G, Cengiz M, Eser M, Soner B*, et al.* (2011). Doxorubicin induced nephrotoxicity: protective effect of nicotinamide. *International journal of cell biology* **2011**.

Bartel DP (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**(2)**:** 281-297.

Bartel DP (2009). MicroRNAs: target recognition and regulatory functions. *Cell* **136**(2)**:** 215-233.

Bernard AM, Vyskocil A, Mahieu P, Lauwerys R (1987). Assessment of urinary retinol-binding protein as an index of proximal tubular injury. *Clinical chemistry* **33**(6)**:** 775-779.

Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**(6818)**:** 363-366.

Billy E, Brondani V, Zhang H, Müller U, Filipowicz W (2001). Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proceedings of the National Academy of Sciences* **98**(25)**:** 14428-14433.

Blowey DL (2005). Nephrotoxicity of over-the-counter analgesics, natural medicines, and illicit drugs. *Adolesc Med Clin* **16**(1)**:** 31-43.

Bonci D, Coppola V, Musumeci M, Addario A, Giuffrida R, Memeo L*, et al.* (2008). The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. *Nature medicine* **14**(11)**:** 1271-1277.

Bonventre JV, Vaidya VS, Schmouder R, Feig P, Dieterle F (2010). Next-generation biomarkers for detecting kidney toxicity. *Nat Biotechnol* **28**(5)**:** 436-440.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* **72:** 248-254.

Brase JC, Wuttig D, Kuner R, Sultmann H (2010). Serum microRNAs as non-invasive biomarkers for cancer. *Mol Cancer* **9:** 306.

Broker LE, Kruyt FA, Giaccone G (2005). Cell death independent of caspases: a review. *Clin Cancer Res* **11**(9)**:** 3155-3162.

Cannell IG, Bushell M (2010a). Regulation of Myc by miR-34c: A mechanism to prevent genomic instability? *Cell Cycle* **9**(14)**:** 2726-2730.

Cannell IG, Kong YW, Johnston SJ, Chen ML, Collins HM, Dobbyn HC*, et al.* (2010b). p38 MAPK/MK2-mediated induction of miR-34c following DNA damage prevents Myc-dependent DNA replication. *Proc Natl Acad Sci U S A* **107**(12)**:** 5375-5380.

Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K*, et al.* (2008). Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res* **18**(10)**:** 997-1006.

Chen XM (2009). MicroRNA signatures in liver diseases. *World J Gastroenterol* **15**(14)**:** 1665-1672.

Chertow GM, Burdick E, Honour M, Bonventre JV, Bates DW (2005a). Acute kidney injury, mortality, length of stay, and costs in hospitalized patients. *J Am Soc Nephrol* **16**(11)**:** 3365-3370.

Chertow GM, Burdick E, Honour M, Bonventre JV, Bates DW (2005b). Acute kidney injury, mortality, length of stay, and costs in hospitalized patients. *Journal of the American Society of Nephrology* **16**(11)**:** 3365-3370.

Chung AC, Huang XR, Meng X, Lan HY (2010). miR-192 mediates TGF-beta/Smad3-driven renal fibrosis. *J Am Soc Nephrol* **21**(8)**:** 1317-1325.

Coca SG, Yalavarthy R, Concato J, Parikh CR (2008). Biomarkers for the diagnosis and risk stratification of acute kidney injury: a systematic review. *Kidney Int* **73**(9)**:** 1008-1016.

Collins AJ, Foley RN, Chavers B, Gilbertson D, Herzog C, Johansen K*, et al.* (2012). 'United States Renal Data System 2011 Annual Data Report: Atlas of chronic kidney disease & end-stage renal disease in the United States. *American journal of kidney diseases: the official journal of the National Kidney Foundation* **59**(1 Suppl 1)**:** A7, e1.

Coresh J, Wei GL, McQuillan G, Brancati FL, Levey AS, Jones C*, et al.* (2001). Prevalence of high blood pressure and elevated serum creatinine level in the United States: findings from the third National Health and Nutrition Examination Survey (1988-1994). *Archives of internal medicine* **161**(9)**:** 1207.

Davies DF, Shock NW (1950). Age changes in glomerular filtration rate, effective renal plasma flow, and tubular excretory capacity in adult males. *Journal of Clinical Investigation* **29**(5)**:** 496.

Elmore S (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol* **35**(4)**:** 495-516.

Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M*, et al.* (2006). miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell metabolism* **3**(2)**:** 87-98.

Fabbri M, Croce CM, Calin GA (2008). MicroRNAs. *Cancer J* **14**(1)**:** 1-6.

Ferguson MA, Vaidya VS, Bonventre JV (2008). Biomarkers of nephrotoxic acute kidney injury. *Toxicology* **245**(3)**:** 182-193.

Gandhi TK, Burstin HR, Cook EF, Puopolo AL, Haas JS, Brennan TA*, et al.* (2000). Drug complications in outpatients. *Journal of General Internal Medicine* **15**(3)**:** 149-154.

Geevasinga N, Coleman PL, Webster AC, Roger SD (2006). Proton pump inhibitors and acute interstitial nephritis. *Clinical Gastroenterology and Hepatology* **4**(5)**:** 597-604.

Gilad S, Meiri E, Yogev Y, Benjamin S, Lebanony D, Yerushalmi N*, et al.* (2008). Serum microRNAs are promising novel biomarkers. *PLoS One* **3**(9)**:** e3148.

Girard M, Jacquemin E, Munnich A, Lyonnet S, Henrion-Caude A (2008). miR-122, a paradigm for the role of microRNAs in the liver. *J Hepatol* **48**(4)**:** 648-656.

Greene SB, Herschkowitz JI, Rosen JM (2010). The ups and downs of miR-205: identifying the roles of miR-205 in mammary gland development and breast cancer. *RNA Biol* **7**(3)**:** 300-304.

Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I*, et al.* (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.

Grosshans H, Slack FJ (2002). Micro-RNAs: small is plentiful. *J Cell Biol* **156**(1)**:** 17-21.

Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ (2001). Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* **293**(5532)**:** 1146-1150.

Han WK, Wagener G, Zhu Y, Wang S, Lee HT (2009). Urinary biomarkers in the early detection of acute kidney injury after cardiac surgery. *Clin J Am Soc Nephrol* **4**(5)**:** 873-882.

Han WK, Waikar SS, Johnson A, Betensky RA, Dent CL, Devarajan P*, et al.* (2008). Urinary biomarkers in the early diagnosis of acute kidney injury. *Kidney Int* **73**(7)**:** 863-869.

Hanigan MH, Devarajan P (2003). Cisplatin nephrotoxicity: molecular mechanisms. *Cancer Therapy* **1:** 47.

Hanke M, Hoefig K, Merz H, Feller AC, Kausch I, Jocham D*, et al.* (2010). A robust methodology to study urine microRNA as tumor marker: microRNA-126 and microRNA-182 are related to urinary bladder cancer. *Urol Oncol* **28**(6)**:** 655-661.

He C, Xiong J, Xu X, Lu W, Liu L, Xiao D*, et al.* (2009). Functional elucidation of MiR-34 in osteosarcoma cells and primary tumor samples. *Biochem Biophys Res Commun* **388**(1)**:** 35-40.

He L, Hannon GJ (2004). MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* **5**(7)**:** 522-531.

He L, He X, Lowe SW, Hannon GJ (2007). microRNAs join the p53 network--another piece in the tumour-suppression puzzle. *Nat Rev Cancer* **7**(11)**:** 819-822.

Ho AS, Huang X, Cao H, Christman-Skieller C, Bennewith K, Le QT*, et al.* (2010). Circulating miR-210 as a Novel Hypoxia Marker in Pancreatic Cancer. *Transl Oncol* **3**(2)**:** 109-113.

Ho J, Ng KH, Rosen S, Dostal A, Gregory RI, Kreidberg JA (2008). Podocyte-specific loss of functional microRNAs leads to rapid glomerular and tubular injury. *J Am Soc Nephrol* **19**(11)**:** 2069-2075.

Hoste EA, Kellum JA (2006). Acute kidney injury: epidemiology and diagnostic criteria. *Current opinion in critical care* **12**(6)**:** 531-537.

Huang V, Place RF, Portnoy V, Wang J, Qi Z, Jia Z*, et al.* (2012). Upregulation of Cyclin B1 by miRNA and its implications in cancer. *Nucleic Acids Res* **40**(4)**:** 1695-1707.

Hutvágner G, McLachlan J, Pasquinelli AE, Bálint É, Tuschl T, Zamore PD (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* **293**(5531)**:** 834-838.

Ichimura T, Hung CC, Yang SA, Stevens JL, Bonventre JV (2004). Kidney injury molecule-1: a tissue and urinary biomarker for nephrotoxicant-induced renal injury. *Am J Physiol Renal Physiol* **286**(3)**:** F552-563.

Inui M, Martello G, Piccolo S (2010). MicroRNA control of signal transduction. *Nat Rev Mol Cell Biol* **11**(4)**:** 252-263.

Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ (2002). Mechanisms of hepatotoxicity. *Toxicol Sci* **65**(2)**:** 166-176.

Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P (2005). Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* **309**(5740)**:** 1577-1581.

Kellum JA (2008). Acute kidney injury. *Crit Care Med* **36**(4 Suppl)**:** S141-145.

Kellum JA, Bellomo R, Ronco C (2012). Kidney attack. *JAMA* **307**(21)**:** 2265-2266.

Kellum JA, Levin N, Bouman C, Lameire N (2002). Developing a consensus classification system for acute renal failure. *Current opinion in critical care* **8**(6)**:** 509-514.

Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, Plasterk RH (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. *Genes & development* **15**(20)**:** 2654-2659.

Kohli HS, Bhaskaran MC, Muthukumar T, Thennarasu K, Sud K, Jha V*, et al.* (2000). Treatment-related acute renal failure in the elderly: a hospital-based prospective study. *Nephrology Dialysis Transplantation* **15**(2)**:** 212-217.

Kong YW, Ferland-McCollough D, Jackson TJ, Bushell M (2012). microRNAs in cancer management. *Lancet Oncol* **13**(6)**:** e249-258.

Kosaka N, Iguchi H, Ochiya T (2010a). Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. *Cancer science* **101**(10)**:** 2087-2092.

Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T (2010b). Secretory mechanisms and intercellular transfer of microRNAs in living cells. *Journal of Biological Chemistry* **285**(23)**:** 17442-17452.

Koyner JL, Vaidya VS, Bennett MR, Ma Q, Worcester E, Akhter SA*, et al.* (2010). Urinary biomarkers in the clinical prognosis and early detection of acute kidney injury. *Clin J Am Soc Nephrol* **5**(12)**:** 2154-2165.

Kroh EM, Parkin RK, Mitchell PS, Tewari M (2010). Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). *Methods* **50**(4)**:** 298-301.

Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001). Identification of novel genes coding for small expressed RNAs. *Science* **294**(5543)**:** 853-858.

Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T (2002). Identification of tissue-specific microRNAs from mouse. *Curr Biol* **12**(9)**:** 735-739.

Lasser KE, Allen PD, Woolhandler SJ, Himmelstein DU, Wolfe SM, Bor DH (2002). Timing of new black box warnings and withdrawals for prescription medications. *JAMA* **287**(17)**:** 2215-2220.

Lazarou J, Pomeranz BH, Corey PN (1998). Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. *JAMA* **279**(15)**:** 1200-1205.

Lee RC, Ambros V (2001). An extensive class of small RNAs in Caenorhabditis elegans. *Science* **294**(5543)**:** 862-864.

Lee WM (2003). Acute liver failure in the United States. *Semin Liver Dis* **23**(3)**:** 217-226.

Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J*, et al.* (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**(6956)**:** 415-419.

Levey AS, Coresh J, Balk E, Kausz AT, Levin A, Steffes MW*, et al.* (2003). National Kidney Foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Ann Intern Med* **139**(2)**:** 137-147.

Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB (2003). Prediction of mammalian microRNA targets. *Cell* **115**(7)**:** 787-798.

Liang M, Liu Y, Mladinov D, Cowley AW, Jr., Trivedi H, Fang Y*, et al.* (2009). MicroRNA: a new frontier in kidney and blood pressure research. *Am J Physiol Renal Physiol* **297**(3)**:** F553-558.

Liangos O, Perianayagam MC, Vaidya VS, Han WK, Wald R, Tighiouart H*, et al.* (2007). Urinary N-acetyl-beta-(D)-glucosaminidase activity and kidney injury molecule-1 level are associated with adverse outcomes in acute renal failure. *J Am Soc Nephrol* **18**(3)**:** 904-912.

Liangos O, Tighiouart H, Perianayagam MC, Kolyada A, Han WK, Wald R*, et al.* (2009). Comparative analysis of urinary biomarkers for early detection of acute kidney injury following cardiopulmonary bypass. *Biomarkers* **14**(6)**:** 423-431.

Lindeman R, Tobin J, Shock N (1985). Longitudinal studies on the rate of decline in renal function with age. *Journal of the American Geriatrics Society* **33**(4)**:** 278.

Liu M, Chien C-C, Burne-Taney M, Molls RR, Racusen LC, Colvin RB*, et al.* (2006). A pathophysiologic role for T lymphocytes in murine acute cisplatin nephrotoxicity. *Journal of the American Society of Nephrology* **17**(3)**:** 765-774.

Lund AH (2010). miR-10 in development and cancer. *Cell death and differentiation* **17**(2)**:** 209-214.

Majid S, Dar AA, Saini S, Yamamura S, Hirata H, Tanaka Y*, et al.* (2010). MicroRNA-205-directed transcriptional activation of tumor suppressor genes in prostate cancer. *Cancer* **116**(24)**:** 5637-5649.

Majno G, Joris I (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol* **146**(1)**:** 3-15.

McClellan WM, Knight DF, Karp H, Brown WW (1997). Early detection and treatment of renal disease in hospitalized diabetic and hypertensive patients: important differences between practice and published guidelines. *American Journal of Kidney Diseases* **29**(3)**:** 368-375.

Mehta R, Kellum J, Shah S, Molitoris B, Ronco C, Warnock D*, et al.* (2007). Acute Kidney Injury Network: report of an initiative to improve outcomes in acute kidney injury. *Critical care* **11**(2)**:** R31.

Mehta RL, T Pascual M, Soroko S, Savage BR, Himmelfarb J, Ikizler TA*, et al.* (2004). Spectrum of acute renal failure in the intensive care unit: the PICARD experience. *Kidney international* **66**(4)**:** 1613-1621.

Meijer HA, Kong YW, Lu WT, Wilczynska A, Spriggs RV, Robinson SW*, et al.* (2013). Translational repression and eIF4A2 activity are critical for microRNA-mediated gene regulation. *Science* **340**(6128)**:** 82-85.

Mishra J, Ma Q, Prada A, Mitsnefes M, Zahedi K, Yang J*, et al.* (2003). Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. *J Am Soc Nephrol* **14**(10)**:** 2534-2543.

Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL*, et al.* (2008). Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* **105**(30)**:** 10513-10518.

Molitoris BA, Levin A, Warnock DG, Joannidis M, Mehta RL, Kellum JA*, et al.* (2007). Improving outcomes from acute kidney injury. *Journal of the American Society of Nephrology* **18**(7)**:** 1992-1994.

Morley SJ, Jeffrey I, Bushell M, Pain VM, Clemens MJ (2000). Differential requirements for caspase-8 activity in the mechanism of phosphorylation of eIF2alpha, cleavage of eIF4GI and signaling events associated with the inhibition of protein synthesis in apoptotic Jurkat T cells. *FEBS letters* **477**(3)**:** 229-236.

Nathwani RA, Pais S, Reynolds TB, Kaplowitz N (2005). Serum alanine aminotransferase in skeletal muscle diseases. *Hepatology* **41**(2)**:** 380-382.

Naughton CA (2008). Drug-induced nephrotoxicity. *Am Fam Physician* **78**(6)**:** 743-750.

Nogae S, Miyazaki M, Kobayashi N, Saito T, Abe K, Saito H*, et al.* (1998). Induction of apoptosis in ischemia-reperfusion model of mouse kidney: possible involvement of Fas. *J Am Soc Nephrol* **9**(4)**:** 620-631.

Obrador GT, Ruthazer R, Arora P, Kausz AT, Pereira BJ (1999). Prevalence of and factors associated with suboptimal care before initiation of dialysis in the United States. *Journal of the American Society of Nephrology* **10**(8)**:** 1793-1800.

Ozer J, Ratner M, Shaw M, Bailey W, Schomaker S (2008). The current state of serum biomarkers of hepatotoxicity. *Toxicology* **245**(3)**:** 194-205.

Ozer JS (2010). A guidance for renal biomarker lead optimization and use in translational pharmacodynamics. *Drug Discov Today* **15**(3-4)**:** 142-147.

Ozer JS, Dieterle F, Troth S, Perentes E, Cordier A, Verdes P*, et al.* (2010). A panel of urinary biomarkers to monitor reversibility of renal injury and a serum marker with improved potential to assess renal function. *Nat Biotechnol* **28**(5)**:** 486-494.

Pabla N, Dong Z (2008). Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. *Kidney international* **73**(9)**:** 994-1007.

Park BK, Kitteringham NR, Maggs JL, Pirmohamed M, Williams DP (2005). The role of metabolic activation in drug-induced hepatotoxicity. *Annu Rev Pharmacol Toxicol* **45:** 177-202.

Park BK, Pirmohamed M, Kitteringham NR (1998). Role of drug disposition in drug hypersensitivity: a chemical, molecular, and clinical perspective. *Chem Res Toxicol* **11**(9)**:** 969-988.

Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B*, et al.* (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* **408**(6808)**:** 86-89.

Pirmohamed M, Breckenridge AM, Kitteringham NR, Park BK (1998). Adverse drug reactions. *BMJ* **316**(7140)**:** 1295-1298.

Pirmohamed M, James S, Meakin S, Green C, Scott AK, Walley TJ*, et al.* (2004). Adverse drug reactions as cause of admission to hospital: prospective analysis of 18 820 patients. *BMJ* **329**(7456)**:** 15-19.

Pulsinelli WA, Brierley JB (1979). A new model of bilateral hemispheric ischemia in the unanesthetized rat. *Stroke* **10**(3)**:** 267-272.

Rabb H, Mendiola CC, Saba SR, Dietz JR, Smith CW, Bonventre JV*, et al.* (1995). Antibodies to ICAM-1 protect kidneys in severe ischemic reperfusion injury. *Biochem Biophys Res Commun* **211**(1)**:** 67-73.

Remuzzi G, Ruggenenti P, Perico N (2002). Chronic renal diseases: renoprotective benefits of renin–angiotensin system inhibition. *Annals of internal medicine* **136**(8)**:** 604-615.

Renolleau S, Aggoun-Zouaoui D, Ben-Ari Y, Charriaut-Marlangue C (1998). A model of transient unilateral focal ischemia with reperfusion in the P7 neonatal rat: morphological changes indicative of apoptosis. *Stroke* **29**(7)**:** 1454-1460; discussion 1461.

Ricci Z, Cruz D, Ronco C (2008). The RIFLE criteria and mortality in acute kidney injury: A systematic review. *Kidney Int* **73**(5)**:** 538-546.

Rowe JW, Andres R, Tobin JD, Norris AH, Shock NW (1976). The effect of age on creatinine clearance in men: a cross-sectional and longitudinal study. *Journal of Gerontology* **31**(2)**:** 155-163.

Ruggenenti P, Schieppati A, Remuzzi G (2001). Progression, remission, regression of chronic renal diseases. *The Lancet* **357**(9268)**:** 1601-1608.

Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnica-Worms H*, et al.* (1997). Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. *Science* **277**(5331)**:** 1497-1501.

Schmittgen TD, Jiang J, Liu Q, Yang L (2004). A high-throughput method to monitor the expression of microRNA precursors. *Nucleic Acids Res* **32**(4)**:** e43.

Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V (2004). Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol* **5**(3)**:** R13.

Shelton LM, Park BK, Copple IM (2013). Role of Nrf2 in protection against acute kidney injury. *Kidney international*.

Shlipak MG, Sarnak MJ, Katz R, Fried LF, Seliger SL, Newman AB*, et al.* (2005). Cystatin C and the risk of death and cardiovascular events among elderly persons. *New England Journal of Medicine* **352**(20)**:** 2049-2060.

Simpson IJ, Marshall MR, Pilmore H, Manley P, Williams L, Thein H*, et al.* (2006). Proton pump inhibitors and acute interstitial nephritis: report and analysis of 15 cases. *Nephrology* **11**(5)**:** 381-385.

Smyth RM, Gargon E, Kirkham J, Cresswell L, Golder S, Smyth R*, et al.* (2012). Adverse drug reactions in children--a systematic review. *PLoS One* **7**(3)**:** e24061.

Star RA (1998). Treatment of acute renal failure. *Kidney Int* **54**(6)**:** 1817-1831.

Starkey Lewis PJ, Dear J, Platt V, Simpson KJ, Craig DG, Antoine DJ*, et al.* (2011). Circulating microRNAs as potential markers of human drug-induced liver injury. *Hepatology* **54**(5)**:** 1767-1776.

Sun Y, Koo S, White N, Peralta E, Esau C, Dean NM*, et al.* (2004). Development of a micro-array to detect human and mouse microRNAs and characterization of expression in human organs. *Nucleic Acids Res* **32**(22)**:** e188.

Tian Z, Greene AS, Pietrusz JL, Matus IR, Liang M (2008). MicroRNA-target pairs in the rat kidney identified by microRNA microarray, proteomic, and bioinformatic analysis. *Genome Res* **18**(3)**:** 404-411.

Tonelli M, Wiebe N, Culleton B, House A, Rabbat C, Fok M*, et al.* (2006). Chronic kidney disease and mortality risk: a systematic review. *J Am Soc Nephrol* **17**(7)**:** 2034-2047.

Uchino S, Kellum JA, Bellomo R, Doig GS, Morimatsu H, Morgera S*, et al.* (2005). Acute renal failure in critically ill patients. *JAMA: the journal of the American Medical Association* **294**(7)**:** 813-818.

Vaidya VS, Ozer JS, Dieterle F, Collings FB, Ramirez V, Troth S*, et al.* (2010). Kidney injury molecule-1 outperforms traditional biomarkers of kidney injury in preclinical biomarker qualification studies. *Nat Biotechnol* **28**(5)**:** 478-485.

Vakkila J, Lotze MT (2004). Inflammation and necrosis promote tumour growth. *Nat Rev Immunol* **4**(8)**:** 641-648.

Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* **9**(6)**:** 654-659.

Vandeputte C, Guizon I, Genestie-Denis I, Vannier B, Lorenzon G (1994). A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. *Cell Biol Toxicol* **10**(5-6)**:** 415-421.

Waldman SA, Terzic A (2008). MicroRNA signatures as diagnostic and therapeutic targets. *Clin Chem* **54**(6)**:** 943-944.

Wang K, Zhang S, Marzolf B, Troisch P, Brightman A, Hu Z*, et al.* (2009). Circulating microRNAs, potential biomarkers for drug-induced liver injury. *Proc Natl Acad Sci U S A* **106**(11)**:** 4402-4407.

Wang K, Zhang S, Weber J, Baxter D, Galas DJ (2010). Export of microRNAs and microRNA-protective protein by mammalian cells. *Nucleic Acids Res* **38**(20)**:** 7248-7259.

Weickmann JL, Glitz DG (1982). Human ribonucleases. Quantitation of pancreatic-like enzymes in serum, urine, and organ preparations. *J Biol Chem* **257**(15)**:** 8705-8710.

Welch C, Chen Y, Stallings RL (2007). MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. *Oncogene* **26**(34)**:** 5017-5022.

Wightman B, Ha I, Ruvkun G (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)**:** 855-862.

Wingard JR, Kubilis P, Lee L, Yee G, White M, Louise W*, et al.* (1999). Clinical significance of nephrotoxicity in patients treated with amphotericin B for suspected or proven aspergillosis. *Clinical infectious diseases* **29**(6)**:** 1402-1407.

Wong QW, Lung RW, Law PT, Lai PB, Chan KY, To KF*, et al.* (2008). MicroRNA-223 is commonly repressed in hepatocellular carcinoma and potentiates expression of Stathmin1. *Gastroenterology* **135**(1)**:** 257-269.

Yamakuchi M, Ferlito M, Lowenstein CJ (2008). miR-34a repression of SIRT1 regulates apoptosis. *Proc Natl Acad Sci U S A* **105**(36)**:** 13421-13426.

Yang BF, Lu YJ, Wang ZG (2009a). MicroRNAs and apoptosis: implications in the molecular therapy of human disease. *Clin Exp Pharmacol Physiol* **36**(10)**:** 951-960.

Yang L, Zheng S, Epstein PN (2009b). Metallothionein over-expression in podocytes reduces adriamycin nephrotoxicity. *Free radical research* **43**(2)**:** 174-182.

Yao X, Panichpisal K, Kurtzman N, Nugent K (2007). Cisplatin nephrotoxicity: a review. *The American journal of the medical sciences* **334**(2)**:** 115-124.

Yu H, Yanagisawa Y, Forbes M, Cooper E, Crockson R, MacLennan I (1983). Alpha-1-microglobulin: an indicator protein for renal tubular function. *Journal of clinical pathology* **36**(3)**:** 253-259.

Zhang L, Cooper AJ, Krasnikov BF, Xu H, Bubber P, Pinto JT*, et al.* (2006). Cisplatin-induced toxicity is associated with platinum deposition in mouse kidney mitochondria in vivo and with selective

inactivation of the α-ketoglutarate dehydrogenase complex in LLC-PK1 cells. *Biochemistry* **45**(29)**:** 8959-8971.

Zhang QL, Rothenbacher D (2008). Prevalence of chronic kidney disease in population-based studies: systematic review. *BMC Public Health* **8:** 117.