Characterisation of microRNAs in the heart

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ABSTRACT

MicroRNAs (miRNAs) are endogenous, non-coding RNA species that regulate gene expression at the post-transcriptional level. Recent studies have shown that miRNAs are important for cardiac hypertrophy and heart failure, and are critical determinants of tissue metabolism.

To investigate the role(s) of miRNAs in the insulin resistant heart, left ventricular biopsies were collected from patients with normal ventricular function with or without type 2 diabetes, and patients with left ventricular dysfunction (LVD). Using TaqMan based reverse transcriptase PCR, quantitative expression levels of 155 mature miRNAs in normal and diabetic hearts were determined. Five miRNAs were significantly upregulated in the diabetic human heart. Among these, miR-223 was upregulated in both diabetic heart and patients with LVD. Adenoviral-mediated overexpression of miR-223 increased baseline glucose uptake in cardiac myocytes in vitro with an effect size similar to that observed for insulin stimulation. This increase was associated with increase in Glut4 protein expression but independent of PI3K/Akt signalling and AMPK activity. In contrast to findings in other cells, in cardiac myocytes miR-223 did not downregulate protein levels of Mef2c or Igf1r, and an unexpected increase in NfIa protein was observed, where all three genes are miR-223 targets in immune cells.

Systemic inhibition of miR-223 in vivo decreased blood glucose level 48 hours after administration and increased Glut4 protein level in the skeletal muscle, however Glut4 levels were decreased in the heart. Cardiac-specific transgenic mice overexpressing miR-223 showed no detectable changes in Glut4 protein level and cardiac insulin signalling at baseline.

Collectively, these data characterise the expression of miRNAs in the human heart, demonstrate that miRNAs regulate gene targets in a cell/tissue type specific manner, they can unexpectedly increase protein expression in cardiac myocytes, and miR-223 regulates cardiac glucose metabolism through a non-canonical pathway, which may have implications for future investigations and treatment of insulin resistance.
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<td>4E-BP1</td>
<td>4E-binding protein-1</td>
</tr>
<tr>
<td>Akt</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>AGO</td>
<td>argonaute</td>
</tr>
<tr>
<td>AMPK</td>
<td>5' adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>ANF</td>
<td>atrial natriuretic factor</td>
</tr>
<tr>
<td>AP1</td>
<td>Adaptor protein 1</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphotase</td>
</tr>
<tr>
<td>ARE</td>
<td>AU-rich element</td>
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<tr>
<td>ASO</td>
<td>antisense oligonucleotides</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BG</td>
<td>blood glucose</td>
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<tr>
<td>BMI</td>
<td>body mass index</td>
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<tr>
<td>BNF</td>
<td>brain natriuretic factor</td>
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<tr>
<td>BUN</td>
<td>blood urea nitrogen</td>
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<tr>
<td>CAD</td>
<td>coronary artery disease</td>
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<td>Cav2</td>
<td>caveolin 2</td>
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<td>Cdk9</td>
<td>cyclin-dependent kinase 9</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DBP</td>
<td>diastolic blood pressure</td>
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<tr>
<td>DIG</td>
<td>digoxygenin</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
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<tr>
<td>DPBS</td>
<td>Dulbecco’s Phosphate Buffered Saline</td>
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<tr>
<td>dsRNA</td>
<td>double-stranded RNA</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EF</td>
<td>ejection fraction</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<tr>
<td>ET-1</td>
<td>endothelin-1</td>
</tr>
<tr>
<td>FA</td>
<td>fatty acid</td>
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<tr>
<td>FC</td>
<td>fold change</td>
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### Abbreviations

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<td>FCS</td>
<td>foetal calf serum</td>
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<td>FXR1</td>
<td>fragile-X mental-retardation–related protein 1 (FXR1)</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GGA</td>
<td>golgi-localised γ-ear-containing Arf-binding protein</td>
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<td>GK</td>
<td>goto–Kakizaki rats</td>
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<td>GLUT</td>
<td>glucose transporter</td>
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<td>Hb</td>
<td>haemoglobin</td>
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<tr>
<td>HbA1c</td>
<td>glycated haemoglobin</td>
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<tr>
<td>HDAC4</td>
<td>histone deacetylase 4</td>
</tr>
<tr>
<td>HR</td>
<td>heart rate</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<td>insulin receptor</td>
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<td>IRAP</td>
<td>insulin-regulated aminopeptidase</td>
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<td>insulin-responsive compartments</td>
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<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LNA</td>
<td>locked nucleic acid</td>
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<tr>
<td>LV</td>
<td>left ventricular</td>
</tr>
<tr>
<td>LVD</td>
<td>left ventricular dysfunction</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>miRISC</td>
<td>microRNA induced silencing complex</td>
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<td>miRNA</td>
<td>microRNA</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>NFI</td>
<td>nuclear factor I</td>
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<td>NRVM</td>
<td>neonatal rat ventricular myocyte</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PDK1</td>
<td>phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PE</td>
<td>phenylephrine</td>
</tr>
<tr>
<td>PET</td>
<td>position emission tomography</td>
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<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
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<tr>
<td>PITA</td>
<td>Probability of Interaction by Target Accessibility</td>
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<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>PMSF</td>
<td>phenylmethylsulphonylfluoride</td>
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<td>Pri-miRNA</td>
<td>primary-miRNA</td>
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<td>q-PCR</td>
<td>quantitative PCR</td>
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<td>qRT-PCR</td>
<td>quantitative reverse transcriptase PCR</td>
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<td>RasGAP</td>
<td>Ras GTPase-activating protein</td>
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<td>Rheb</td>
<td>Ras homolog enriched in brain</td>
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<td>RNA induced silencing complex</td>
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<td>RNA interference</td>
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<td>RNP</td>
<td>ribonucleoprotein complex</td>
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<td>RT</td>
<td>reverse transcriptase</td>
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<td>SBP</td>
<td>systolic blood pressure</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNAs</td>
</tr>
<tr>
<td>SM</td>
<td>skeletal muscle</td>
</tr>
<tr>
<td>SNARE</td>
<td>SNAP and NSF attachment receptors</td>
</tr>
<tr>
<td>SOC</td>
<td>super optimal broth with catabolite repression</td>
</tr>
<tr>
<td>SPRY1</td>
<td>sprouty homologue 1</td>
</tr>
<tr>
<td>SRF</td>
<td>serum response factor</td>
</tr>
<tr>
<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TAC</td>
<td>transverse aortic constriction</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>THRAP1</td>
<td>thyroid hormone receptor-associated protein 1</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>TR</td>
<td>thyroid hormone receptor</td>
</tr>
<tr>
<td>TSC2</td>
<td>tuberous sclerosis factor</td>
</tr>
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<td>uniform resource locator</td>
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<tr>
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<td>untranslated region</td>
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<tr>
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<td>vesicle-associated membrane protein-2</td>
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<td>WBC</td>
<td>white blood count</td>
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STATEMENT OF CONTRIBUTION

Technical assistance in the preparation of primary cultures of neonatal cardiac myocytes was provided by Ms Rachel Buchan and Ms Sharifah Syed Salim. Technical assistance on in vivo experiments was provided by Mr Phillip Muckett.

Experiments on AMPK activity were done in collaboration with Dr Nell Marty (Cell Stress Group, Clinical Sciences Centre, MRC; Section 4.3.5, Figure 4.7). Glut4 full cDNA overexpression in COS7 cells and subsequent immunoblotting analysis was done by Ms Rachel Buchan (Section 5.3.4, Figure 5.3).

Cardiac magnetic resonance imaging was done in collaboration with Dr Marzena Wylezinska-Arridge and Dr Willy Gsell (Biological Imaging Centre, Clinical Sciences Centre, MRC, Chapter 6, Section 6.3.2.5, Figure 6.14).

All other experimental work, and all data analyses, were performed by myself unless otherwise stated.
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Regulatory RNA in Biology and Human Health, Miami Winter Symposium 2008, USA

H Lu, P Punjabi, F Rahman, P Camici, T Aitman, SA Cook.
Characterisation of microRNAs in the human heart
British Cardiovascular Society Annual Scientific Conference 2007, Glasgow, UK
CHAPTER ONE

Introduction

A class of small non-coding RNA molecules, termed microRNA (miRNA), have been implicated to have major roles in heart development, cardiac hypertrophy and the pathophysiology of metabolic disease. This chapter outlines the functional and biological effects of miRNA, describes the role for miRNA in the development of cardiac hypertrophy and insulin resistance, and discusses the potential role of miRNA in the pathogenesis of human heart diseases and its therapeutic implications.

1.1 miRNA

miRNAs are a newly discovered RNA species. This section describes the biological processing mechanisms and molecular functions of miRNA known to date; and outlines experimental methodologies developed for studying miRNA.

1.1.1 Expression and processing

miRNAs are endogenously expressed ~22-nucleotide non-coding RNAs that primarily regulate gene expression at the translational level by binding to complementary sequences in phylogenetically conserved targets in the 3’ untranslated regions (UTRs) of protein encoding messenger RNAs (mRNAs; Bartel, 2004). To date, almost 700 miRNAs have been identified in Humans (miRBASE: http://miRNA.sanger.ac.uk/).

Figure 1.1 depicts the miRNA processing pathway in a mammalian cell (adapted from Kim, 2005). Similar to mRNA in protein coding genes, miRNAs are transcribed as primary-miRNA (pri-miRNA) by RNA polymerase II from a template DNA sequence. These templates can be embedded among introns or protein exons of coding genes or located intergenically between known protein coding genes (Cai et al., 2004; Lee et al., 2004). While in the nucleus, pri-miRNA transcript is cleaved into a short hairpin
Figure 1.1 miRNA biogenesis pathway in mammalian cells. Primary-miRNA transcript is cleaved into a short hairpin pre-miRNAs (approx 70-100nt) by the nuclear RNase III, Drosha. After being transported to the cytoplasm, the hairpin sequence is processed further by cytosolic RNase III Dicer to generate mature miRNA:miRNA* duplex (miRNA* denotes the RNA strand that contains the reverse compliment sequence to the mature miRNA). The mature miRNA then associates with RNA induced silencing complex (RISC) and bind to the 3’ untranslated region (UTR) of target mRNA.
pre-miRNA (approx 70-100nt) by the nuclear RNase III, Drosha (Lee et al., 2003). A nuclear transport factor, Exportin 5, then actively transports the pre-miRNA out of the nucleus into the cytoplasm (Yi et al., 2003). Here the hairpin sequence is processed further by cytosolic RNase III, Dicer, generating double stranded miRNA:miRNA* duplexes, where miRNA* denotes the RNA strand that contains the reverse complement sequence to the mature miRNA. Depending on the relative stability of the two ends of the duplex, the mature miRNA strand is nearly always the one whose 5’ end is less tightly paired (Khvorova et al., 2003). Once processed, the mature miRNA associates with a ribonucleoprotein complex, the miRNA induced silencing complex (miRISC), and binds to the 3’ untranslated region (UTR) of target mRNA, at which time the miRNA* sequence is removed and degraded (Figure 1.1).

1.1.2 Gene regulation: mechanisms and function

Once bound to a target mRNA, miRNAs are thought to regulate gene expression through a number of mechanisms (Figure 1.2). The classical silencing mechanism of miRNA occurs at the translational level. This can be achieved through three distinct ways: (1) inhibition of translation elongation (Petersen et al., 2006); (2) premature termination of translation as a result of ribosome ‘drop-off’ (Petersen et al., 2006); and (3) inhibition of translation initiation through blocking translation completely by targeting either the capping or the adenylation of the target mRNA (Mathonnet et al., 2007; Wakiyama et al., 2007). All mechanisms results in a decrease in levels of protein the mRNA encodes. Apart from translational interference, miRNA mediated gene silencing can also occur through degradation of target mRNA, which affects protein as well as mRNA transcript levels. This effect was shown in a study using genome wide microarray analysis of mammalian cells where upon exogenous miRNA expression, large numbers of transcripts were downregulated (Lim et al., 2005). In addition to
Figure 1.2 miRNA mediated gene regulation. Mature miRNA associates with argonaute (AGO) proteins to form miRNA induced silencing complex and bind to the target mRNA. Upon binding, the miRISC can induce mRNA silencing by a number of mechanisms, this includes inhibitions of capping or tailing of the mRNA, inhibition of initiation or elongation of protein translation, or promoting mRNA degradation.
regulation at the post-transcriptional level, miRNA may also alter gene expression by interfering with transcription. Studies of double stranded small interfering RNAs (siRNA) have been shown to result in changes in chromatin structure by triggering DNA methylation. This shows that double-stranded RNAs (dsRNA) can form a negative feedback loop to modulate the accessibility of cis-regulatory DNA elements in close proximity to the mRNA target’s physical position (Kawasaki and Taira, 2006; Mathieu and Bender, 2004). These findings may also apply to miRNA-mediated gene inhibitory mechanisms, but have yet to be proven.

Since their discovery fifteen years ago, miRNAs have been generally considered as negative regulators of gene expression. However, more recent studies of cell cycle variation and the cellular stress response have revealed that mRNAs repressed by miRNAs in one cellular state can be reactivated in another state and that miRNAs can also ‘paradoxically’ activate translation (Bhattacharyya et al., 2006; Vasudevan et al., 2008). Vasudevan et al. showed upon cell cycle arrest, miR-396-3 directs the recruitment of microRNP (micro-ribonucleoprotein complex) associated factors, such as argonaute (AGO) and fragile-X mental-retardation–related protein 1 (FXR1), to the AU-rich element (ARE) in the 3’UTR of tumour necrosis factor-α (TNFα) mRNA to activate translation. Interestingly, they also showed let-7, a well studied miRNA, can not only repress translation in proliferating cells but can also induce translation of target mRNA during cell-cycle arrest (Vasudevan et al., 2007).

Collectively, these findings demonstrate differing mechanisms for miRNA mediated gene regulation, which suggest miRNAs can regulate gene expression through a broad range of mechanisms depending on the specific miRNA, the mRNA target, the cell type
and cell cycle involved, or the interaction of miRNA with other unknown RNA binding proteins. The relative importance of each mechanism and the processes controlling miRNA specificity remain to be determined.

1.1.3 Biological functions of miRNAs

Binding of miRNAs to target sequence is often not fully complementary, this allows miRNAs to interact with multiple targets as opposed to a single or limited transcript(s) (Brennecke et al., 2005), which suggests miRNAs may have a wide range of biological functions. Indeed, miRNAs are important regulators for a diverse range of biological processes, such as cell and tissue differentiation, apoptosis, metabolism and cancer formation (Baehrecke, 2003; Chen et al., 2004; Esquela-Kerscher and Slack, 2006; Gregory and Shiekhattar, 2005; Poy et al., 2004). A miRNA expression study by Krichevsky and colleagues showed the expression of 8 mature miRNA sequences were significantly differentially regulated during brain development, of which miR-9 and miR-131 were dysregulated in presenilin-1 null mice with severe brain developmental defects (Krichevsky et al., 2003). In the pancreas, miR-375 suppresses glucose-induced insulin secretion by inhibiting myotrophin (Poy et al., 2004). In addition, miRNA-122 is a regulator of lipid metabolism and inhibition of miR-122 expression resulted in decreased plasma cholesterol levels, increased hepatic fatty-acid oxidation, and a decrease in hepatic fatty-acid and cholesterol synthesis rates in mice (Esau et al., 2006). These studies indicate miRNAs are important regulators of both pre- and post-natal tissue physiology.

1.1.4 miRNA nomenclature

The rapid growth of the miRNA field has driven the development of a dedicated miRNA database (miRBase, http://miRNA.sanger.ac.uk) which provides access to miRNA sequence information, target prediction programs, and allows for online
submission for newly discovered miRNAs (miRBase Registry). An annotation scheme has been implemented for assigning unique miRNA identifiers. Newly identified miRNAs are named by a sequential number (e.g. miR-1, miR-2… miR-133, etc), unless they are orthologues of known miRNAs, in which case the same number is used. For animals, the species from which the miRNA derives is shown as abbreviated three letter prefixes, for instance, hsa-miR-218 (Homo sapiens) or rno-miR-22 (Rattus norvegicus). Mature miRNA sequences that differ by one or two nucleotides (nt) are grouped as a family, with an alphabetical suffix denoting individual sequences (e.g. miR-30b, miR-30c). If two mature miRNAs are processed from a common hairpin precursor, they are distinguished on the basis of which arm they derive from, for example, miR-126-5p (from 5’ arm) and miR-126-3p (from 3’ arm). An asterisk denotes the antisense sequence produced from miRNA processing (Figure 1.1, e.g. miR-223*), these sequences usually have a short half-life and are degraded once the complementary mature miRNA enters the miRISC. Different genomic loci that give rise to identical mature miRNA sequences are designated with number suffix, for example, mmu-mir-16-1 and mmu-mir-16-2 in mouse (Ambros et al., 2003; Griffiths-Jones et al., 2006).

1.1.5 miRNA target prediction

miRNA function is mediated by inhibiting translation of target mRNAs, thus, identification of the target is an essential step towards understanding the biological role(s) of a miRNA. How do miRNAs recognise their target mRNAs? In plants, most miRNAs exhibit extended sequence complementarity to mRNA targets’ 3’UTRs (Rhoades et al., 2002). In animals however, most miRNA target sites contain seven nucleotides that base pair with positions 2-8 from the 5’ end of the miRNA. The complementarity of this seed with the target site is crucial for target recognition. Studies in HeLa cells have shown that mismatches within the core seed binding region inhibit
miRNA binding to target mRNAs, and prevent either target cleavage or translational repression regardless of the degree of complementarity elsewhere in the miRNA binding site. However, core seed pairing supplemented by just a few flanking base pairings appears to be sufficient to mediate translational repression in cooperation with other RISCs bound to the target mRNA (Lewis et al., 2003).

Applying this knowledge of sequence complementarity and other determinants of miRNA-mRNA interactions, a number of mathematical algorithms for miRNA target prediction have been developed. The basic principles of in silico predictions rely on: (1) complementarity of miRNA sequence to the 3’UTR of the target mRNA, (2) strong binding of the 5’ end of the miRNA to target compared to 3’ end, (3) the degree of sequence conservation of the target recognition site on the mRNA across species (Krek et al., 2005), (4) thermodynamic stability of the miRNA-target duplex (Kertesz et al., 2007), and (5) accessibility of the mRNA target site, where miRNA binding can be hindered by strong secondary RNA structure.

TargetScan (http://www.targetscan.org; Lewis et al., 2005; Lewis et al., 2003), a web-based program for the prediction of vertebrate miRNA target prediction, searches the 3’UTR of the mRNA for 7-nt complementary seed sequences, it allows the G:U wobble base pairing and optimises base pairing by analysing the 3’UTR of the mRNA with the RNA-fold program. Prioritised target sites are tested further for sequence conservation across human, mouse, rat, chicken and dog genomes, and conserved sequences flanking the seed match are considered to increase the specificity of the predication. All predicted UTRs are then scored and ranked. According to its developers, the false positive rate was estimated ~22% for targets conserved in mammals.
In 2004, John and colleagues (John et al., 2004) developed miRanda (http://www.miRNA.org) for prediction of miRNA targets in mouse, rat and man. In addition to sequence complementarity and cross species conservation, miRanda also takes into account the thermodynamics of the miRNA:mRNA duplex, the physical position of the predicted site on the 3’UTR and the number of sites identified on each mRNA. Cross species analysis using miRanda found ~2000 human genes contain target sites that are conserved among mammals. Of these, ~250 human genes are conserved among mammals and fish (Betel et al., 2008; John et al., 2004).

PicTar (http://pictar.mdc-berlin.de/), the probabilistic identification of combinations of target site, was developed by Krek and colleagues (2005). This algorithm accounts for synergistic effects of multiple binding sites of one miRNA or several miRNAs acting together. In this model, miRNAs compete with each other for binding, and the synergistic effect of multiple binding sites of one miRNA or several miRNAs acting together is taken into consideration. Cross-species comparisons are used to reduce the chance of false positives, where candidate target genes are defined as UTRs with a minimal (user-defined) number of evolutionarily conserved putative binding sites. PicTar then scores the candidate sequences for each species separately. The resulting scores are combined to obtain the final PicTar score for a gene. Estimations using published miRNA targets indicated PicTar could have ~30% false discovery rate (Grun et al., 2005).

A systematic analysis on miRNA target site accessibility was performed by Kertesz and colleagues in 2007 (Kertesz et al., 2007). It was shown, mutations that diminish target accessibility reduce miRNA induced translational repression, and this effect was
comparable to mutations that disrupt sequence complementarity. The authors devised a model for miRNA-target interaction that computes the difference between the free energy gained from the formation of the miRNA-target duplex and the energy lost by unpairing the target site nucleotides. In doing so, the model generates an energy-based score for the miRNA-target interaction (ΔΔG), which was strongly correlated with the experimentally measured degree of repression. Based on this model, a genome wide miRNA target prediction program was developed (PITA – Probability of Interaction by Target Accessibility; http://genie.weizmann.ac.il/pubs/mir07/index.html). PITA uses standardised settings based on sequence complementarity to identify initial seeds for each miRNA in 3' UTRs, then applies the free-energy model to each putative site, and finally combines sites for the same miRNA to obtain a total interaction score for the miRNA and UTR. Different to other programs described above, PITA allow user defined settings for seed size and wobble base pairing. In addition to the pre-defined 3’UTR sequences, users can also upload cDNA sequences to predict potential miRNA binding.

Apart from the freely available web-based programs described here, many more programs for miRNA target prediction have been developed. However, due to the complexity and limited knowledge of miRNA binding motifs, computational predictions based on sequence alignments are not as straight forward as formerly anticipated and different algorithms identify disparate targets (Baek et al., 2008; Kertesz et al., 2007). Thus, experimental validation of the predicated targets is essential for identifying the true mRNA target and determining the function of the miRNA (Wang, 2006).
1.1.6 Experimental detection of miRNAs

As a result of the physical properties of miRNAs, several methods have been developed to purify and detect miRNAs. These include: small RNA purification methods; PCR-based techniques which can detect low copy number with high sensitivity and specificity for both the precursor and the mature form (Chen et al., 2005; Schmittgen et al., 2004); and hybridization techniques such as Northern Blotting, in situ hybridization, bead-based flow cytometry and microarray expression profiling (Kloosterman et al., 2006; Liu et al., 2004; Lu et al., 2005).

1.1.6.1 Purification of small RNAs

Mature miRNA sequences are around 22nt in length. Conventional RNA purification methods using spin columns (e.g. QIAGEN RNeasy Kit) predominantly isolate RNA species larger than 200nt, and most small RNAs are lost during the purification process. Methods for the enrichment of small RNA species that are less than 200nt were therefore developed. Similar to conventional RNA extraction procedures, the sample (tissue or cultured cells) is homogenised in a lysis buffer, followed by acid phenol based organic extraction step to separate DNA and protein from total RNA (Boom et al., 1990; Chomczynski and Sacchi, 1987). To isolate RNA that is highly enriched for small RNA species, the large RNA species is first precipitated and immobilised, then, small RNA are precipitated from the filtrate using a higher ethanol concentration. A number of small RNA purification kits have been developed based on this principle (e.g. Ambion/Applied Biosystems, mirVana miRNA Isolation kit; QIAGEN, miRNeasy Mini Kit).
1.1.6.2 miRNA detection using quantitative reverse transcriptase-PCR

The stem-loop quantitative reverse transcriptase PCR (qRT-PCR; Applied Biosystems) assay is designed specifically for the quantification of mature miRNAs. It is a TaqMan® based two-step procedure (reverse transcription and real time quantitative PCR) where the RT primer has a stem-loop structure (Figure 1.3). Stem-loop RT primers are thought to be better than conventional primers in terms of RT efficiency and specificity. The assays are highly specific for individual miRNAs and are not affected by genomic DNA contamination. The manufacturer claims that precise quantification can be achieved with as little as 25 pg of total RNA. Compared to more traditional methods such as Northern Blotting, this method enables fast and sensitive miRNA expression profiling. In addition, stem-loop RT-PCR can be used for the quantification of other small RNA molecules such as siRNAs (Chen et al., 2005). The stem-loop qRT-PCR method was developed by Applied Biosystems. An early access assay kit containing a 180-assay panel was released in early 2005 (TaqMan® miRNA Assays – Human Panel Early Access Kit, PN 4365381), which contained assays for most of the known human miRNAs at the time.

1.1.6.3 miRNA detection using locked nucleic acid

Locked nucleic acid (LNA) is a class of nucleic acid analogues that exhibit high affinity for hybridization towards complementary DNA and RNA. The ribose ring of a LNA is “locked” by a methylene bridge connecting the 2’-O atom with the 4’-C atom (Figure 1.4). LNA nucleosides consists six common bases (T, C, G, A, U and mC), which enables Watson-Crick base pairing with complementary DNA and RNA nucleotides. Because the molecule is ‘locked’ by the methylene bridge, the LNA is constrained in the ideal conformation for Watson-Crick base pairing, this improves mismatch
Figure 1.3 Two-step stem-loop quantitative reverse transcription PCR (qRT-PCR; Chen et al., 2005).
Figure 1.4 Molecular structures of DNA, LNA (Locked Nucleic Acid) and RNA
discrimination and increases the thermal stability of the resulting duplex (Petersen and Wengel, 2003). Since the early application of LNAs for Northern Blotting (Valoczi et al., 2004), LNA probes have been widely used for miRNA detection procedures such as *in situ* hybridisation (Bak et al., 2008; Moschos et al., 2007; Schratt et al., 2006; Wienholds et al., 2005) and LNA based miRNA microarray expression profiling (da Costa Martins et al., 2008; Valadi et al., 2007).

### 1.1.7 Modulation of endogenous miRNA function using synthetic oligonucleotides

Applying the advances of RNA interference (RNAi) approaches, miRNA function can now be modulated using single stranded antisense oligonucleotides (ASOs). During RNAi, small interfering RNA (siRNA) molecules bind to target mRNA by full sequence complementarity, which induces endogenous sequence-specific degradation of the target mRNA. Using a similar approach, synthetic miRNA-specific reverse complement oligonucleotides can down-regulate the expression of endogenous miRNA (Krutzfeldt et al., 2005) or competitively bind to miRNA and prevent the association of miRISC (van Rooij et al., 2008a).

To date, a number of anti-miRNA ASOs have been successfully used to inhibit miRNA activity *in vitro* and *in vivo*. Antagomirs were one of the first such ASOs described. They are synthesised with 2’-O-methoxyethyl modified nucleotides on a phosphorothioate backbone and contain hydroxyprolinol-linked cholesterol at the 3’ end (Krutzfeldt et al., 2005). These modifications reduce RNase-mediated degradation and facilitate cellular uptake of the oligonucleotide. Krutzfeldt and colleagues showed the intravenous administration of antagomirs to mice significantly decreased targeted miRNA levels (Krutzfeldt et al., 2005). 2’O-Methoxyethyl phosphorothioate modified
nucleotides without 3’ cholesterol conjugation have also been used to efficiently inhibit miRNA in liver (Esau et al., 2006). In addition, oligonucleotides consisting of locked-nucleic-acid (LNA) modified bases were also used to target and inhibit miRNAs. Elmen and colleagues (Elmen et al., 2008) showed mature miR-122, important for lipid metabolism, was depleted in non-human primates upon intravenous injections of anti-miR-122 LNA oligonucleotides, and this led to a significant decrease of plasma cholesterol level. LNA modified oligonucleotides are now been evaluated for human clinical trials of miRNA inhibition (van Rooij et al., 2008a). These findings suggest ASOs are good resources for miRNA functional studies in vitro and in vivo, and as for siRNAs, may be important for therapeutic applications.

1.2 Role of miRNA in the heart

Heart disease is a major cause of mortality and morbidity world wide. It is a significant health burden, one of the largest problems faced by the National Health Service in the United Kingdom, accounting for a substantial proportion of healthcare resources (Luengo-Fernandez et al., 2006). Common forms of heart disease such as cardiomyopathy, hypertensive heart disease, heart failure and ischemic heart disease, are caused by a combination of genetic and environmental factors. Much effort has been invested into the study of heart diseases at the cellular level, but the molecular mechanisms underlying these common diseases remain poorly defined.

The role of miRNAs in the heart was poorly understood at the outset of this study. However, over the last few years, studies of miRNAs in the developing and adult heart have identified critical roles for miRNAs in normal cardiac function and pathological conditions. Heart-specific Dicer knockout mice showed disrupted expression of cardiac contractile proteins and profound sarcomere disarray, which leads to dilated
cardiomyopathy, left ventricular dysfunction (LVD) and post-natal lethality. Intriguingly, Dicer expression is decreased in end-stage human dilated cardiomyopathy and LVD (Chen et al., 2008). In other studies, expression profiling revealed miRNA expression patterns of foetal heart and that of the adult are different. Interestingly, in both human and animal models of cardiac hypertrophy and heart failure, miRNAs that are normally only expressed in the foetal heart are re-expressed in the failing heart (Thum et al., 2007). Over 100 miRNAs have been shown to be differentially regulated in both human and animal models of heart disease, but only a small number of miRNAs have been studied at a functional level (reviewed in Latronico et al., 2007 and Thum et al., 2008a). A few examples of miRNAs that have been shown to have important roles in cardiogenesis and disease pathophysiology are described in this section (Table 1.1).

1.2.1 miR-1 and miR-133 in cardiogenesis and hypertrophy

miR-1 and miR-133 are muscle specific miRNAs that have important regulatory roles in embryonic heart development and post-natal cardiac physiology. Studies in the mouse embryo revealed that both miRNAs are directly controlled by muscle differentiation transcription factors including MyoD, Mef2 and serum response factor (SRF). Sequence analysis of these transcription factor binding sites in the miRNA promoter regions showed this regulatory mechanism is highly conserved across species, including humans (Zhao et al., 2005). Although miR-1 and miR-133 are expressed together from the same genomic locus in a tissue-specific manner, they have different roles in skeletal muscle development (Chen et al., 2006). miR-1 promotes myogenesis through inhibition of histone deacetylase 4 (HDAC4), a transcriptional repressor of muscle gene expression, whereas miR-133 enhances myoblast proliferation by repressing its own transcription factor SRF via a negative regulatory loop. miR-1 is also involved in cardiac differentiation, by targeting and inhibiting Hand2, a transcription factor...
Table 1.1: miRNA functions in the heart.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Function</th>
<th>Validated target(s)*</th>
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<td>Inhibition of proliferation</td>
<td>Hand2</td>
</tr>
<tr>
<td></td>
<td>Myogenesis</td>
<td>HDAC4</td>
</tr>
<tr>
<td>miR-133</td>
<td>Promotion of proliferation</td>
<td>SRF</td>
</tr>
<tr>
<td></td>
<td>Control of cardiac conductance</td>
<td>HERG</td>
</tr>
<tr>
<td></td>
<td>Inhibition of cardiac hypertrophy</td>
<td>RhoA, Cdc-42, WHSC2</td>
</tr>
<tr>
<td>miR-21</td>
<td>Reduce cardiac ERK-MAPK and inhibit cardiac fibrosis</td>
<td>SPRY1</td>
</tr>
<tr>
<td>miR-29</td>
<td>Inhibits cardiac fibrosis</td>
<td>COL1A1, COL1A2, COL3A1, FBN1</td>
</tr>
<tr>
<td>miR-208</td>
<td>Stress-dependent cardiac remodelling</td>
<td>THRAP1</td>
</tr>
<tr>
<td></td>
<td>Regulation of β-MHC expression</td>
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</table>

*Gene names: **Hand2**, heart and neural crest derivatives expressed 2; **HDAC4**, histone deacetylase 4; **SRF**, serum response factor; **HERG**, potassium voltage-gated channel, subfamily H, member 2; **RhoA**, ras homolog gene family, member A; **Cdc-42**, cell division cycle 42; **WHSC2**, Wolf-Hirschhorn syndrome candidate 2; **SPRY1**, sprouty homolog 1; **COL1A1**, collagen type I, alpha 1; **COL1A2**, collagen type I, alpha 2; **COL3A1**, collagen type III, alpha 2; **FBN1**, fibrillin 1; **THRAP1**, thyroid hormone receptor associated protein 1
important for ventricular cardiac myocyte hyperplasia (Zhao et al., 2005). In addition, a study by Lim and colleagues using microarray technology showed overexpression of miR-1 in HeLa cells caused a shift in gene expression profile towards the heart and skeletal muscle (Lim et al., 2005), indicating miRNAs can down-regulate a large number of target mRNA transcripts and define tissue-specific gene expression.

Both miR-1 and miR-133 are also involved in the development of post-natal cardiac myocyte pathophysiology. Care et al. (2007) showed a decrease in miR-1 and miR-133 expression in cultured cardiac myocytes treated with phenylephrine (PE) and in the ventricular tissue of mice induced with transverse aortic constriction (TAC), a widely used in vivo model of cardiac hypertrophy. Interestingly, expression levels of these miRNAs are also decreased in the ventricles of exercise-trained wild type mice and in the cardiac-specific Akt transgenic mouse (Care et al., 2007). Therefore, miR-1 and miR-133 are implicated in cardiac myocyte hypertrophy irrespective of the nature of underlying cause. Further studies of miR-133 have shown overexpression of miR-133a-2 in cultured cardiac myocytes abolishes the hypertrophic response to PE stimulation, and in vivo administration of antagonir-133 via osmotic pump in mice induced hypertrophy. In silico and experimental target analyses of miR-133 revealed that RhoA, a GDP-GTP exchange protein regulating cardiac hypertrophy, Cdc42, a signal transduction kinase implicated in hypertrophy, and Nelf-A/WHSC2, a nuclear factor involved in cardiogenesis, are all targets of miR-133 (Care et al., 2007).

In a time course study performed by Sayed et al., more than 50 miRNAs were differentially regulated during the development of pressure overload-induced cardiac hypertrophy (Sayed et al., 2007). Amongst these miRNAs, miR-1 was downregulated
soon after TAC (day 1), and its expression was at the lowest at day 7, following which it recovered and was normal by day 14 post-TAC. Viral overexpression of miR-1 inhibited serum-induced hypertrophic growth and growth-related target genes, including Ras GTPase-activating protein (RasGAP), cyclin-dependent kinase 9 (Cdk9), fibronectin, and Ras homolog enriched in brain (Rheb) in cultured neonatal cardiac myocytes. In separate studies by Yang et al. and Luo et al., miR-1 and miR-133 were shown to have an important role in arrhythmogenesis through inhibition of HCN2 and HCN4, which encode the hyperpolarization-activated channels in the heart (Luo et al., 2008; Yang et al., 2007). HCN2 and HCN4 proteins are upregulated during hypertrophy, and this was accompanied by pronounced reduction of miR-1 and miR-133 levels. Forced expression of both miRNAs prevented the overexpression of HCN2 and HCN4 in hypertrophied cardiac myocytes (Luo et al., 2008), suggesting a causal relationship between miR-1 and miR-133 and HCN2/HCN4.

### 1.2.2 A role for miR-208 following hypertrophic stimulation in vivo

Cardiac hypertrophy is associated with upregulation of the foetal genes such as atrial natriuretic factor (ANF), brain natriuretic factor (BNF) and β-myosin heavy chain (β-MHC). In the heart, the genes encoding the two isoforms of MHC (α and β) are located in tandem, separated by 4.5kb of intergenic sequence, with β-MHC gene located 5’ upstream of the α-MHC gene (Mahdavi et al., 1984). miR-208 is encoded in intron 27 of the human and mouse α-MHC genes, and controls regulation of β-MHC only in conditions of stress, not during normal cardiac development (van Rooij et al., 2007). The miR-208 knockout mouse is viable without an obvious cardiac phenotype at baseline apart from the upregulation of fast skeletal muscle contractile proteins and ‘stress proteins’ (ANF and BNF) at baseline. These genes are not direct targets of miR-208. When the knockout animals were subjected to TAC, they failed to produce
hypertrophic and fibrotic responses to pressure overload. In addition, apart from the expected upregulation of stress markers (such as ANF and BNP), an increase in β-MHC was not observed. In contrast, α-MHC was increased, rather than reduced. The protein product of a predicted miR-208 target, thyroid hormone receptor (TR)-associated protein 1 (THRAP1), the TR coregulator, was increased in miR-208 knockout mice. Treating the knockout animals with stress stimuli that cause reduction of α-MHC transcription also reduced the level of the pri-miR-208, which in turn relieves transcriptional repression on its target mRNA, thrap1. The resulting increase in THRAP1 protein influences the TR-regulated expression of both α- and β-MHC. Therefore, at least in mouse heart, miR-208 appears to mediate the switch from the expression of α-MHC to that of β-MHC during stress or thyroid hormone induced cardiac growth.

1.2.3 A Role for miR-29 in cardiac fibrosis

The expression patterns of miRNAs in mice and humans after myocardial infarction (MI) were examined by van Rooij et al. (2008b), which revealed dynamic regulation of miRNA expression during post-MI remodelling in both humans and mouse. Five miRNAs were found to have altered expression levels (miR-21, miR214 and miR-223 were upregulated; miR-29b and miR-149 were downregulated). Among the differentially regulated miRNAs, all members of the miR-29 family were downregulated. In vitro experiments confirmed miR-29 targets a group of key mRNA that encode fibrosis related proteins such as collagens and fibrillins, and the expression level of miR-29 in cardiac fibroblasts were considerably (5- to 12-fold) higher than in the cardiac myocytes. Systemic in vivo silencing of miR-29b using miR-29b specific antagomirs led to the induction of collagen mRNA expression in the heart. These data
suggest miR-29 functions as a negative regulator of collagen gene expression in the heart.

1.2.4 Gene regulation by miR-21 in cardiac fibroblasts

In addition to miR-29, miR-21 also has an important role in cardiac fibroblast function/activity of the failing heart. Thum and colleagues investigated the expression profile of miRNAs in a transgenic mouse model of heart failure (β₁-adrenergic receptor transgenic mice; Engelhardt et al., 1999), and identified that miR-21 expression is the most differentially regulated out of all miRNAs studied. Further experiments confirmed miR-21 to be significantly upregulated in other animal models of heart disease as well as in human heart failure (Thum et al., 2008b). Expression analysis across cell types using quantitative-PCR (q-PCR) showed miR-21 to be most highly expressed in cardiac fibroblasts and with low levels of expression in cardiac myocytes. While miR-21 levels increased in fibroblasts during heart failure, it was unchanged in cardiac myocytes. In functional studies, miR-21 regulated fibroblast survival and growth factor secretion via the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway, which is implicated in interstitial fibrosis. This control is mediated through the direct inhibition of a potent inhibitor of the ERK pathway, SPRY1 (sprouty homologue 1) by miR-21. *In vivo* silencing of miR-21 in a mouse pressure-overload-induced disease model (TAC) reduced cardiac ERK/MAPK activity and inhibited interstitial fibrosis and attenuated cardiac dysfunction.

1.3 Role for miRNAs in Type 2 Diabetes and diabetic heart

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterised by whole body insulin resistance, relative insulin deficiency and hyperglycaemia. In insulin resistant conditions, tissues fail to respond to insulin stimulation, there is a decrease in glucose uptake in skeletal muscle and blood glucose levels are increased (hyperglycaemia;
Shulman, 2000). As an insulin sensitive organ, the heart is affected by insulin resistance states. Patients with T2DM are at increased risk of developing heart diseases such as coronary artery disease (CAD) and LVD (Grundy et al., 1999; Nathan et al., 1997). As mentioned in the previous section, miRNAs have shown to have important functions in cardiac development and cardiac hypertrophy, however, their involvement in the diabetic heart and insulin resistance related LVD is still largely unknown. A number of miRNAs have recently been implicated in the regulation of insulin secretion and glucose homeostasis, suggesting their possible roles in T2DM and insulin resistance.

1.3.1 miR-375 regulates exocytosis of insulin in the pancreas

miR-375 is an important regulator of insulin exocytosis in the pancreatic islet. Poy and colleagues discovered that overexpression of miR-375 in pancreatic cell lines decreased glucose-induced insulin secretion with no alterations in glucose-mediated production of ATP. Furthermore, repression of miR-375 increased glucose-stimulated secretion of insulin. Myotrophin, a protein implicated in exocytosis, was identified as a direct target of miR-375. Interaction of miR-375 and the 3’UTR of myotrophin resulted in the repression of myotrophin translocation and inhibited insulin secretion (Poy et al., 2004). In addition to exocytosis, myotrophin also activate nuclear factor-κB (NFκB, p65) in cardiac myocytes (Gupta et al., 2002), which is associated with cardiac hypertrophy. NFκB activity in the pancreatic beta cells also associates with cytoskeleton remodelling, which results in increased glucose-stimulated insulin secretion (Hammar et al., 2005). Further investigations on the interaction of miR-375 or other miRNAs with myotrophin and NFκB in other systems such as the heart may provide insights to the function of miRNA in the insulin resistant heart.
1.3.2 miR-29 and insulin resistance

Two members of miR-29 family (miR-29a and miR-29b) were identified as upregulated in muscle, fat and liver of Goto–Kakizaki (GK) rats, a non-obese model of type 2 diabetes, as compared to control animals (He et al., 2007). Overexpression of miR-29a/b/c in 3T3-L1 adipocytes, a murine insulin-responsive fat cell line, inhibited insulin-stimulated glucose uptake. High levels of miR-29 led to insulin resistance mimicking the insulin resistance in cells incubated with high glucose and high insulin. miR-29a and miR-29b levels were upregulated in the presence of high glucose (hyperglycemia) and high insulin (hyperinsulinemia) in 3T3-L1 adipocytes. Insig1 (insulin-induced gene 1), an ER membrane protein that is involved in the control of cholesterol biosynthesis and Cav2 (caveolin 2) have been validated as targets of miR-29a/b/c (He et al., 2007).

1.3.3 miR-133 in diabetic heart

Apart from its role in cardiac hypertrophy, miR-133 was also identified to be differentially expressed in diabetic hearts (Xiao et al., 2007b). The upregulation of miR-133 in diabetic rabbit hearts was induced by the serum response factor (SRF), which was robustly activated in the diabetic heart. Delivery of exogenous miR-133 into the rabbit myocytes and cell lines led to post-transcriptional repression of HERG, the ether-a-go-go-related gene that encodes cardiac potassium channel responsible for rapid delayed rectifier K+ current (I(Kr)). This caused substantial depression of I(Kr), and the effect was abrogated by inhibition of miR-133. Silencing of SRF downregulated miR-133 expression and increased I(Kr) density. Repression of ERG by miR-133 is likely to be reflected by the differential changes of ERG protein and transcript, and thereby depresses I(Kr), which contributes to repolarization and the associated arrhythmias in diabetic hearts (Xiao et al., 2007b).
Collectively, these findings suggest the importance of miRNA mediated gene regulation in insulin resistance and possibly type 2 diabetes. However the role of miRNA in insulin signalling and glucose metabolism in the heart is still to be identified.

1.4 Insulin resistance and diabetic heart disease

Type 2 diabetic patients can develop cardiac function abnormalities even in the absence of predisposing conditions such as hypertension and CAD (Shishehbor et al., 2003). One of the earliest cardiac abnormalities that these patients develop is left ventricular diastolic dysfunction, which has been reported in 60% of patients with well-controlled T2DM (Poirier et al., 2001). In insulin resistant conditions, cardiac energy metabolism is impaired, left ventricular hypertrophy may develop and fibrosis is common, which causes increase in stiffness of the ventricular wall and impaired relaxation, leading to diastolic dysfunction (Gonzalez-Vilchez et al., 2005; Zarich et al., 1988). Additionally, systolic abnormalities may develop; these include, shortened systolic ejection time, diminished ejection fraction (EF) and increased systolic dimension and mass (Gonzalez-Vilchez et al., 2005; Sykes et al., 1977). If insulin resistance and the symptoms of LVD are left untreated, heart function can deteriorate further and heart failure symptoms and signs worsen. This section reviews current findings on insulin signalling in the heart and the molecular effects of insulin resistance on cardiac metabolism.

1.4.1 Insulin signalling in the heart

Insulin plays a key role in regulating glucose uptake and utilisation in the heart. Under normal physiological conditions, when blood glucose levels are high, insulin induces cardiac myocyte glucose uptake by binding to the tetrameric insulin receptor (IR), which consists of two extracellular α-subunits and two transmembrane β-subunits. The binding of insulin to the extracellular subunits of the receptor induces the activation of the tyrosine kinase activity of the β-subunits (Ottensmeyer et al., 2000), triggering the
autotransphosphorylation of the receptor where one β-subunit phosphorylates the other on several tyrosine residues. Once activated, the IR recruits and phosphorylates a number of downstream targets including the IR substrate (IRS) family, which in turn recruits and activates the phosphoinositide 3-kinases (PI3K) at the plasma membrane (Bertrand et al., 2008; Hirsch et al., 2007). PI3K is a heterodimeric protein composed of a p110 catalytic and a p85 regulatory subunit; it phosphorylates PI phosphates at the 3’ position. The main product of PI3K is PI3,4,5P$_3$ (PIP3) produced from the PI4,5P$_2$. Increase in PIP3 concentration in the plasma membrane induces the translocation of phosphoinositide-dependent kinase 1 (PDK1) and Akt (also known as Protein Kinase B, PKB) to the plasma membrane. PDK1 phosphorylates and activates Akt (Thr 308), which in turn, promotes the translocation of insulin sensitive glucose transporter (GLUT4) from the cytoplasm to the sarcolemmal membrane, to allow glucose entry into the myocyte (Zorzano et al., 1997; Figure 1.5).

Although the IR/PI3K/Akt pathway is well defined, in the heart, the downstream elements regulating glucose uptake are less well understood. Indeed, it was only recently shown that the Rab GTPase-activating protein (GAP) Akt substrate 160 (AS160) is required for membrane trafficking of the GLUT4 containing vesicles. Insulin induces phosphorylation of AS160, which leads to its inactivation, this prevents Rab GAP function and promotes GLUT4 translocation to the plasma membrane (Kramer et al., 2006; Miinea et al., 2005). Apart from signalling through Akt, insulin can also regulate glucose uptake via other PDK1 substrates, including the atypical protein kinase C (PKC) λ/ζ family, which has been implicated in GLUT4 membrane translocation (Farese et al., 2005). Other signalling pathways regulating GLUT4 translocation following insulin stimulation have also been described (Chang et al., 2004,
Figure 1.5 The insulin signalling pathway
2007; Watson and Pessin, 2007), however these mechanisms have not been studied in detail in the heart.

Apart from regulating glucose uptake, insulin signalling also controls protein synthesis in cardiac myocytes through modulation of several key translation factors and ribosomal proteins. Upon activation, Akt phosphorylates and inactivates the tuberous sclerosis factor (TSC2), a GAP of the G protein Rheb, which induces the activation of Rheb and in turn induces the phosphorylation and activation of mTOR (Inoki et al., 2002; Potter et al., 2002). The 4E-binding protein-1 (4E-BP1) and p70 ribosomal S6 protein kinase (p70S6K) are the main downstream targets of mTOR, where phosphorylation of both elements promotes protein translation (Pham et al., 2000; Proud and Denton, 1997; Rolfe et al., 2005). In addition, Akt can also inhibit GSK-3β (by phosphorylation of serine 9; Cross et al., 1995), which in turn activates eIF2B (eukaryotic initiation factor 2B), and thus induces protein synthesis (Welsh et al., 1997). GSK-3β and mTOR also play key roles in the development of cardiac hypertrophy (Frey and Olson, 2003) and this emphasises the central role for insulin in the regulation of cardiac hypertrophy.

1.4.1.1 Altered signalling in insulin resistant states

Insulin resistance is characterised by perturbed insulin-induced activation of the PI3K/Akt signalling pathway. Dysregulated insulin signalling can be mediated through diacylglycerol accumulation, which activates PKCs that in turn provoke serine phosphorylation of IRS1, as a result inhibiting the PI3K/Akt pathway (Savage et al., 2005). Or, insulin signalling by itself can activate negative feedback, where Ser/Thr kinases including mTOR and PKCαζ mediate phosphorylation (at serine/threonine residues) and inhibition of IRS proteins (Liu et al., 2001; Zick, 2005). Abnormal fatty acid metabolism also plays a major role in cellular insulin resistance (Shulman, 2000).
1.4.2 Glucose uptake and cardiac metabolism

The heart requires large amount of energy and oxygen to sustain its uninterrupted contraction/relaxation cycle. About 2% of total cellular ATP is consumed in each heart beat in the rat (Jacobus, 1985). In the normal heart, ATP generation comes from mitochondrial oxidation of different substrates, consisting 60-70% fatty acid (FA), 20% glucose and 10% lactate (Neely et al., 1972; Saddik and Lopaschuk, 1991). However, when circulating glucose and insulin concentrations rise, glucose becomes the favoured oxidised substrate of the heart. In T2DM, glucose uptake, glycolysis and pyruvate oxidation are impaired, lack of insulin function augments lipolysis and release of FA from the adipose tissue. The heart rapidly adapts to use FA exclusively for ATP production; this adaptation has a major role in the chronic development of diabetic cardiomyopathy.

Under normal physiological conditions, glucose is one of the major carbohydrates utilised by the heart. Cardiac glucose uptake is dependent on the transmembrane glucose gradient and the content of sarcolemmal glucose transporters (GLUT1 and GLUT4). GLUT1 is mostly localised in the sarcolemmal membrane and is responsible for basal cardiac glucose uptake. GLUT4 is the insulin-sensitive adult glucose transporter, and the majority of this isoform is located in an intracellular compartment under basal conditions. The translocation of GLUT4 from the cytosol to the plasma membrane requires insulin stimulation (Luiken et al., 2004b). Independent of insulin stimulation, GLUT4-mediated glucose uptake can also be induced by AMPK, which promotes GLUT4 redistribution to the sarcolemmal membrane (Li et al., 2004; Yang and Holman, 2005). Following translocation, glucose is converted to pyruvate through glycolysis, and the pyruvate produced is converted to acetyl-CoA by decarboxylation,
which enters the Citric Acid Cycle to be broken down into H₂O and CO₂ for ATP generation. Oxidation of one glucose molecule provides 30 ATP with 6 oxygen consumed.

1.4.2.1 Altered cardiac metabolism during insulin resistance

As compared with glucose, FA is the preferred substrate for ATP production in the normal heart. The ability to metabolise glucose provides an alternative energy source for the heart, especially under stressed conditions such as ischemia and pressure overload. However, in insulin resistant states, cardiac myocytes fail to take up circulating glucose due to the diminished response to insulin stimulation and the heart remains dependent on FA metabolism for energy generation. In patients with T2DM, enhanced lipolysis in adipose tissue and higher lipoprotein synthesis by the liver increases circulating free FA and triglycerides, this results in increased FA delivery to the cardiac myocytes. Although the heart adapts to utilise FA, when FA supply exceeds the oxidative capacity, the FA is converted to triglycerides or ceramide, which may worsen myocardial insulin resistance (Hajduch et al., 2008).

The reliance on lipid metabolism in insulin resistant states increases the intracellular glycogen pool through increased glycogen synthesis and/or impaired glycogenolysis (Higuchi et al., 1995; Laughlin et al., 1990). If glycogen accumulation persists, it may also directly impair insulin signalling (Jensen et al., 2006). The identification of a glycogen binding domain in AMPK suggests a possible role for the kinase in the regulation of glycogen metabolism (Hudson et al., 2003). Indeed, additional studies show that AMPK mutations can affect glycogen metabolism (Arad et al., 2002), whereas primary defects in glycogen metabolism activate AMPK while decreasing glycogen synthase activity (Nielsen et al., 2002).
1.4.3 Regulation of GLUT4 translocation and expression

As a result of its hydrophilic properties, glucose cannot pass through a lipid bilayer by diffusion. Instead, glucose enters the cell through glucose transporter-mediated import. Like many other inducible substrate transporters in the heart (e.g. long-chain fatty acids transporter fatty acid translocase FAT/CD36; Luiken et al., 2004a), GLUT4 is located in both sarcolemma and intracellular compartments. Following insulin stimulation, GLUT4 located in the intracellular pool is activated and translocated to the sarcolemma. In cardiac myocytes, GLUT4 is stored in two distinct compartments, the non-endosomal compartment (mainly for storage) and the endosomal compartment within the recycling endosomes (Fischer et al., 1997). Most GLUT4 molecules are stored in the non-endosomal compartment. Following insulin stimulation, GLUT4 vesicles translocate from the non-endosomal compartment to the recycling endosome and from there to the plasma membrane.

1.4.3.1 Translocation of GLUT4

Translocation of GLUT4 from the cytoplasm to the plasma membrane is a vesicle-mediated process. Irrespective of the specific signal transduction pathways required, they must impinge upon the GLUT4 storage compartments to induce GLUT4 trafficking. Thus, regulation of substrate uptake can occur via vesicle biogenesis, and its translocation to the cell membrane or exocytosis. Glucose transporter vesicles form the major part of insulin-responsive compartments (IRC). Although the mechanisms of GLUT4 vesicle biogenesis in the heart remain unclear, studies in adipocytes and muscle cells have shown these compartments are enriched for GLUT4, IRAP (insulin-regulated aminopeptidase) and VAMP2 (vesicle-associated membrane protein-2), but are absent of the small vesicle transport marker cellugyrin and endosome markers EEA1 and transferring receptor (Martin et al., 1996; Zeigerer et al., 2002). Coat and adaptor
proteins (e.g AP1) are recruited during vesicle formation, which drive the formation of the budding compartments on the donor membranes of the Golgi apparatus. In turn, the coat and adaptor proteins associate with donor membranes through interactions with cytoplasmic tails of cargo proteins (Gillingham et al., 1999; Li and Kandror, 2005). In addition, Golgi-localised γ-ear-containing Arf-binding protein (GGA) of coat adaptor proteins has been identified as a requirement for the sorting of GLUT4 protein from the Golgi to the IRC, but not from the IRC to the plasma membrane under the stimulation of insulin (Hou et al., 2006; Li and Kandror, 2005).

Despite intense investigation, the precise sub-cellular location and trafficking pathway of GLUT4 containing vesicles are still largely unclear. Studies in cultured adipocytes using GLUT4-GFP (green fluorescent protein) fusion proteins suggested the GLUT4 pool is peri-nuclear localised, and undergoes enhanced exocytosis and trafficking to the plasma membrane by insulin regulation (Zeigerer et al., 2002). A more recent study suggests GLUT4 vesicles are continuously trafficking to and from plasma membrane in absence of insulin. During insulin stimulation, these vesicles have increased pausing at the plasma membrane with higher probability of membrane fusion (Lizunov et al., 2005). The processes of docking, pausing (or tethering) and fusion of vesicles is again poorly understood. The docking of GLUT4 vesicles by the plasma membrane appears to require the exocytosis complex, where three components, Exo70, Sec6 and Sec8, redistributes to the plasma membrane in response to insulin (Ewart et al., 2005). Once at the membrane, SNARE (SNAP and NSF attachment receptors) complex proteins are required for the fusion of the vesicles. (Hong, 2005), of which VAMP2 is the predominant SNARE required for insulin stimulated GLUT4 translocation (Randhawa et al., 2000).
1.4.3.2 Expression of GLUT4

In addition to GLUT4 translocation, glucose uptake can also be regulated by controlling the expression of the GLUT4 protein. Regulation of GLUT4 gene transcription is conferred within a 2.4kb promoter region of the DNA. This region contains a MEF2 binding domain at position between -466 and -457, relative to transcription initiation site, which are essential for regulation of tissue specific expression of the gene (Thai et al., 1998). Two other regulatory factor binding sites have also been identified: thyroid hormone receptor binding site located between -457 and -426, and MyoD binding site, which is located next to the MEF2 site. It was found in rat cardiac muscle that full activation of GLUT4 transcription required the cooperation of these three elements and the presence a 82bp enhancer (at -502/-420) (Santalucia et al., 2001).

Insulin increases glucose uptake by stimulating GLUT4 translocation. In insulin resistant states, insulin-stimulated glucose uptake is decreased. GLUT4 protein and mRNA levels are decreased in adipose tissue during insulin resistance, however, regulation of GLUT4 expression by insulin is only observed in certain muscle cell types (Camps et al., 1992), suggesting insulin is only able to regulate GLUT4 gene expression in specific cell types/states. Insulin resistance associated with T2DM does not appear to be linked to changes in GLUT4 expression in skeletal muscle (Dohm et al., 1991; Handberg et al., 1990; Pedersen et al., 1990), and reduction in glucose uptake in the skeletal muscle in diabetic patients is not solely a result of deficient expression of GLUT4 (Friedman et al., 1992), but predominantly due to the failure of insulin stimulated recruitment of GLUT4 to the cell surface. In the heart however, studies in cultured rat cardiac myocytes revealed an increase in glut4 transcription and mRNA levels following insulin stimulation (Petersen et al., 1995). Promoter studies in human Glut4 have identified a fragment of DNA located in the promoter region (between -1154
and -730) that allows insulin dependent regulation in adipose tissue and cardiac muscle (Olson and Pessin, 1995). Indeed, members of nuclear factor I (NFI) family of transcription factors have been identified to mediate GLUT4 expression in adipocytes by binding to the insulin response element in the presence of insulin stimulation (Cooke and Lane, 1999b). However, transcription factors and enhancers involved in insulin mediated transcription of GLUT4 in the heart are still yet to be identified.

1.5 Aims of the Study

Previous studies have shown that miRNAs have important role(s) in heart development, cardiac hypertrophy and the development of metabolic disease. This PhD thesis characterises miRNAs in the insulin resistant heart, and determines a role of miRNAs in the pathophysiology of cardiac insulin resistance using in vitro and in vivo methodologies.

The specific aims of the study were:

1. To establish the experimental methodologies for miRNA studies in the host laboratory
2. To determine the expression of miRNAs in the normal human heart
3. To identify differentially expressed miRNAs in the insulin resistant human heart.
4. To prioritise miRNA(s) of interest and characterise their role in the heart.
5. To validate mRNA targets of miRNA(s) of interest using bioinformatic and experimental approaches.
6. To determine the function of miRNA(s) of interest using in vitro and in vivo methodologies
CHAPTER TWO

General materials and methods

Details of buffers and media used in this dissertation can be found in Appendix A. Maps of all plasmids can be found in Appendix B. Sequence information on primers can be found in Appendix C.

2.1 RNA isolation, quantification and quality check

2.1.1 RNA isolation

Total RNA was extracted using Trizol reagent (Invitrogen, Paisley, UK), and small RNA samples were then purified using mirVana™ miRNA Isolation kit (Ambion, Applied Biosystems, Austin, Texas, USA). This procedure isolated total small RNA species (<200nt) from large RNAs as separate samples, in accordance with the manufacturer’s protocol. In all instances, RNA was extracted from 20 – 50 mg of human or rodent tissue. Tissue samples were first homogenised in 1ml of Trizol, then treated with chloroform (200µl). The Phenol-chloroform phase and the aqueous phases were separated by centrifugation (12 000 × g, 4°C, 20 min). Total RNA was precipitated from the aqueous phase using isopropyl alcohol (0.5ml, Sigma) and washed with 75% ethanol (v/v; 1ml). After air-drying, the pellet was dissolved in RNase free water (Sigma). To extract RNA from cultured cells, 1ml of Trizol agent was added directly to the cells that were washed in cold phosphate buffered saline (PBS). For preparation of RNA samples enriched with small RNA and large RNA respectively, total RNA samples from the Trizol extraction were mixed with the Lysis/Binding Buffer and miRNA Homogenate Additive, then mixed with 100% ethanol (1/3 volume for large RNA and 2/3 volume for small RNA) to precipitate RNAs of difference sizes using two glass fibre filters. While attached to the filters, each RNA sample was washed with
Washing Buffers 1, 2 and 3. Each RNA sample was then eluted in 60µl of Elution Solution.

For preliminary assessment, sample concentration were measured by spectrophotometry (NanoDrop Technology, Delaware, USA) and the RNA quality was determined by the 260/280 absorbance ratio (acceptable ratio >1.9). For the initial study of miRNA expression in human heart samples, RNA integrity was assessed using both the RNA 6000 Nano (for total RNA samples) and the Picochip (for small RNA samples; Agilent 2100 Bioanalyser; Agilent Technologies) according to the manufacture’s protocols. 250ng (Nanochip) or 2.5ng (Picochip) of each sample was used for the analyses. In preparation for qRT-PCR analysis, RNA concentrations of human samples were also determined using RiboGreen (Invitrogen) based on standard curve analysis (Section 2.1.2). Quantified samples were diluted with RNase free water accordingly.

**2.1.2 RNA quantification using RiboGreen®**

RiboGreen® RNA quantitation reagent (Invitrogen) is a fluorescent RNA stain used for RNA quantification. The quantification was carried out following the manufacturer’s protocol. In brief, RiboGreen reagent and RNA samples were diluted in nuclease free 1×TE (Tris-EDTA, Invitrogen) buffer respectively. The standard curve samples were also prepared in 1×TE buffer consisting 7 concentration points ranging from 3µg/ml to 47ng/ml (Figure 2.1). Small RNA and large RNA stock samples were used for the standard curve accordingly for small RNA and large RNA quantifications. For each sample 100µl of diluted RNA was mixed with 100µl of diluted RiboGreen®. Mixtures were incubated for 5 min at room temperature before analysing on the fluorescent plate reader (FLUOStar OPTIMA, BMG Labtech, Aylebury UK). RNA sample concentrations were calculated from the standard curve based on the equation:
Figure 2.1 Standard curve of small RNA concentration in relation to RiboGreen fluorescent level. The graph plots the mean fluorescent level (determined by optical density) against the RNA concentration of each sample. Small RNAs were purified using mirVana miRNA Isolation Kit (Ambion) and concentrations of 47, 94, 187, 375, 750, 1500 and 3000ng/ml were used for the standard curve. 100µl of diluted RNA samples were mixed with 100µl of Ribogreen (1×). Levels of fluorescence were read in duplicate. Fluorescent level of both the standard curve samples and RNA samples for quantification were read on one plate using FLUOstar OPTIMA spectrophotometer.
\[ y = mx + c, \] where \( y \) = fluorescence, \( m \) = slope of standard curve, \( x \) = concentration in ng/ml, and \( c \) = y intercept.

### 2.1.3 Agilent Bioanalyser

The Agilent 2100 Bioanalyzer (Agilent Technology) is a microfluidics-based platform that can be used for the analysis of DNA, RNA, protein and cells. It performs capillary electrophoresis on a microchip, which uses microchannels (Ogura et al., 1998; Woolley and Mathies, 1994). The Bioanalyzer applies electrophoretic and electro-osmotic forces to drive fluids through capillaries to produce a virtual gel image and an electropherogram of the sample. Total RNA integrity check is one of the major applications of the Bioanalyzer. Using the RNA 6000 Chip, a high quality total RNA sample produces two dominating but distinct bands representing the 18S and 28S ribosomal RNA (rRNA), with a ratio 28S/18S of 2. However, a degraded total RNA sample produces more fragmented bands with a much lower 28S/18S ratio (Auer et al., 2003). In addition to total RNAs, the RNA 6000 chip can also be used to detect the integrity of samples enriched with small RNAs (Goff et al., 2005). Indeed, a RNA Chip designed for small RNA analysis was developed in 2007 (Agilent Technology). This allows the separation of various species of small RNA, including miRNAs, thus enables the user to identify small RNAs in total RNA extracts as well as optimising miRNA extraction procedures.

In this study, to assess RNA quality, 1µl of RNA (2.5ng or 250ng) from each sample was tested using the Agilent Bioanalyser RNA 6000 pico or nano assay. Before use, the Bioanalyser electrodes were cleaned by applying 350µl of RNAZap (Ambion, Huntington, UK) for one min followed by 350µl of water for 10 seconds. The electrodes were then allowed to dry. Filtered gel matrix was prepared by pipetting 550µl of RNA matrix into a spin filter and centrifuging at 1500 \( \times \) g for 10 min. This was aliquoted into
65µl aliquots for use. Immediately prior to running the Bioanalyser assay 1µl of vortexed RNA dye concentrate was added to a 65µl aliquot of filtered gel matrix and centrifuged at 13,000 × g for 10 min. 9µl of this gel dye mix was used to prepare the RNA 6000 nanochip in the chip priming station. Once the chip was primed another 9µl was pipetted into each well of the chip marked with a G. 5µl of RNA 6000 Nano Marker was added to the remaining 13 wells of the chip. 1µl of each sample and 1µl of RNA 6000 Ladder were denatured at 70°C for 2 min and placed on ice before being added to the wells on the chip. The chip was then vortexed for 1 min and using the *Eukaryotic total RNA Nano* assay. Procedures for using the RNA 6000 Pico assay are the same as the Nano assay, except Pico Assay specific reagents and settings were used and only 2.5ng of each RNA sample were tested.

**2.2 qRT-PCR**

**2.2.1 TaqMan based qRT-PCR for miRNA using stem-loop RT primers**

Specific mature miRNA expression levels were quantified by TaqMan® MicroRNA Assays (Applied Biosystems) using stem-loop primers for reverse transcription (RT) followed by q-PCR. For RT, each reaction contained 1mM dNTPs, 50U of MultiScibe™ Reverse Transcriptase, 3.76U of RNase Inhibitor, 3µl of RT-primer (5× concentration) and 5ng of small RNA sample, quantified by the standard curve method using RiboGreen (Section 2.1.2). Reactions were incubated for 30 min at 16°C, 30 min at 42°C and 3 min at 85°C. The q-PCR reaction was set up based on the concentrations and volumes recommended in the TaqMan 2× Universal PCR Master Mix (No AmpErase UNG) and TaqMan MicroRNA Assay protocols (Applied Biosystems). The reaction was performed on the 7500 Fast Real Time PCR Sequence Detection machine (Applied Biosystems) under the following conditions: 10 min at 95°C followed by 40
cycles of 15 seconds at 95°C, 60 seconds at 60°C, with data collection at end of each cycle. Each RT step was performed in duplicate, and for each RT reaction, the q-PCR step was performed twice. For qRT-PCR performed for validation and subsequent functional analyses, individual TaqMan MicroRNA Assays (purchased from Applied Biosystems) were used (Table 2.1). 5ng of small RNA sample was used in each reaction. All TaqMan probes were FAM labelled.

2.2.2 Two-step qRT-PCR mRNA assays

Total RNA was extracted, purified and quantified as described in Section 2.1. Samples were DNase treated by mixing 1µg of total RNA with 1µl of DNase I (Invitrogen), 1µl of 10×DNase I Reaction Buffer (Invitrogen) in a 10µl reaction and incubating at room temperature for 15 min. cDNA were synthesised using iScript™ cDNA Synthesis Kit (Bio-Rad) following manufacturer’s protocol. In brief, each 20µl reaction contained 1µg of total RNA, 4µl of 5×iScript Reaction Mix, 1µl of iScript Reverse transcriptase. The reaction mix was incubated at 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. For SYBR® Green based q-PCR, the 25µl reaction contained 12.5µl of SYBR® Green JumpStart Taq Ready Mix (Sigma), 200nM of each primer and 10ng cDNA (from iScript cDNA synthesis). Cycling conditions were: 94°C for 2 min followed by 40 cycles of 94°C for 15 seconds, 60°C for 1 min and 72°C for 1 min, with data collection at the end of each cycle. Dissociation analysis (melt curve) was performed at the end of each SYBR PCR run to verify the presence of a single amplification product. PCR primers were designed by using Primer 3 (http://frodo.wi.mit.edu; Misener and Krawetz, 2000). Primer pair selection criteria were: 17-22 nucleotides (nt) in length, at least one GC clamp on the 3’ end; GC content between 40 and 60%; non-complementary 3’ ends in primer pairs; and the difference of melting temperatures (Tm) is < 1°C. Primers were designed using mRNA transcript sequences, with primers
Table 2.1 List of Individual TaqMan MicroRNA Assays used*

<table>
<thead>
<tr>
<th>miRNA ID</th>
<th>Product Number</th>
<th>Target Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Let-7a</td>
<td>4373169</td>
<td>UGAGGUAGGUAGGUUGUAUAGUU</td>
</tr>
<tr>
<td>miR-16</td>
<td>4365552</td>
<td>UGUCAGUUUGUCAAAAUACCCCC</td>
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<tr>
<td>miR-29b</td>
<td>4373288</td>
<td>UAGCACCUUUGAAAUCAGUGUU</td>
</tr>
<tr>
<td>miR-30b</td>
<td>4373290</td>
<td>UGUAACAUCCUACACUCAGCU</td>
</tr>
<tr>
<td>miR-34a</td>
<td>4395168</td>
<td>UGGCAGUGUCUUAGCUGGUUGU</td>
</tr>
<tr>
<td>miR-125b</td>
<td>4373148</td>
<td>UCCUGAGACCCUAACUUGUGA</td>
</tr>
<tr>
<td>miR-187</td>
<td>4373307</td>
<td>UCGUGUCUUGUGUUGCAGCCGG</td>
</tr>
<tr>
<td>miR-199a</td>
<td>4373272</td>
<td>CCCAGUGUUCAGACUACCUGUUC</td>
</tr>
<tr>
<td>miR-214</td>
<td>4373085</td>
<td>ACAGCAGGCACAGACAGGCAG</td>
</tr>
<tr>
<td>miR-218</td>
<td>4373081</td>
<td>UUGUGCUUGAUCUAACCAUGU</td>
</tr>
<tr>
<td>miR-223</td>
<td>4373075</td>
<td>CGUGUAUUUGACAAGCUGAGU</td>
</tr>
<tr>
<td>miR-296</td>
<td>4373066</td>
<td>AGGGCCCCCCCUCCAAUCUGU</td>
</tr>
<tr>
<td>miR-335</td>
<td>4373045</td>
<td>UCAAGAGCAUAACGAAAAAUGU</td>
</tr>
<tr>
<td>miR342</td>
<td>4395371</td>
<td>UCUCACACAGAAUUCGCACCCGU</td>
</tr>
</tbody>
</table>

*At the time of thesis preparation, target sequences of each miRNA were conserved across human, mice and rat.
annealing to sites flanking exon-exon boundaries. Oligonucleotide primers were synthesised by Sigma-Genosys (Dorset, UK).

Primers used for SYBR q-PCR:

Glut4 (ENSRNOT00000023256):
Forward: 5’-GATTCTGCTGCCCTTCTGTC-3’ (bind to positions 792 to 811)
Reverse: 5’-CAGCTCAGCTAGTGCGTCAG-3’ (binds to positions 917 to 936)
Amplicon size: 145bp

For TaqMan based q-PCR, reactions were set up as per the product information sheets (2× Universal PCR Master Mix, No AmpErase UNG; Applied Biosystems). Primer and TaqMan® probe mix was manufactured by Applied Biosystems (pre-made, TaqMan® Gene Expression Assays). Each reaction contained 10µl of TaqMan 2× Universal PCR Master Mix (No AmpErase UNG), 1µl of 20× Primer and TaqMan® probe mix, 2µl of cDNA sample (5ng/ul), and made up to 20µl volume with nuclease free water.
Incubation conditions were: 95°C for 10 min, followed by 40 cycles of 30 seconds at 95°C and 1 min at 60°C, with data collection at the end of each cycle. For each q-PCR run, reactions containing no cDNA sample were used as no template control. All reactions were performed in duplicate, including negative controls. All TaqMan probes were 5’-FAM labelled, and all reagents and enzymes used for TaqMan based q-PCR analysis were supplied by Applied Biosystems.

TaqMan primer and probe sets used:
DICER1 (Assay ID: Hs00229023_ml)
Mef2c (Assay ID: Rn01494042_gl)

2.2.3 Data analysis
QRT-PCR results were analysed using the 7500 Fast System Software version 1.3.0 (Applied Biosystems). The threshold Cycle number (Ct) was determined for each
reaction at an appropriate threshold level (above baseline, within the exponential growth region of the amplification region of the amplification curve) and a baseline selected automatically by the software. Expression fold changes were determined by the ∆Ct method (Pfaffl, 2001). The analysis was performed based on the assumption of equal amplification efficiency among all miRNA species detected. Statistical analyses were performed using MS Office Excel and GraphPad Prism.

2.3 Northern Blotting for miRNA detection

Given that miRNAs are considerably shorter than other RNA species such as mRNAs, modifications have been made to the traditional Northern Blotting procedure for effective detection of miRNAs, however, overall the same principles apply.

Total RNA was extracted using Trizol (Section 2.1.1) and 10~30µg of RNA sample in a volume less than 10µl were mixed with equal volumes of bromophenol blue/xylene cyanol loading buffer (Gel loading buffer II, Ambion) and denatured for 10 min at 80°C. SpeedVac was used to concentrate the sample if the samples exceeded 10µl. Samples were loaded on 15% denaturing acrylamide gel with 8M urea (Sigma) and resolved at 150 volts until the bromophenol blue dye front reaches the bottom of the gel. The position of bromophenol blue and xylene cyanol in 15% gels corresponds to approximately 10 to 30nt respectively. The gel was then stained in 1× TBE buffer containing 4µg/ml of ethidium bromide for 10 min, and viewed under UV to verify equal loading using the 5S rRNA bands (121nt). After destaining, the RNA was transferred to positively charged nylon membrane (BrightStart, Applied Biosystems – Ambion) using a semi-dry transfer cell (Bio-Rad), and the membrane was UV-cross linked at 1200uJ (×100) for 30 seconds using the Stratalinker® UV Crosslinker (Stratagene, La Jolla, CA, USA). Membranes were prehybridised in Ultrahyb Oligo
solution (Applied Biosystems – Ambion) for 1 hour at 42°C. DNA oligonucleotides, complementary to the mature miRNAs were obtained from Sigma-Genosys (Dorset, UK), were 5'-end labelled with Redivue adenosine 5'[-32P] triphosphate, triethylammonium salt (PerkinElmer, Waltham, Massachusetts, USA) using mirVana Probe and Marker Kit (Ambion, see Section 2.3.1) and added to the hybridization buffer. The blot was hybridized overnight at 42°C and then washed twice with 2× sodium chloride sodium citrate buffer (SSC) containing 0.5% sodium dodecyl sulfate (w/v, SDS) and exposed to X-ray film (Sigma) between intensifying screens for 18-24 hours at -80°C. Blots were stripped using 0.5% (w/v) SDS for 1 hour at 60°C and reprobed for 5S rRNA after prehybridization as loading controls.

Denaturing acrylamide gel was prepared with components listed below:

<table>
<thead>
<tr>
<th></th>
<th>x1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Urea</strong></td>
<td>7.2g</td>
</tr>
<tr>
<td><strong>10x TBE</strong></td>
<td>1.5ml</td>
</tr>
<tr>
<td><strong>40% Acrylamide (acry:bis acry=19:1)</strong></td>
<td>5.6ml</td>
</tr>
<tr>
<td><strong>DEPC treated water</strong></td>
<td>To 15ml</td>
</tr>
<tr>
<td>Mix well until urea is dissolved, then add</td>
<td></td>
</tr>
<tr>
<td><strong>10% APS</strong></td>
<td>75ul</td>
</tr>
<tr>
<td><strong>TEMED</strong></td>
<td>15ul</td>
</tr>
<tr>
<td>Mix well and pour gel immediately</td>
<td></td>
</tr>
</tbody>
</table>

### 2.3.1 Probe Labelling

DNA oligonucleotides probes were 5’-labelled with Redivue adenosine 5'[-32P] triphosphate, triethylammonium salt (#EG502Z250UC PerkinElmer, Waltham, Massachusetts, USA) using mirVana Probe and Marker Kit (Ambion) according to the following recipe:
The mixture was then incubated at 37°C for 1 hour, and kept on ice or stored at -20°C until needed.

Sequence for miR-223 probe: 5’-GGGGTATTTTGACAAACTGACA-3’

Sequence for miR-296 probe: 5’-GGAGAGCCTCCACCCAACCCTC-3’

Sequence for 5S rRNA: 5’-TTAGCTTCCGAGATCA-3’

### 2.4 Cloning

All restriction endonucleases used in this study were purchased from New England Biolabs.

#### 2.4.1 DNA amplification (PCR) and agarose gel electrophoresis

PCR primers were designed by using Primer 3 (http://frodo.wi.mit.edu; Misener and Krawetz, 2000). Primer pair selection criteria were: 17-22 nucleotides (nt) in length, at least one GC clamp on the 3’ end; GC content between 40 and 60%; non-complementary 3’ ends in primer pairs; and the difference of melting temperatures (Tm) is < 1°C. Oligonucleotide primers were synthesised by Sigma-Genosys (Dorset, UK). PCR reactions were performed in 10µl volumes and contained a final concentration of 1× reaction buffer, 5mM of MgCl₂, 0.2mM dNTPs, 1 mM of each oligonucleotide
primer and 0.75U of *Taq* polymerase (Bioline, London, UK). Genomic DNA was used at a final concentration of 5ng/µl. Reactions containing water instead of DNA were set as negative controls. PCR was carried out using Tetrad thermal cycler (MJ Research, Rayne, UK). Standard amplification conditions were 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. For PCR using AmpliTaq Gold (Applied Biosystems, Warrington, UK), the initial denaturation step was increased to 12 min. DNA products were resolved by agarose gel electrophoresis. For sequence information on all primers used for DNA amplification, please refer to Appendix C.

Agarose gel electrophoresis was carried out using horizontal submarine agarose gels. Gels were made by dissolving agarose powder (Invitrogen, Paisley, UK) in 1× TAE (Tris-acetate-EDTA, 80°C) buffer. Agarose was cooled to 60°C before pouring. For agarose gels used for DNA gel purification, 1% to 2% (w/v) standard agarose and NuSieve agarose (Cambrex BioScience, Rockland, ME, USA) was combined in a ratio of 1:1. Ethidium bromide was added to the gel and running buffer at the final concentration of 10µg per 100ml of gel or running buffer. DNA samples were loaded with 1/5 volume of loading buffer (Orange G) and electrophoresis carried out at 100 volts for 35 min. Quick-Load 2-Log DNA Ladder (New England Biolabs) was used as DNA size markers. DNA was visualised using the InGenius gel documentation and analysis system (Syngene, Cambridge, UK).

**2.4.2 TA cloning**

PCR products amplified by *Taq* polymerase have a single deoxyadenosine (A) at 3’ ends due to *Taq*’s non-template-dependent terminal transferase activity. This can be used to clone the product into a plasmid vector that has a single overhanging 3’ deoxythymidine
For cloning into the pCR 2.1-TOPO® vector (Invitrogen, Appendix B), 2µl of PCR product was mixed with 1µl of Salt Solution, 1µl of pCR 2.1-TOPO® vector and 2µl of water in a final volume of 6µl. The mixture was incubated at room temperature for 5 min. Then 2µl of this ligation mixture was transformed into chemically competent *E. coli* cells. For cloning into pTargeT™ Vector (Promega, Madison, WI, USA; Appendix B) the 10µl ligation mixture consisted 1µl of 10×ligation buffer, 3U of T4 DNA ligase (Promega), 1µl (60ng) of pTargeT™ Vector, and the appropriate amount of PCR product calculated according to the insert/vector molar ratio of 3:1 using the equation:

\[
\frac{ng\ of\ vector \times kb\ size\ of\ insert}{kb\ size\ of\ vector} \times \frac{3}{1} = ng\ of\ insert
\]

The ligation mix was then incubated at 4°C overnight. 2µl of the ligation reaction was then transformed into chemically competent *E. coli* cells.

### 2.4.3 Transformation of chemically competent *E. coli* cells

Chemically competent ONE SHOT® TOP10 *E. coli* were purchased from Invitrogen. For each transformation, one vial (~50µl) of cells was allowed to thaw on ice for 5 min. 2µl of ligation mixture was added to the competent cells, and incubated on ice for 20 min. The cells were then heat shocked at 42°C for 30 seconds and returned on ice immediately. 250µl of room temperature SOC medium (super optimal broth with catabolite; Invitrogen) was added to the transformed cells, and incubated at 37°C with shaking (~200rpm) for 1 hour. 50µl of each transformation was plated onto pre-warmed LB (Lysogeny Broth) agar plate containing ampicillin (50µg/ml) and x-gal (5-bromo-4-chloro-3-indolyl beta-galactoside, 40mg/ml), and incubated at 37°C overnight. Cells were plated onto pre-warmed LB agar plate containing ampicillin (50µg/ml) or kanamycin (50µg/ml), if blue/white colony selection is required, IPTG (0.5mM) and x-gal (5-bromo-4-chloro-3-indolyl beta-galactoside, 80µg/ml) are also added to the agar
Chapter Two

plate. Plates were then incubated at 37°C overnight. For both pCR2.1-TOPO® and pTargeT™ vector transformations, white colonies were picked for screening of the insert; colonies were screened by PCR using forward primer binding to the T7 promoter (5’-TAATACGACTCACTATAGG-3’) and the reverse primer of the PCR product. Clones positive for the insert were sequenced to confirm the insert sequence and orientation.

2.4.4 Restriction digests
All restriction digests were performed using restriction endonucleases supplied by New England Biolabs (Hitchin, UK). All reactions were carried out in a 20µl reaction volume consisting 20U of each restriction enzyme used, 0.5-2µg of DNA, 2µg of Bovine Serum Albumin (BSA), and 2µl of 10× reaction buffer that is recommended for the enzymes used in the reaction. For double digests, the compatibility of restriction enzymes was determined using NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/index.php). All reactions were incubated for 2 hours at 37°C, and enzymes were denatured at 65°C for 15 min. Digested products were resolved by agarose gel electrophoresis.

2.4.5 Standard protocol for directional cloning
Both the vector and the TA plasmid containing the inserts were cut with two different restriction enzymes that generated non-complementary sticky ends at either end of each restriction fragment. Both fragments were resolved on an agarose gel and DNA bands representing the fragments were excised out of the gel and DNA was purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The insert was ligated to the vector in a 20µl reaction containing 400 Units of T4 DNA ligase (New England Biolabs), 2µl of 10× buffer, 200-300ng of cut vector, and the appropriate amount of insert DNA according to the 3:1 insert to vector molar ratio (calculated using the equation in Section 2.4.2). The ligation mix was incubated at room temperature for
Chapter Two

12 min and kept at 16°C until use. 2µl of the ligation mix were transformed into TOP10 chemically competent cells (Invitrogen) as described in Section 2.4.3. Positive clones were screened by PCR using primers that was originally used for insert amplification. Clones positive for the insert were sequenced to confirm the insert sequence and orientation.

2.4.6 DNA Sequencing

For sequencing of PCR products, DNA from 50µl reactions were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). For sequencing of plasmid DNA, plasmids were prepared using Wizard Plus SV Minipreps DNA Purification System (Promega). Purified DNA samples were quantified by Spectrophotometry (Nanodrop Technology). Each sequencing template reaction contained ~200ng of DNA, 3.2pmol of primers and water in a total volume of 10µl. Samples were sent to the Genomics Core Laboratory (Clinical Sciences Centre, MRC) for cycle sequencing, purification and electrophoresis according to the protocols below:

Samples are cycle sequenced using Big Dye v3.1 chemistry (Applied Biosystems, Warrington, UK). 5µl of the template reaction was added to 0.9µl of BigDye, 2µl of water and 2µl of 5× buffer. Reactions were cycled under the following PCR conditions: 95°C for 1 min, followed by 30 cycles of 95°C for 10 seconds, 55°C for 15 seconds and 60°C for 4 min, then hold at 4°C. The PCR reactions were then cleaned up using an EDTA-Ethanol precipitation: 5µl of 125mM EDTA and 30µl of 100% Ethanol was added to 10µl of sequencing reaction and incubated at room temperature for 15mins before centrifugation at 3,000g for 30mins. The pellets are washed once with 100% ethanol and then twice with 70% ethanol. DNA was resuspended in 10-12µl of HiDi Formamide, and run on the Applied Biosystems Ltd 3730xl DNA Analyzer.
All DNA templates were sequenced in the sense and anti-sense strand. DNA sequences were analysed using the Sequencher software (Gene Codes Corporation, An Arbor, MI, USA).

2.4.7 Mini plasmid preparation

All mini plasmid preparations (miniprep) were carried out using the Wizard Plus SV Minipreps DNA Purification System (Promega) following manufacturer’s protocol. 1 to 5ml of LB culture containing the appropriate antibiotics were inoculated with a single colony containing the plasmid of interest, and incubated overnight at 37°C with shaking (~200rpm). Bacteria were harvested by centrifugation for 5 min at 10 000 × g at room temperature. Each cell pellet was resuspended with 250µl of Cell Resuspension Solution by vortexing, and cells were lysed with 250µl of Cell Lysis Solution and incubation at room temperature for 5 min. After treatment with 10µl Alkaline Phosphatase Solution, the mixture was neutralised with 350µl of Neutralization Solution and centrifuged at 14 000 × g at room temperature for 10 min. The cleared supernatant was then applied to a spin column for DNA binding. The bound DNA was then washed twice with Column Wash Solution, then plasmid DNA is eluted from the column with water. Plasmid DNA was then quantified by spectrophotometry (Nanodrop).

2.4.8 Maxi plasmids preparation

Plasmids required for further experiment purposes in large quantities were prepared using the QIAfilter Plasmid Maxi Purification Kit (QIAGEN, Crawley, UK) following manufacturer’s protocol. 250ml of LB medium was inoculated with miniprep culture of the plasmid of interest, and incubated at 37°C overnight with shaking (~300rpm). Bacterial cells were harvested by centrifugation at 6000 × g for 15 min at 4°C. Bacterial pellet was resuspended in 10ml of Buffer P1 (containing RNase A), and mixed with 10ml of Buffer P2 and incubated at room temperature for 5 min to allow lysis of the
cells. 10ml of chilled Buffer P3 was then added to the lysate and the mixture was filtered through the QIAfilter Cartridge to remove the precipitate that contains protein, genomic DNA and detergent. The cleared lysate was then applied to pre-equilibrated QIAGEN-tip 500 to allow DNA binding to the resin by gravity flow. The QIAGEN-tip was then washed twice with 30ml of Buffer QC before the plasmid DNA was eluted in 15ml of Buffer QF, and DNA was precipitated with 0.7 volumes (10.5ml) of room temperature isopropanol, and centrifuged at 15 000 × g for 10 min. The DNA pellet was then washed with 5ml of room temperature 70% ethanol and centrifuged again at 15 000 × g for 10 min. After 10 min of the air-drying, the plasmid DNA was dissolved in TE buffer. DNA was quantified by spectrophotometry (Nanodrop).

2.5 Cell culture

2.5.1 Preparation of Neonatal Rat Ventricular Myocytes

The neonatal rat ventricular myocyte (NRVM) culture was first described by Harary and Farley (Harary and Farley, 1963a, b); since then, they have been widely used as an in vitro model system. The non-dividing and spontaneously beating NRVMs enable the study of the morphological, biochemical and electrophysiological characteristics of the heart for studies of hypertrophy, ischaemia, hypoxia etc (Bahi et al., 2006; Long et al., 1992; Yamashita et al., 1994).

NRVM cultures were prepared by an adaptation of the method of (Iwaki et al., 1990). One to two day old neonatal Sprague-Dawley rats (Harlan, Oxford, UK) were sacrificed by decapitation. Hearts were removed and collected in ADS buffer (Appendix A) kept on ice. Once all dissections were complete, the atria were removed. Hearts were then minced in ADS buffer, and then the minced tissue pieces were transferred to a 100ml bottle. For the initial digest, 10ml of enzyme mix (collagenase type 2, 115U/ml,
Worthington Biochemical Corp #CLS-2, and Pancreatin, 0.6mg/ml, Sigma-Aldrich #P3292, dissolved in ADS buffer and filtered through 20µm syringe filter) was added to the tissue and the mixture was incubated with shaking (160 strokes per min) for 5 min at 37°C. The supernatant was discarded. The tissues were then digested for 5 more times with 6-10ml of enzyme mix at shaking speed of 130-140 strokes per min. At the end of each digest, the supernatant was collected and centrifuged at 1000rpm for 5 min. The cell pellet were resuspended with foetal calf serum (FCS) and kept at 37°C. Cells from all digest were pooled in one tube after the last digest, and this was centrifuged at 1000rpm for 7 min. To remove the fibroblasts, cells were then resuspended in plating media (Appendix A) and pre-plated onto non-gelatin coated Primaria dishes (BD Biosciences, San Jose, CA, USA) and incubated for 1 hour at 37°C. After pre-plating, un-attached cells were washed off the plate surface with plating media, and cells were plated onto gelatin coated Primaria dishes at a density of $1.5 \times 10^3$ cells per mm$^2$ and incubated at 37°C with 5% CO$_2$ overnight. After incubation, cells were treated with serum free maintenance media (80% Dulbecco's Modified Eagle's Medium, 20% Media 199) supplemented with 1% penicillin and streptomycin (pen/strep) solution (Sigma) 24 hours prior to further experiments.

2.5.2 COS7 cells

COS7 cells were maintained using DMEM (Dulbecco’s Modified Eagle Medium, Invitrogen) supplemented with 10% (v/v) FCS and 1% (v/v) pen/strep solution at 37°C, 5% CO$_2$. Cells were seeded onto appropriate culture plates at the density of ~500 cells/mm$^2$ 18 hours prior to transfection. Cells were passaged at the ratio of 1 to 10 when density reached 80% confluency.
2.5.3 Transfection protocol

All transfections were performed using lipid based transfection reagent Lipofectamine™ 2000 (Invitrogen) following the manufacturer’s protocol. In brief, cells were seeded and serum-starved 24 hours before transfection. For each transfection, the specified amount of DNA or RNA oligonucleotides was mixed with Opti-MEM (Invitrogen), and separately, Lipofectamine was diluted in Opti-MEM (Table 2.2). The mixtures were incubated at room temperature for 5 min, and then combined together by adding the DNA/RNA mix to the Lipofectamine solution; this was mixed gently and incubated at room temperature for 20 min. This transfection mixture was then added to the cells. Cells were then incubated for 4 hours under normal maintenance conditions, and cell culture media changed where necessary. Cells were harvested 24-48 hours after transfection.

2.6 Protein extraction and quantification

For isolation of whole cell protein extracts from human or animal tissue, ~50mg of tissue was homogenised in 1ml of 1× cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with 1mM PMSF (phenylmethylesulphonylfluoride) and 1× protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Mannheim, Germany). Lysates were centrifuged at 14 000 × g for 10 min at 4°C. Supernatant was collected and protein concentration was determined by Bradford assay using Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA, USA) against BSA standard curve (5µg/ml ~ 40µg/ml). For protein extraction from cultured cells, cells were washed with cold PBS and treated with 200µl of 1× cell lysis buffer with PMSF. Collected Lysates were centrifuged as above.
Table 2.2 Amount of transfection reaction reagents used for various culture vessels

<table>
<thead>
<tr>
<th>Culture Vessel</th>
<th>Volume of plating media</th>
<th>Volume of Opti-MEM</th>
<th>Volume of Lipofectamine 2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well plate</td>
<td>100µl</td>
<td>2×25µl</td>
<td>0.5µl</td>
</tr>
<tr>
<td>24-well plate</td>
<td>500µl</td>
<td>2×50µl</td>
<td>2µl for DNA 1µl for RNA</td>
</tr>
<tr>
<td>6-well plate/35mm dish</td>
<td>2ml</td>
<td>2×250µl</td>
<td>8µl</td>
</tr>
<tr>
<td>60mm dish</td>
<td>4ml</td>
<td>2×500µl</td>
<td>16µl</td>
</tr>
</tbody>
</table>
For membrane protein extraction (P1), whole mouse LV was homogenised in a buffer containing 20mM Hepes, 250mM sucrose, 1mM EDTA, 1mM PMSF and a cocktail of protease inhibitors (5mM benzamidine, 1µM aprotinin, 1µM pepstatin, 1µM leupeptin). The homogenate was centrifuged at 2000 × g for 10 min (4°C), and pellet was discarded and the supernatant was centrifuged again at 9000 × g for 20 min at 4°C. The pellet (membrane protein) was resuspended PBS supplemented protease inhibitor cocktail.

### 2.7 Immunoblotting

Protein samples were resolved by sodium dodecyl sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) using mini PROTEAN 3 system (Bio-Rad). Gels were prepared with components listed below:

<table>
<thead>
<tr>
<th>For two gels:</th>
<th>Resolving Gel (10%)</th>
<th>Stacking Gel (4%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% acrylamide (w/v)</td>
<td>3.75ml</td>
<td>0.75ml</td>
</tr>
<tr>
<td>2% bis (w/v)</td>
<td>2.00ml</td>
<td>0.41ml</td>
</tr>
<tr>
<td>20% (w/v) SDS</td>
<td>75µl</td>
<td>37.5µl</td>
</tr>
<tr>
<td>1M Tris pH8.8</td>
<td>5.63ml</td>
<td>-</td>
</tr>
<tr>
<td>1M Tris pH6.8</td>
<td>-</td>
<td>0.95ml</td>
</tr>
<tr>
<td>10% (w/v) Ammonium Persulphate (APS)</td>
<td>113µl</td>
<td>112.5µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>15µl</td>
<td>7.5µl</td>
</tr>
<tr>
<td>Distilled De-ionised Water</td>
<td>3.42ml</td>
<td>5.23ml</td>
</tr>
<tr>
<td>Total Volume</td>
<td>15ml</td>
<td>7.5ml</td>
</tr>
</tbody>
</table>

Protein samples were prepared to 1~3µg/µl with NuPage LD Sample Buffer and NuPage Reducing Reagent (Invitrogen) and boiled for 5 min at 95°C before loading onto the gel. 10µg~30µg of protein were loaded in each lane. Gels were run in Tris-Glycine-SDS Buffer (Bio-Rad) for 30 min at 100V then at 150V for 60-80 min,
depending on the molecular weight of protein. Precision Plus Protein Standards were used as molecular size marker (Bio-Rad).

When SDS-PAGE was complete, resolved proteins were transferred (wet) to Protran® Nitrocellulose Transfer Membrane (Whatman, Maidstone, UK) by electrophoresis using Bio-Rad mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) at 60V for 1 to 2 hours, depending on the molecular weight of protein. Membranes were blocked in hybridisation buffer at room temperature for 20 min, and hybridised with specified primary antibody in hybridisation buffer at 4°C overnight. Membrane was washed three times in Tris buffered saline (TBS) supplemented with 0.05% (v/v) Tween 20 (Sigma), then probed with secondary antibody at room temperature for 1 hour in TBS supplemented with 1% (w/v) milk and 0.05% (v/v) Tween 20. The membrane was washed three times again in TBS with Tween 20. Proteins were visualised using chemiluminescence (20× LumiGLO Reagent and 20× Peroxide, Cell Signaling). Signals were detected by exposing X-ray film (Kodak Scientific Imaging) to the membrane for 30 seconds to 10 min, depending on the intensity of the signal. List of antibodies used can be found in Table 2.3. Details of buffers can be found in Appendix A.

2.8 Statistics

All data are represented as means ± SEM and statistical significance were tested by Student’s t-Test or ANOVA using MS Excel or GraphPad Prism 4, unless otherwise stated. Specific statistical analyses are described in detail in the relevant Chapter where the test is applied.
### Table 2.3 List of antibodies used.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Working dilution</th>
<th>Host</th>
<th>Supplier &amp; catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Akt</td>
<td>1 in 1000</td>
<td>Rabbit</td>
<td>Cell Signaling 9272</td>
</tr>
<tr>
<td>Anti-phospho-Akt (ser473)</td>
<td>1 in 1000</td>
<td>Rabbit</td>
<td>Cell Signaling 9271</td>
</tr>
<tr>
<td>Anti-GSK3β</td>
<td>1 in 1000</td>
<td>Rabbit</td>
<td>Cell Signaling 9315</td>
</tr>
<tr>
<td>Anti-phospho-GSK3β (ser9)</td>
<td>1 in 1000</td>
<td>Rabbit</td>
<td>Cell Signaling 9336</td>
</tr>
<tr>
<td>Anti-insulin receptorβ</td>
<td>1 in 1000</td>
<td>Rabbit</td>
<td>Upstate (Millipore) 33460</td>
</tr>
<tr>
<td>Anti-Glut4</td>
<td>1 in 2500</td>
<td>Rabbit</td>
<td>Abcam ab645</td>
</tr>
<tr>
<td>Anti-Glut1</td>
<td>1 in 1000</td>
<td>Rabbit</td>
<td>Abcam ab32551</td>
</tr>
<tr>
<td>Anti-NFIA</td>
<td>1 in 1000</td>
<td>Rabbit</td>
<td>Abcam ab11988</td>
</tr>
<tr>
<td>Anti-Mef2c</td>
<td>1 in 500</td>
<td>Goat</td>
<td>Santa Cruz sc-13266</td>
</tr>
<tr>
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<td>Upstate (Millipore) 06-248</td>
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<td>Mouse</td>
<td>Abcam ab8226</td>
</tr>
<tr>
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<td>Mouse</td>
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CHAPTER THREE

Expression profiling of miRNAs in the human heart

3.1 Introduction
At the outset of this study, miRNAs had been shown to have a major role in gene expression control during many biological processes, and when dysregulated, could contribute to disease pathophysiology (Alvarez-Garcia and Miska, 2005). Several expression profiling studies of miRNAs in animal models of heart disease and end-stage heart failure have been reported since 2006, and a number of miRNAs are dysregulated in the diseased heart (reviewed by Latronico et al., 2007). However, miRNA expression studies of the insulin resistant human heart have not been reported, to date. In this Chapter, the expression levels of 155 miRNAs in left ventricular biopsy samples from patients with T2DM and control samples are examined using TaqMan-based stem-loop qRT-PCR. As LVD is also associated with insulin resistance independent of T2DM, biopsy samples from patients with LVD were also collected and levels of miRNAs differentially regulated in the T2DM group were further assayed in the LVD group.

3.2 Materials and Methods

3.2.1 Study population and sample procurement
Left ventricular biopsies were obtained from patients undergoing coronary artery bypass graft surgery who were suitable for inclusion (in collaboration with Prof PG Camici and Mr Punjabi; approved by Research Ethics Committee, Hammersmith Hospital; reference number: LREC 2003/6699). Inclusion criteria: listed for coronary bypass surgery, age 35-75 years, creatinine < 200 µmol/l, an echocardiogram and coronary angiogram within 3 months. Exclusion criteria were myocardial infarction within 3 months, left ventricular ejection fraction <15%, claustrophobia, viability in less than
35% of the left ventricle as determined by cardiac MRI or positron emission tomography (PET), coincident left ventricular dysfunction and T2DM, or an estimated operative mortality of >15%. Patients were assigned to one of three groups: control, T2DM or LVD. Patients were considered to have LVD, if their ejection fraction was less than 40%; biopsies were taken from normally contracting regions with no evidence of infarction. All patients with T2DM had previously been diagnosed with the condition and all had normal systolic left ventricular function (Table 3.1). All patients were managed with optimal medical therapy which was discontinued the evening prior to surgery. The study protocol was approved by the Administration of Radioactive Substances Advisory Committee in compliance with guidelines. Written consent was obtained from all patients.

### 3.2.2 RNA isolation, quantification and quality check

Tissues samples were processed and RNA was extracted as described in detail in Chapter 2, Section 2.1. RNA samples were quantified using NanoDrop (Section 2.1.1) and the RiboGreen (Section 2.1.2). Sample quality was assessed using the Bioanlyser (Section 2.1.3).

### 3.2.3 qRT-PCR

#### 3.2.3.1 miRNA specific qRT-PCR with stem-loop RT primers

For the initial miRNA expression profiling of the human biopsy samples, specific mature miRNA expression levels were quantified by TaqMan® MicroRNA Assays Human Panel Early Access kit (Applied Biosystems) using stem-loop primers for reverse transcription (RT) followed by real-time PCR. Hsa-let-7a and hsa-miR-16 served as positive, ubiquitously expressed controls, and non-human cel-miR-2, cel-lin-4 and ath-miR159a were used as negative controls. For each 96-well reaction plate, the expression of miR-16 (TaqMan MicroRNA Assay, Applied Biosystems, Table 2.1) was
Table 3.1 Patient characteristics (n=6 per condition). Data are represented as mean ± SD. ** *P*<0.001, relative to control. T2DM, type 2 diabetes mellitus; LVD, left ventricular dysfunction; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; EF, ejection fraction; BG, blood glucose; Hb, haemoglobin; HbA1c, glycated haemoglobin; WBC, white blood count; BUN, blood urea nitrogen

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<td>70 ± 10.7</td>
<td>30 ± 9.0**</td>
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</table>
measured in triplicate in a rat RNA sample, which is used as an amplification efficiency control. For RT, each reaction contained 1mM dNTPs, 50U of MultiScibe™ Reverse Transcriptase, 3.76U of RNase Inhibitor, 3µl of RT-primer (5×) and 5ng of RNA sample. Reactions were incubated for 30 min at 16°C, 30 min at 42°C and 3 min at 85°C. The q-PCR reaction was set up based on the recommended concentrations and volumes from the TaqMan 2× Universal PCR Master Mix (No AmpErase UNG) and TaqMan miRNA Assays protocol (Applied Biosystems). The reaction was performed using the 7500 Fast Real Time PCR Sequence Detection machine (10 min at 95°C followed by 40 cycles of 15 seconds at 95°C, 60 seconds at 60°C), with data collection at end of each cycle. For the Human LV miRNA expression screen, biopsy sample size and technical complexity of the assay required pooling of the RNA samples of each patient group. Each RT step were performed in duplicate, and for each RT reaction, the q-PCR step was performed twice. For qRT-PCR validation experiments, 5ng of RNA sample was used in each reaction, and each RT step were performed once and the q-PCR step was performed twice. All TaqMan probes used were FAM labelled.

### 3.2.3.2 U6 snRNA and 5S rRNA levels

Levels of U6 small nuclear RNA (snRNA) and 5S ribosomal RNA (rRNA, mirVana™ qRT-PCR Primer Set for Normalization, Ambion) selected for data normalisation were determined using mirVana qRT-PCR miRNA Detection Kit (Ambion/Applied Biosystems) in small RNA samples (20ng/reaction). Reagents were supplied as part of the Detection Kit, apart from SYBR Green I (10,000×) and Taq polymerase (5U/µl), which were obtained from Invitrogen. The two-step qRT-PCR was performed according to manufacturer’s protocol. In brief, each RT reaction contained 2µl of 5× mirVana RT buffer, 1µl of 1× mirVana RT primer, 2µl of small RNA (10ng/µl), 0.4µl of ArrayScript Enzyme Mix, made up to 10µl total volume with nuclease free water. The reaction was
incubated (37°C, 30 min; 95°C, 10 min), followed by q-PCR using 5µl of 5× mirVana PCR buffer (contains 1.5× SYBR Green I), 0.2µl of Taq polymerase (5U/µl, Invitrogen), 0.5µl of 1× PCR primers, 10µl of the RT reaction, and nuclease free water (25µl final reaction volume). The reaction was performed using the 7500 Fast Real Time PCR Sequence Detection machine (Applied Biosystems) under the following conditions: 95°C for 3min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds, with data collection at end of each cycle. Dissociation analysis (melt curve) was performed immediately following the amplification cycles (95°C for 15 seconds, 60°C for 15 seconds, followed by a slow ramp to 95°C) to verify the presence of a single amplification product.

3.2.3.3 Data analysis
QRT-PCR results were analysed with the 7500 Fast System Software version 1.3.0 (Applied Biosystems). The threshold Cycle number (Ct) was determined for each reaction at a baseline selected automatically by the software and a threshold level where the line intercepts the linear phase of amplification curve of all samples. Expression fold changes were determined by the ∆Ct method (Pfaffl, 2001). For the initial human LV miRNA expression profiling data, the mean of Ct values of miR-16 levels in the rat sample was used as internal control for amplification efficiency to normalise Ct values obtained from all human miRNAs. Fold change (FC) of each miRNA was calculated using average ∆Ct values of the four technical replicates between control and T2DM groups. Amplification efficiency was determined by the Ct values of three randomly selected miRNAs at RNA concentrations from 0.008ng/µl to 5ng/µl. The analysis was performed based on the assumption of equal amplification efficiency among all miRNA species detected. Statistical analyses were performed using MS Office Excel and GraphPad Prism. Unsupervised hierarchical clustering was performed using Cluster 3.0.
software (Eisen et al., 1998), a heatmap was generated using TreeView 1.60 (http://rana.lbl.gov/EisenSoftware.htm).

### 3.3 Results

#### 3.3.1 Small RNA Sample preparation and quality control

Human LV biopsy samples were collected at the time of coronary artery bypass surgery (ethics approval reference, LREC 2003/6699, Mr Punjabi). Large and small RNA was extracted from the biopsy samples using the mirVana miRNA isolation kit (Ambion). Samples were divided into three groups: control (n=6), diabetic group (T2DM, n=6) and LVD (n=6). RNA samples were quantified and normalised against small or large RNA standard curves using RiboGreen (Invitrogen). On average, 263±86ng of large RNA and 170±38ng of small RNA were recovered from 1mg of heart tissue. The quality of small RNA extracted was assessed using the Agilent Bioanalyser (Figure 3.1). Three samples from each patient group were analysed, and ~2.5ng of each RNA sample was used. As a result of the RNA extraction method used, the samples are enriched with small RNA species that are <200 nt, as indicated by the predominant peak, compared with the low levels of carryover 18s rRNA (Figure 3.1). When the small RNA peaks were examined at higher resolution, all samples showed similar profiles, indicating no global distortion of the small RNA species in patient subgroups.

#### 3.3.2 Expression of non-coding small RNAs in the heart

Small non-coding RNA species such as 5s rRNA and U6 snRNA have been used as ‘house-keeping’ controls in small RNA qRT-PCR studies (Pineles et al., 2007; Takamizawa et al., 2004). However, analyses of the expression of 5s rRNA and U6 snRNA in the human heart small RNA samples revealed these RNA species were differentially expressed among patient groups (Figure 3.2). Expression levels of U6 snRNA and 5S rRNA were determined in the human biopsy samples (control n = 6,
Figure 3.1 Electropherogram of small RNA samples extracted from human heart biopsies. Small RNA species were extracted using mirVana microRNA Isolation Kit (Ambion); ~2.5ng used from each sample for the analyses; samples were analysed with Agilent RNA 6000 Pico Chip with RNA 6000 ladder, which is shown in red (2100 Bioanalyser, Agilent) a. The predominant small RNA peaks and small amount of retained 18s rRNA peaks are detected. b. High resolution view of small RNA peaks. Three samples from each patient group are shown. T2DM, type 2 diabetes mellitus; LVD, left ventricular dysfunction.
Figure 3.2 Optimizing qRT-PCR normalization. In some studies of miRNA expression, qRT-PCR data were normalized to 5S rRNA or U6 snRNA expression levels. However, in this study, these small RNA species were found to be differentially regulated between patient groups. Fold change (FC) was calculated by comparing the mean Ct value of the diseased groups with that of the control (Control = 6, LVD = 6, T2DM = 6). Data are represented as means ± SEM. Statistical analysis revealed significant variation between the patient groups ($p = 0.021$, two-way ANOVA).
T2DM n = 6, LVD n = 6; 20ng small RNA per reaction) by qRT-PCR (SYBRGreen, Ambion). Compared to controls, the expression of both 5S and U6 small RNAs were increased in patients with T2DM (1.7 fold and 2.6 fold respectively; \( p = 0.021 \), two-way ANOVA). In patients with LVD there was a similar pattern as observed for the T2DM group, with both small RNAs upregulated (1.2 fold, 5s rRNA; 2.3 fold, U6 snRNA; \( p = \) non-significant for both). There was statistically significant variation between the patient groups \(( p = 0.021 ; \) Two-way ANOVA). This finding was somewhat unexpected, as 5S and U6 genes have been widely used as internal ‘house keeping’ controls. Based on these observations, 5S rRNA and U6 snRNA were not used for data normalisation and all subsequent qRT-PCR analyses were normalised to total small RNA concentrations.

### 3.3.3 miRNA expression profiling in control and T2DM patients

The miRNA expression profiles of heart samples from control and T2DM patients were then assayed. Samples from each group were pooled, and expression of 155 miRNAs were assayed using stem-loop qRT-PCR. For each patient group, the expression of each miRNA was determined using 4 technical replicates. Given expression of negative controls were detectable at PCR cycle number 35, only miRNAs with \( \text{Ct} \) values <35 were considered to be expressed. Based on this criteria, out of 155 miRNAs represented in the qRT-PCR panel 115 miRNAs were expressed (Table 3.2). As shown by the analysis of three representative miRNAs of high, intermediate and low expression levels, the amplification efficiencies of the qRT-PCR reactions were equal (Figure 3.3). This enabled the relative quantification of relative miRNA expression levels in the control group over a range of ~4 orders of magnitude (Table 3.2). The most highly expressed miRNAs detected were miR-133a and b, which are known to be highly expressed in skeletal muscle and the heart (Care et al., 2007; Rao et al., 2006).
Table 3.2 miRNA expression in the human heart. Levels of 115 miRNAs are shown in raw Ct values and relative expression levels, where relative expression levels of miRNA were determined in relation to the expression level of the miRNA with the lowest detectable miRNA (hsa-miR-215). miRNAs are ranked based on their Ct values in the control group (the higher the Ct value, the lower the expression level). miRNAs with Ct > 35 were excluded as negative controls were detectable at this level.

<table>
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<th>miRNA</th>
<th>Ct</th>
<th>Standard Deviation</th>
<th>Relative expression levels</th>
<th>miRNA</th>
<th>Ct</th>
<th>Standard Deviation</th>
<th>Relative expression levels</th>
<th>miRNA</th>
<th>Ct</th>
<th>Standard Deviation</th>
<th>Relative expression levels</th>
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Figure 3.3 Amplification efficiencies of mature miRNA qRT-PCR assays A, Standard curves of miR-30b, miR-187 and miR-223 were produced using human LV small RNA samples at concentrations 5, 1, 0.2, 0.04, 0.008ng/ul. Linear regression is each line is shown ($R^2$). B, Derived amplification efficiencies ($E = 10^{\text{slope}}$; % efficiency = (E-1) $\times$100%; see Section 3.2.3.3).
3.3.4 Differential expression of miRNAs between control and T2DM patients

Expression levels of miRNAs were then compared between control patients and patients with T2DM based on the ΔCt method (expression levels were normalised against total RNA concentrations). Figure 3.4 shows the heatmap of unsupervised hierarchical clustering analysis on the fold change (log₂ transformed) in miRNA expressions in the T2DM group compared to the control. Differentially expressed miRNAs are highlighted in clusters A and B, with A showing upregulated miRNAs (e.g. hsa-miR-342, hsa-miR-223) and B showing downregulated miRNAs (e.g. hsa-miR-214, hsa-miR-199a) in the T2DM group compared to the control. The significance of the changes of individual miRNA levels were tested by t-test, assuming unequal variance between two samples.

Figure 3.5 plots the p-value against the FC observed in T2DM samples compared to the controls. To prioritise robustly expressed miRNAs with fold changes that are likely to have biologically relevant effects, a filtering cut-off was set as Ct<35 and FC < -2 or > 2. This revealed that, out of the 115 miRNAs that had Ct values less than 35, 10% miRNAs were differentially regulated by these criteria as compared with the control group (p≤0.05). Out of these 11 miRNAs, seven were upregulated and four were downregulated. However, the shortcomings of pooled sample analysis are widely recognised, thus fold changes in pooled samples were validated by direct biological validation in individual samples. Owing to the small sample size and limited statistical power, multiple testing was not performed on preliminary data obtained from the pooled samples.

To examine the expression of the 11 miRNAs identified as differentially regulated in the pooled screen in more detail, the expression of these miRNAs were analysed in
Figure 3.4 Heatmap of miRNA expression levels in hearts of control and T2DM patients. Unsupervised hierarchical clustering analysis was performed using log$_2$ transformed fold-change in expression of T2DM group compared to the control (4 technical replicates in each group). Low expressions are shown in blue and yellow indicate high expression. Four technical replicates were obtained in each group. Clustering was performed using Cluster 3.0, heatmap was generated using TreeView 1.60 (see Section 3.2.3.3).
Figure 3.5 Plot of nominal $p$-value against fold change for miRNAs in T2DM patients vs. control. miRNAs that lie in the shaded areas have fold changes of 2 or more, and nominal $p<0.01$. $p$-values were calculated using two-tailed $t$-test, assuming unequal variance between two samples. Multiple testing was not used. Instead, fold changes in pooled samples presented here were validated by direct biological validation in individual samples.
Table 3.3 Expression levels of miRNAs prioritised in pooled sample studies in individual patient samples. qRT-PCR were performed in individual patient samples, fold change (FC) of the diabetic group (n = 6) compared to the control samples (n = 6) were determined by the ΔCt method. p-values were obtained by t-test (see Appendix D).

<table>
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<tr>
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<th>Individual sample analysis</th>
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individual patient samples by qRT-PCR (Table 3.3, Appendix D). Out of the 11 miRNAs, fold changes of four miRNAs (miR-34a, miR-187, miR-223 and miR-302a) were confirmed in the independent samples. Although hsa-miR-125b was found to be downregulated in the pooled analysis, it was significantly upregulated in the individual sample study.

### 3.3.5 Expression levels of miRNAs differentially regulated in T2DM patients in patients with LVD

Similar to diabetic cardiomyopathy, LVD is characterized by myocardial insulin resistance (Dutka et al., 2006; Paternostro et al., 1996). To explore further miRNA expression in the insulin resistant heart, the study was extended to samples from patients with LVD. Expression levels of the miRNAs confirmed as differentially regulated in the T2DM group were examined in individual LVD samples by qRT-PCR (Figure 3.6). Levels of miR-34a and miR-187 were unchanged, whereas expression of miR-302a was 2.0 fold lower in LVD group samples than the controls ($p=\text{ns}$, Figure 3.6C) and miR-125b was 1.4 fold higher in LVD ($p=\text{ns}$; Figure 3.6E). miR-223 was the only consistently differentially expressed miRNA in the insulin resistant heart with 2.2-fold and 2.0-fold increase in LVD and T2DM patient groups respectively (LVD: $p=0.056$; T2DM: $p=0.021$; Figure 3.6D).

### 3.3.6 Expression of DICER1 in the human LV

DICER, the ribonuclease essential for the processing of mature miRNAs has an important role in the development of dilated cardiomyopathy and LVD (Chen et al., 2008; da Costa Martins et al., 2008; Zhao et al., 2007). The expression level of DICER1, the gene that encodes for the RNase III DICER protein, was determined in the human samples. Gene expression was determined by qRT-PCR. Compared to the control group, DICER1 expression is down-regulated in LVD (FC=-2.15, $p=0.071$) but unchanged in
Figure 3.6 Differential miRNA expression in T2DM and LVD compared with control in individual samples. Expression levels of a. hsa-miR-34a; b. hsa-miR-187; c. hsa-miR-223; d. hsa-miR-302a and e. hsa-miR-125b were determined in individual patient samples by qRT-PCR. Fold changes compared to the control group were calculated based on the ∆Ct method. Data represented as means of biological replicate of each patient group ± SEM (Control: n=6; T2DM: n=6; LVD: n=6), ** p = 0.001; * p < 0.05 and ∆ p = 0.056 (one-tailed student’s t-test).
Figure 3.7 qRT-PCR analysis of *Dicer 1* mRNA expression in human LV. 150ng of large RNA samples extracted from biopsy samples were used for each reaction. Gene expression of each group was detected from each sample individually (Control = 6, LVD = 6, T2DM = 6). Fold changes were calculated by comparing the mean Ct values of diseased groups with that of the control. Data represented as means ± SEM.
diabetic hearts (FC=1.09; Figure 3.7). DICER protein analysis by immunoblotting was not possible due to the limited amount of biopsy samples for protein preparation and the poor quality of the DICER antibody (data not shown).

3.4 Discussion

In cardiac hypertrophy and heart failure there is profound dysregulation of miRNAs and a causative role of some specific miRNAs have been confirmed in these pathologies (Sayed et al., 2007; Thum et al., 2008a; van Rooij et al., 2008b). However the regulation of miRNAs in the insulin resistant heart, has not been characterised. In order to determine the expression of mature miRNAs in the insulin resistant human heart, small RNAs were isolated from LV biopsy samples collected from control patients and from two patient groups characterised by myocardial insulin resistance (T2DM and LVD) at the time of cardiac surgery.

Microarray-based, large scale miRNA expression analysis technologies were not available at the onset of the study and hence a 96-well-plate format qRT-PCR method was used to determine miRNA expression levels in the human heart. To control for amplification variation between reaction plates, three internal control reactions amplifying rno-miR-16 from a common rat heart small RNA sample were included on each plate. A scaling factor was calculated based on the averages of the three control reactions on each plate. Ct values of the rest of the plate was then normalised using this scaling factor, this allows for the comparison of expression levels among reaction plates. Similar to observations of mRNA studies, finding a ‘housekeeping’ gene that is expressed at a constant level is also difficult for small RNA studies. Here, the ‘house keeping’ genes (5s rRNA and U6 snRNA, Figure 3.2) were differentially expressed among patient groups. Therefore, to calculate the relative expression levels (fold
change), Ct values were normalised to total small RNA concentrations, using a standard curve method (Cook et al., 2002; Pfaffl, 2001). This method allows for more accurate correction of the amount of template in each reaction (Bustin, 2002).

Expression levels of 115 miRNAs in the non-diabetic human heart with normal ventricular function were detected over 4 magnitudes of expression (Table 3.2). Based on the initial screen of pooled samples, ~10% of the miRNAs assayed exhibited changes in expression between the diabetic group as compared to the expression in the normal LV (Figure 3.4 and 3.5). For the initial pooled screen, because of small sample size and limited statistical power multiple testing was not used. If the multiple testing is applied, all variation would be lost. To control for this, differential expression detected in the pooled screen were validated by direct biological validation in individual samples. Not all differences could be validated in individual samples (Table 3.3 and Figure 3.6), indicating, as expected, expression profiling of pooled samples requires validation in individual samples. However, the preliminary pooled screen was unavoidable in this study as human heart biopsy samples are of limited amount and did not provide sufficient RNA to perform the initial analysis in individual samples.

Compared to previous studies of end-stage LVD in humans (Thum et al., 2008a), there were less pronounced changes in miRNA levels and no change in DICER in the diabetic heart (Figure 3.7). Downregulation of DICER (FC=-2.15, P = 0.071) in the LVD group is in agreement with a previous report, where DICER protein levels were found to be significantly lower in the failing heart compared to the non-failure control. Interestingly, Dicer1 deletion in mice has severe structural and functional abnormalities in the heart (Chen et al., 2008). Decrease in Dicer1 may lead to a global down-regulation of mature miRNAs and this could be coupled with an increase in accumulation of pre-miRNA
sequences. Further large scale expression profiling studies on both pre- and mature miRNAs in LVD or heart failure samples could shed more light on the role of DICER in gene expression control via regulation of miRNA processing.

Four miRNAs were confirmed as significantly upregulated in the diabetic heart. Among these, miR-34a, miR-125b, miR-187 and miR-223 have been reported to be involved in carcinogenesis (Johnnidis et al., 2008; Nikiforova et al., 2008; Scott et al., 2007; Yamakuchi et al., 2008), whereas miR-302a may have a role in stem-cell differentiation (Card et al., 2008; Greco and Rameshwar, 2007). None of these miRNAs have been previously implicated in insulin resistance. A recent study in a mouse model of myocardial infarction revealed that miR-223 is the most highly upregulated miRNA species in both border zone and remote myocardium 14 days after infarction (van Rooij et al., 2008b).

In this chapter, miR-223 was the only miRNA identified as differentially regulated in both T2DM and LVD. As both LVD and diabetic patients are insulin resistant at the myocardial level and diabetic hearts are prone to heart failure (Cook et al., 2005), the potential role for miR-223 in cardiac insulin resistance was explored further.
CHAPTER FOUR

Expression of miR-223 in the heart and its role in insulin signalling

4.1 Introduction

The diabetic and failing heart are both associated with myocardial insulin resistance and perturbed cardiac glucose metabolism. The insulin signalling pathway is the major regulator of cardiac glucose metabolism. Under normal physiological conditions, insulin induces cardiac myocyte glucose uptake through binding to the insulin receptor (IR) and activating the phosphatidylinositol 3-kinase (PI3K) pathway (Bertrand et al., 2008; Hirsch et al., 2007), which leads to the activation of protein kinase B (Akt) and translocation of insulin sensitive glucose transporter (GLUT4) from the cytosol to the sarcolemmal membrane (Pham et al., 2000; Zorzano et al., 1997). However, in insulin resistant states cardiac myocytes have a diminished response to insulin stimulation and reduced glucose uptake. Although the detailed mechanisms underlying insulin resistance remain to be fully characterised, miRNAs have already been implicated in glucose metabolism and insulin signalling in non-cardiac tissues. In the pancreas, miR-375 was described to suppress glucose-induced insulin secretion (Poy et al., 2004); and members of the miR-29 family were upregulated in the skeletal muscle of the diabetic rat, and their overexpression induced insulin resistance in the cultured adipocytes (He et al., 2007).

In chapter three, miR-223 was identified as the only miRNA upregulated in both diabetic and failing hearts, suggesting a possible role for this miRNA in the pathophysiology of myocardial insulin resistance. Previous studies of miR-223 have...
focused on the role of this miRNA in establishing and maintaining the cell lineage of immune cells. The expression of miR-223 was first described in murine bone marrow, where it was found to be expressed in myeloid cell lineages (Chen et al., 2004). It was subsequently shown to be an essential modulator of myeloid differentiation in human (Fazi et al., 2005). In humans, the gene expressing miR-223 is located on the X chromosome, and in immune cells, its expression is regulated by two transcription factors C/EBPα and NFI-A. In addition to mammalian myelopoiesis, miR-223 is also involved in triggering the innate immune response in the lung (Moschos et al., 2007).

To date, a functional role for miR-223 in the heart has not been reported, although it was found to be upregulated following myocardial infarction (van Rooij et al., 2008b). This chapter describes the effect of miR-223 on cardiac glucose metabolism and insulin signalling in vitro.

4.2 Materials and Methods

For descriptions of materials and methods used for PCR, standard cloning, qRT-PCR, Northern Blotting, tissue culture, protein extraction and quantification, immunoblotting and antibody used in this chapter, please refer to Chapter 2. For maps of all plasmids used, please refer to Appendix B.

4.2.1 Histology and in situ hybridisation

In situ hybridisation was performed as previously described (Moschos et al., 2007). In Brief, 10 µm thick cryosections were cut from OCT (Tissue-Tek, Surrey UK) embedded murine heart tissue, fixed in 4% (w/v) paraformaldehyde (Sigma; 10 min) and washed in Dulbecco’s Phosphate Buffered Saline (DPBS, Sigma). Sections were incubated with 10 µg/ml proteinase K (Sigma) and re-fixed in 4% (w/v) paraformaldehyde. After washing in DPBS, sections were pre-hybridised in hybridisation buffer (50% formamide (Sigma), 5× SSC buffer (Sigma), 250 µg/ml yeast RNA (Ambion), 1× Denhardt's
Chapter Four

solution (Sigma) in DEPC (Sigma) treated water) in a humidifying chamber. Sections were then incubated with 2 µM miRNA-specific Locked nucleic acid (LNA) probe or scrambled probe (Exiqon, Denmark) labelled with digoxygenin (DIG) in hybridisation buffer and incubated in a humidifying chamber at 50°C for 18h. Sections were washed twice in buffer (50% (v/v) formamide, 5× SSC buffer in DEPC-treated water) and in DPBS. Sections were incubated in blocking buffer (10% (v/v) sheep serum; Sigma; 1h) and then with sheep anti-DIG fAb fragments (Roche Diagnostics) labelled with alkaline phosphotase (AP) diluted in 10% (v/v) sheep serum (2h at room temperature). Sections were incubated with AP-substrate BCIP/NBT substrate kit (Vector Laboratories, Peterborough, UK; 12h) in a humidifying chamber, and then mounted with aqueous mounting media (Hydromount, National Diagnostics, Hull, UK). For routine histological staining, frozen tissue sections were cut at 10 µm thickness and fixed in 95% ethanol (Fisher Scientific, Loughborough UK) for 10 min and stained with haematoxylin and eosin. Sequences of LNA-enhanced probes:

Anti-rno-miR-223: GGGGTATTTGACAAACTGACA
Scrambled control: TTCACAATGCGTTATCGGATGT

4.2.2 Adenoviral-mediated overexpression of miRNAs in vitro

4.2.2.1 Generation of adenovirus

Primary miRNA sequences for miR-223 and miR-296 were amplified by PCR from Brown Norway (BN) rat genomic DNA.

Primers used for miR-223 were:
Forward: 5’actggaattcGGTGTCTTAGCCAGTCATGT3’
Reverse: 5’actggcggccgecCTCTGAAGGATTGCTATAGTT3’

Primers used for miR-296 were:
Forward: 5’actggaattcTTGTGTTGGGGAGACAGAGG3’
Reverse: 5’ actgccgccgcACCCCAAGAGCACTAACTGG3’

All primers were tagged with restriction enzyme site sequences at the 5’ end to allow for directional cloning. EcoRI sites (GAATTC) were added to the forward primers, and NotI sites (GCGGCCGC) to the reverse primer. PCR products were digested with EcoRI and NotI, and cloned into the EcoRI/NotI site of VQ Ad5CMV K-NpA plasmid (Appendix 3), supplied by ViraQuest Inc (North Liberty, IA, USA). Positive clones were screened by antibiotic selection and DNA sequencing. Viral particles were generated and titrated commercially by ViraQuest Inc (North Liberty, IA, USA). Method used by ViraQuest Inc for the viral generation was based on the technology described in (Anderson et al., 2000). All viral particles were stored in A195 buffer (Evans et al., 2004).

**4.2.2.2 Adenovirus infection**

For infection of cells, the indicated amount of virus (MOI, multiplicity of infection) was pre-diluted in maintenance media then added directly to the cell culture. For COS7 cells and NRVMs, cells were infected with virus 24 hours after serum starvation. For negative controls, cells were infected with equal concentrations of adenovirus carrying an empty construct (purchased from ViraQuest Inc). All viral expression experiments were performed 24–46 hours after infection. For insulin stimulation experiments, 100nM of insulin was added to the cells for 1 hour.

**4.2.3 Glucose uptake assay**

NRVMs were isolated and serum starved for 24 hours before being infected with adenovirus encoding miR-223 (Ad-miR-223), miR-296 (Ad-miR-296) or control virus at the specified concentrations. Glucose uptake was measured with minor adaptation to the method described by (Moyers et al., 1996). As a positive control for the assay, cells were treated with insulin (10^{-7}M) or vehicle control for 30 min 46 hours after infection.
At the end of stimulation, cells were washed twice with PBS, and incubated for 10 min in glucose free media (Appendix A) containing [³H]2-deoxyglucose (0.2µCi/ml); for detection of non-specific incorporation, 10µM of cytochalasin B was added together with [³H]2-deoxyglucose. Cells were then washed 3× in cold PBS to remove unincorporated [³H]2-deoxyglucose, and permeabilized with 0.1N NaOH. Incorporated radioactivity was measured by scintillation counting (Tri-Carb Liquid Scintillation Analyzer, Perkin Elmer, Waltham, Massachusetts, USA) with correction for non-specific glucose uptake and protein levels. For wortmannin experiments, cells were treated with 100nM of wortmannin 15 min prior to insulin stimulation.

4.2.4 AMPK activity assay

NRVMs were cultured as described in Section 2.5.1. Cells were washed once with ice-cold PBS then quickly harvested in lysis buffer specific for the assay following the ‘fast extraction’ protocol (50mM HEPES pH7.4/7.5, 10% (v/v) glycerol, 1mM EDTA pH8, 1% (v/v) Triton, 0.1mM PMSF, 1mM dithiothreitol, 5mM sodium pyrophosphate, 50mM sodium fluoride). Lysed cells were centrifuged for 20 min at 13 000 × g at 4°C, and the supernatant containing the solubilised fraction was stored at -80°C. Total AMPK activity was measured by incorporation of 32p to SAMS peptide (His-Met-Arg-Ser-A&-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg). This AMPK activity was assayed by Dr N Marty (Cell Stress Group, Clinical Sciences Centre, MRC) as previously described (Cheung et al., 2000; Davies et al., 1989; Woods et al., 1996). In brief, total AMPK was purified from protein lysates by immunoprecipitation using anti-AMPK antibody (rabbit, anti b1 and b2 subunit, produced by Prof Carling’s laboratory, Clinical Sciences Centre MRC; Thornton et al., 1998). Total AMPK activity was measured by incubating the immunoprecipitant with SAMS in a ³²P containing buffer (Davies et al., 1989). Incorporation of ³²P was measured by scintillation counting. Level
of phospho-acetyl-CoA carboxylase was analysed by immunoblotting using Phospho-
Acetyl CoA Carboxylase (serine 79) antibody at 1 in 1000 dilution (3661, Cell
Signalling). Detailed materials and methods for immunoblotting are described in
Section 2.7, Chapter 2.

4.2.5 Immunofluorescence

NRVMs were plated onto gelatine coated 8-well chamber slides at a density of 75 000
cells per well. Cells were treated with serum free maintenance media before infection
with adenovirus for 48 hours. For staining, cells were washed twice with ice-cold PBS
then treated with 4% (w/v) paraformaldehyde for 15 min at 4°C. Cells were then
incubated with 0.1% (v/v) triton X-100 (Sigma) in for 15 min at 4°C. After three washes
in PBS, cells were blocked with 10% (v/v) FCS (in PBS) at room temperature for 20
min, then incubated with Glut4-specific antibody (at 1 in 500 dilution in PBS; Abcam,
Cambridge, UK) for 1 hour. Cells were then washed twice with PBS and incubated with
FITC (Fluorescein isothiocyanate) conjugated anti-rabbit antibody (Invitrogen; 1 in 400)
for 1 hour at room temperature. Cells were then stained with DAPI (VECTASHIELD
Mounting Medium with DAPI) and viewed using Leica TCS SP1 DM IRB confocal
fluorescent microscope.

4.3 Results

4.3.1 Expression of miR-223 in the heart

Previous studies have shown miR-223 to be highly expressed in the bone marrow (Chen
et al., 2004). To determine the expression level of miR-223 in the heart as compared
with other organs, miR-223 expression level was examined across mouse tissues by
qRT-PCR and Northern blot analyses (Figure 4.1). Small RNA samples were prepared
from 8 organs harvested from 8-week old C57Bl6 mice. For qRT-PCR, 5ng of small
RNA was used in each reaction, and relative expression was determined based on the
Figure 4.1 Expression of miR-223 across tissues in the mouse. A, qRT-PCR analysis of miR-223 expression across tissues. Data are presented as FC in relation to the expression level observed in the lowest expressing tissue, the pancreas. Relative expression (FC) were calculated against the pancreatic expression (FC=1). Data are presented as means ± SD, n=2 for each tissue. B, Northern blot of miR-223 expression across tissues. 5S rRNAs are shown to indicate equal loading.
lowest expressing organ (the pancreas, Figure 4.1A). For Northern Blotting, 30µg of RNA sample was used from each tissue, 5’end-32P-labelled DNA oligonucleotides consisting complementary sequences to mmu-miR-223 was used as probe (Figure 4.1B). Both methods showed miR-223 to be most highly expressed in the spleen and lung, moderately expressed in the heart and skeletal muscle, and expressed at low levels in pancreas and brain. To localise miR-223 to a specific cell type in the heart, rat heart cryosections were analysed by in situ hybridization using DIG-labelled LNA enhanced probes (Figure 4.2). This localized miR-223 expression to cardiac myocytes, with prominent peri-nuclear staining, and also in non-myocytes (interstitial and pericardial cells).

### 4.3.2 Adenoviral expression vectors for miRNA overexpression studies

In order to carry out functional studies of miR-223 in vitro, adenoviral vectors expressing miRNA sequences under the control of a CMV promoter were constructed. Pri-miR-233 and pri-miR-296 sequences were cloned into the VQ Ad5CMV K-NpA plasmid following standard cloning methods (Section 2.4, Chapter 2; Figure 4.3). Adenoviral vectors were generated for miR-223 (Ad-miR-223) and a miRNA that is not differentially expressed in the patient groups, miR-296, as control (Ad-miR-296). Viral stock concentrations were $4.0 \times 10^{10}$ and $3.0 \times 10^{10}$ plaque forming units per millilitre (pfu/ml) respectively for Ad-miR-223 and Ad-miR-296. Adenoviral particles carrying empty constructs were used as negative controls for the infection (purchased from ViraQuest Inc, USA). Negative E1 glycoprotein detection confirmed all viral stocks contained no detectable replication competent adenoviral particles (performed by ViraQuest Inc.).
Figure 4.2 In situ hybridization analysis of miR-223 expression in the rat heart. A and C, in situ hybridization at 20× and 40× magnification respectively. B and D, haematoxylin and eosin stain of contiguous sections at 20× and 40× magnification respectively. E, In situ hybridization with negative control (20×). Bar = 50µm (A, B and E); Bar = 25µm (C and D). In situ hybridization was performed using DIG labeled miRCURY™ LNA enhanced probes (Exiqon, Denmark) specific for rat miR-223; negative control contained scrambled sequence.
Figure 4.3 Maps of plasmids used for making miRNA expressing adenovirus. Pri-miRNA sequences were amplified from rat cDNA and cloned into EcoRI and NotI sites of the pVQ-Ad5CMV K-NpA plasmid. Sequence information of insert fragment are presented below the plasmid map. A. for miR-223 B. for miR-296. Pre-miRNA sequences are shown in bold, mature miRNA sequences are underlined.
Figure 4.4 Adenoviral-mediated overexpression of miR-223 and miR-296 in NRVMs. Cultured NRVMs were infected with Ad-miR-223, Ad-miR-296 or control virus (Ctrl) at indicated concentrations for 48 hours. Control virus expresses an ‘empty vector’ which does not carry any transgene. Increase in miR-223 and miR-296 levels were determined by qRT-PCR (A and B) and northern blot (C and D). 5S rRNAs are shown as loading control. Data are shown as mean ± SD, n=2.
To assess the degree of overexpression and to determine the virus concentration to use for *in vitro* functional studies, cultured NRVMs were infected with Ad-miR-223, Ad-miR-296 or control virus at concentrations of 5, 10, 50, 100 MOI (multiplicity of infection) for 48 hours. Small RNA was extracted from the cells, and expression of mature miR-223 was analysed by qRT-PCR and Northern Blotting (Figure 4.4). Fold changes in miR-223 and miR-296 expressions were calculated by comparing the amount of the miRNA in the infected cells to that of the uninfected cells (0MOI). Detectable overexpression was achieved for both miRNAs, even at the lowest viral concentration tested (5 MOI; 17-fold for miR-223 and 50-fold for miR-296). Compared to uninfected cells, infection with the control virus at concentrations greater than 10MOI downregulated the expression of endogenous miR-223 and miR-296 (Figure 4.4). Since control viruses had the minimum effect on endogenous miRNA expression at 5 MOI and both vectors achieved sufficient levels of overexpression at this concentration, 5MOI of virus was used for all subsequent overexpression experiments.

### 4.3.3 Overexpression of miR-223 increases glucose uptake in NRVMs

Based on the observations in the insulin resistant human heart, the hypothesis that miR-223 may play a role in cardiac insulin signalling and/or glucose metabolism was tested. To test this effect, rno-miR-223 was overexpressed in cultured NRVMs using adenovirus, and glucose uptake was determined by incubating the cells with $[^3H]2$-deoxyglucose containing media (Section 4.2.3). In the absence of insulin stimulation, Ad-miR-223 infection significantly increased (1.3-fold, $p<0.05$) glucose uptake, as compared to control virus infected cells (Figure 4.5A). The increase in glucose uptake in unstimulated Ad-miR-223 infected cells was of a similar magnitude to that observed for insulin stimulation of control virus infected cells (1.3 fold, $p<0.01$). No further
Figure 4.5 Glucose uptake assay in cardiac myocytes infected with Ad-miR-223 or control viruses in the presence or absence of insulin (A) or wortmannin (B). NRVMs were infected with Ad-miR-223, Ad-miR-296 or control viral (5 MOI each) vector for 46 hours, then stimulated with insulin (100nM) for 30 min (A) or with wortmannin (100nM) for 15 min (B). Cells were first treated with wortmannin if both stimuli were used. Glucose uptake was then assessed by incubating cells with $[^3]$H]-2-deoxyglucose containing media, incorporated tritium activity was measured by scintillation counting with correction for non-specific glucose uptake and protein levels. All data are represented as means ± SEMs, n=4. *$p<0.05$; **$p<0.01$
increase in glucose uptake was detected in cells treated with miR-223 virus and insulin. Overexpression of miR-296 was used as an additional control and had no effect on glucose uptake in cardiac myocytes. These data show miR-223 affects glucose uptake at baseline, independent of insulin stimulation. To examine whether these effects were dependent on PI3K activity, the glucose uptake assay was repeated with the presence of wortmannin, the PI3K inhibitor (Figure 4.5B). A significant increase in glucose uptake (1.4-fold, \( p<0.05 \)) with miR-223 overexpression remained after inhibition of the PI3K pathway, although total uptake was reduced in all cells treated with wortmannin.

### 4.3.4 Overexpression of miR-223 inhibits insulin stimulated Akt/Gsk3β activation

To dissect the mechanism underlying miR-223-induced glucose uptake, the insulin signalling pathway was examined in NRVMs following miR-223 overexpression, with or without insulin stimulation. By immunoblotting, at baseline, no detectable changes were observed in total levels of AKT, IRβ or IRS1. Similar results were observed for phosphorylated levels of AKT (serine 473) and glycogen synthase kinase 3, β subunit (GSK3β, serine 9). However, following insulin stimulation, the increase in phospho-Akt (serine 473) and Gsk3β (serine 9) was significantly attenuated in Ad-miR-223 infected cells as compared with control (38% and 33% reduction respectively, \( p<0.01 \); Figure 4.6). This suggests miR-223 overexpression inhibits insulin stimulated Akt/Gsk3β phosphorylation in NRVMs.

### 4.3.5 Effects of miR-223 expression on AMPK activity

In addition to the insulin signalling pathway, AMPK activation in response to stress also increases glucose uptake in the heart (Russell et al., 2004). To investigate the effect of miR-223 on AMPK in cardiac myocytes, the beta subunit of AMPK was purified from NRVMs treated with Ad-miR-223, and the phosphorylation activity of the kinase was
Figure 4.6 Immunoblots of insulin signalling proteins in samples from cardiomyocytes infected with Ad-miR-223 or control virus in the presence or absence of insulin. A. Representative immunoblots from three independent experiments with similar results. B and C. Semi-quantitative densitometry of changes in phosphorylation of Akt (Ser473, B) and Gsk3β (Ser9, C). All data are presented as means ± SEMs, n=3; **p<0.01.
Figure 4.7 Effects of miR-223 expression on AMPK activity. Cultured NRVMs were infected with Ad-miR-223 or control adenovirus for 48 hours. A. Total AMPK activity assay. Total AMPKβ activity was measured using SAMS peptide. The amount of phosphorylation was determined by scintillation counting. Results in DPM were normalised against the amount of protein used per assay. B. Immunoblot on phospho-acetyl-CoA carboxylase (ACC; serine 79).
tested using a synthetic peptide (SAMS peptide). Compared to control, no significant change in AMPK activity was found in NRVMs overexpressing miR-223 (Figure 4.7A). Immunoblotting analysis on phospho-Acetyl-CoA carboxylase (ACC), a downstream target of activated AMPK, revealed no change in phosphorylation of the enzyme at the serine 79 residue (Figure 4.7B). Thus, miR-223 overexpression has no detectable effect on AMPK activity in cardiac myocytes.

4.3.6 Overexpression of miR-223 increases total Glut4 protein level

Given that miR-223 expression neither increases insulin signalling nor changes AMPK activity, the role of glucose transporters in miR-223-mediated glucose uptake was investigated. In cardiac myocytes, two proteins are primarily responsible for glucose transportation, Glut1 (basal uptake) and Glut4 (insulin sensitive glucose uptake). In miR-223 overexpressing NRVMs, total Glut1 levels were unchanged, whereas the total Glut4 levels were increased (1.3 fold, \( p<0.001; \) Figure 4.8A). To determine whether this increase is caused by changes in transcription, the level of Glut4 mRNA was analysed by qRT-PCR (Figure 4.8B). No difference in Glut4 mRNA was detected in cells overexpressing miR-223 as compared with control. This suggests that the increase in Glut4 protein induced by miR-223 overexpression is caused through post-transcriptional mechanisms.

4.3.7 Effects of miR-223 expression on Glut4 vesicle proteins and Glut4 localisation

Glucose uptake can be regulated via GLUT4 carrying vesicle biogenesis in addition to changes in insulin signalling and Glut translocation. Levels of two proteins found in GLUT4 vesicles were therefore analysed by immunoblotting: SCAMPs, secretory carrier membrane proteins largely found in GLUT4 carrying vesicles in insulin-sensitive cells (Fischer et al., 1997; Laurie et al., 1993), and UBC9, the small ubiquitin-
Figure 4.8 Levels of Glut4 in miR-223-overexpressing cardiac myocytes. A. Immunoblot of total cellular Glut1 and Glut4 levels in cardiac myocytes. Upper panel, representative immunoblot from three independent experiments with similar results. Lower panel, semi-quantitative densitometry of Glut4 protein levels. B. mRNA level of Glut4 by qRT-PCR. Data are represented as means ± SEMs, ***p<0.001.
Figure 4.9 Effects of miR-223 expression on GLUT4 vesicle protein levels. NRVMs were overexpressed with miR-223 for 46 hours and then stimulated with 100nM of insulin for 30 min. Total protein were extracted from cells and the levels of total SCAMP3 and Ubc9 were analysed by immunoblotting.
Figure 4.10 Cellular localisation of Glut4 in miR-223 overexpressed neonatal rat cardiac myocytes. NRVMs were cultured in chamber slides, infected with Ad-miR-223 and control virus, and stained with Glut4 specific primary antibody and FITC-conjugated anti-rabbit secondary antibody. Cells were visualised at 40x magnification, bar = 20µm. A. and B, DAPI staining on cell nucleus, C. and D. Glut4, E. and F. overlay.
related modifier conjugating enzyme that regulates the GLUT4 turnover in adipose cells (Liu et al., 2007). In NRVMs, no difference in vesicle proteins levels were observed in miR-223 overexpressing cells as compared with control cells (Figure 4.9). Glut4 cellular localisation was then assessed in neonatal cardiac myocytes. Using a Glut4-specific antibody with a FITC-conjugated secondary antibody, the localisation of Glut4 was analysed in miR-223 overexpressing NRVMs by immunofluorescence (Figure 4.10). Glut4 was mostly peri-nuclear localised, and the cytoplasmic localisation of Glut4 was more diffused in miR-223 overexpressing cells than in the controls.

4.4 Discussion

To date, miR-223 has been studied primarily in immune cells. Expression studies have previously described miR-223 as highly expressed in the bone marrow (Chen et al., 2004), however, its levels in other tissues are not well described. In this study, expression analysis across tissues in mouse showed that, apart from bone marrow, miR-223 is most highly expressed in spleen and lung (Figure 4.1), in agreement with a role for miR-223 in immune response and inflammation (Johnnidis et al., 2008; Moschos et al., 2007). Among the tissues tested, miR-223 is moderately expressed in the heart, with prominent localisation peri-nuclear expression in cardiac myocytes and also expression in non-myocytes (Figure 4.2). Levels of intermediate miRNA expression may be particularly important for post-transcriptional gene regulation. Intermediately expressed miRNAs, such as miR-223, may function as a buffer to regulate genetic noise by fine tuning the amount of protein synthesised at the translational level (Hornstein and Shomron, 2006).

miR-223 was upregulated in the hearts of patients with LVD or T2DM, conditions characterised by myocardial insulin resistance and abnormal glucose metabolism. To
investigate whether miR-223 plays a role in glucose metabolism in the heart, the effect of adenoviral-mediated miR-223 overexpression on glucose uptake was analysed in neonatal cardiac myocytes. This revealed overexpression of miR-223 in vitro significantly increases glucose uptake at baseline, independent of PI3K signalling. This increase was similar in magnitude to that induced by insulin stimulation (Figure 4.5). At the same time, high expression of miR-223 suppresses insulin-mediated induction of Akt and Gsk3β (Figure 4.6), suggestive of feedback inhibition of key mediators of insulin signalling. In contrast, miR-223 increased Glut4 protein levels through a post-transcriptional mechanism (Figure 4.8). These data show that miR-223-induced glucose uptake is a result of increased Glut4 expression, and Glut4-mediated glucose uptake, which is independent of insulin signalling. In addition to changes in glucose metabolism, a decrease in Akt phosphorylation suggests miR-223 could cause cells to become less responsive to the insulin stimulation, which is similar to the signalling pattern found in insulin resistant cardiac myocytes. Collectively, miR-223 appears to have dual role of promoting glucose uptake via increase in GLUT4 level and, likely secondarily, inhibiting insulin signalling, possibly due to feedback inhibition.

Apart from insulin signalling, glucose uptake can also be induced by AMPK, which promotes GLUT4 redistribution to the sarcolemmal membrane (Li et al., 2004; Yang and Holman, 2005) in response to stress. To determine if an enhanced AMPK activity is the cause of the observed increase in glucose uptake, the AMPK activity of miR-223 overexpressed NRVMs was measured by the amount of phosphorylation of SAMS peptide (Davies et al., 1989). No difference in phosphorylation was observed between the miR-223 overexpressed NRVMs and control cells (Figure 4.7A); this result was confirmed by immunoblotting on phosphorylated ACC (Figure 4.7B). These data
indicates miR-223 induced glucose uptake was not caused by changes in AMPK activity, and miR-223 expression does not affect AMPK signalling in vitro.

As discussed in Chapter 1, glucose uptake and metabolism are essential for cardiac energy production, especially in ischemic conditions. Previous studies have found that during ischemia, insulin signalling is impaired (Beauloye et al., 2001). However, glucose uptake is increased through AMPK signalling (Russell et al., 2004). In the present study, miR-223 was shown to upregulate glucose uptake at baseline independent of insulin and AMPK signalling, this suggests it may have a more pronounced effect on glucose uptake during metabolic stress such as ischemia and functions as a backup source of glucose supply. Further studies in models (in vivo or ex vivo) of myocardial infarction/ischemia could provide more insights into the metabolic function of miR-223.

Since the increase in glucose uptake to NRVMs was independent of insulin and AMPK signalling, levels of Glut4 proteins were analysed in miR-223 overexpressing cardiac myocytes. Two previously described vesicle proteins that are involved in Glut4 vesicle biogenesis and translocation were examined. No detectable changes were observed in their expression with miR-223 overexpression (Figure 4.9), suggesting miR-223 expression does not have an effect on Glut4 vesicle biogenesis. Although slight differences in the intracellular distribution of Glut4 were observed by immunofluorescence (Figure 4.10), in depth studies on Glut4 carrying vesicles are required to draw firm conclusions of the effects of miR-223 on Glut4 sub-cellular localisation. Previous studies of Glut4 trafficking in adipose cells using Total Internal Reflectance Fluorescent microscopy suggested Glut4 vesicles are continuously trafficking to and from plasma membrane in absence of insulin; whilst following insulin
stimulation, the vesicles have increased pausing at the plasma membrane with higher probability of membrane fusion (Lizunov et al., 2005). Hence, miR-223 could be involved in the regulation and fine-tuning of Glut4 vesicle shuttling to and from the plasma membrane. The effect of miR-223 on Glut4 will be discussed further in Chapter 5.

In this chapter, the potential role of miR-223 in glucose metabolism and insulin signalling was examined in neonatal cardiac myocytes. It was found that miR-223 induces glucose uptake in cardiac myocytes independently of PI3K/Akt activity and AMPK signalling, and involves post-transcriptional upregulation of Glut4 protein levels. In parallel, miR-223 decreased proximal insulin signalling after insulin stimulation, which is similar to the signalling pattern found in insulin resistant cardiac myocytes (He et al., 2006).
CHAPTER FIVE

Prediction and validation of miR-223 targets \textit{in vitro}

5.1 Introduction

miRNA bind to and inhibit target mRNA translation, therefore an approach for miRNA target identification is to use an \textit{in silico} method. As described in Chapter 1, a number of miRNA target prediction program have been developed. However, the interaction between a miRNA and its target is more complex than initially expected and different algorithms identify disparate targets. Thus experimental validation of predicted target genes is essential for identifying true mRNA targets.

Currently, there is no clear consensus as to which experimental approaches should be applied to prove a specific mRNA is the target of a miRNA species. A widely accepted approach uses the luciferase assay, where the 3’UTR sequence of the target mRNA, containing the predicted miRNA target site, is cloned downstream of luciferase, and the luciferase construct is then co-expressed with the miRNA in a cell-line. If an interaction between the miRNA and mRNA exists, luciferase activity is lower in cells co-expressing the miRNA than in control cells. To confirm specificity, luciferase activity should be reinstated when the predicted miRNA binding site is mutated (Fazi et al., 2005; Johnnidis et al., 2008; Poy et al., 2004). As a more direct assay, if an mRNA is the true target of a specific miRNA, alterations of the miRNA’s concentration should correspond with changes in the protein encoded by the mRNA. This can be assayed by overexpressing the miRNA (gain-of-function) in the cell type that is known to express the putative target protein and analysing protein levels by immunoblotting (Poy et al., 2004; Zhao et al., 2007).
To date, using a combination of *in silico* and experimental techniques, three mRNA targets of miR-223 have been identified in granulocytes: NFI-A (Fazi et al., 2005), Mef2c and Igf1r (Johnnidis et al., 2008). During haematopoiesis, NFI-A was found to repress miR-223 expression in non-differentiating granulocytes; and in turn, NFI-A translation is repressed by miR-223 through a negative feedback loop. In other cell types, nuclear factor I is a mediator of insulin and cyclic-AMP stimulated repression of GLUT4 expression (Cooke and Lane, 1999a, b). Mef2c is also targeted by miR-223 in granulocytes, where it promotes myeloid progenitor proliferation. In skeletal muscle, MEF2C regulates GLUT4 expression (Moreno et al., 2003). In the heart, MEF2C is required for cardiogenesis, and inactivation of Mef2c in mouse leads to cardiac morphogenetic defects, vascular abnormalities and embryonic lethality (Morin et al., 2000; Zang et al., 2004). Studies have also found MEF2C and MEF2C-regulated genes are decreased in the failing hearts of diabetic patients (Razeghi et al., 2002). If MEF2C is a true miR-223 target in heart, miR-223 could influence MEF2C-regulated transcription, and establish a role for miR-223 in heart failure patients with diabetes. This chapter describes the predicted mRNA targets for miR-223 and presents results from experimental validations of selected predicted and previously confirmed targets in the cardiac myocytes.

### 5.2 Materials and Methods

For general materials and methods used for tissue culture, adenoviral infection, qRT-PCR, cloning, transfection, protein extraction, immunoblotting and antibodies used in this chapter please refer to Chapter 2. For information on human biopsy samples, please refer to Chapter 3, Section 3.2.1
5.2.1 *In silico* miR-223 target prediction

Five web-based miRNA target prediction programs were used for the prediction of miR-223 target genes (see Table 5.1 for URLs). Following program-specific instructions, mature hsa-miR-223 sequence was used to screen for human genes for potential miR-223 target sites. Where possible, only sites that were conserved across species and contain no G:U wobble base pairing were included. To prioritise target genes that are involved in insulin signalling and/or glucose metabolism, each predicted target was examined in the context of previous data relating to gene function. Targets that were predicted by more than one program and are known to be involved in glucose metabolism were prioritised for further study.

5.2.2 Luciferase reporter assays using psiCHECK-2 vector

The psiCHECK-2 (Promega, Southampton UK; Appendix B) vector enables monitoring of changes in expression of a target gene fused downstream of the *Renilla* luciferase gene. In this project, the plasmid was used to validate the authenticity of the predicted miRNA targets in luciferase assays. In general, 3’UTR sequences flanking the predicted miRNA binding site of potential mRNA targets was amplified by PCR from BN rat cDNA (refer to Appendix C for primer sequence information), and TA cloned into pTarget™ vector (Promega) as described in Section 2.4.2. Clones with the sequence confirmed 3’UTR sequences were selected and the insert was sub-cloned into psiCHECK-2 vector by directional cloning using restriction enzymes *Not*I and *Xho*I (Section 2.4.5). Clones were screened by PCR and DNA sequencing. Selected clones were amplified by maxiprep (Section 2.4.8). For luciferase assay, in each transfection reaction, 100ng of plasmids were transfected into COS7 cells that were seeded onto 24-well plates (1×10^5 cells per well) the day before (as described in Section 2.5.3). After transfection (24 hours), media was removed, cells were washed twice with PBS and
lysed with 1× Passive Lysis Buffer (Promega, 30 min at room temperature). Lysates were transferred into 96-well plates and renilla and firefly luciferase activity were detected using Dual-Glo™ Luciferase Assay System (Promega) using a FLUOstar OPTIMA (BMG Labtech, Aylesbury UK) according to the manufacturer’s instruction. In brief, lysates were first treated with equal volume of Dual-Glo™ Luciferase Buffer to activate firefly bioluminescence. After firefly luciferase activity had been measured, an equal volume of Stop & Glo® buffer was added to allow the detection of Renilla luciferase activity. Data were analysed using MS Excel, Renilla luciferase activity was normalised against the firefly luciferase activity. Four biological replicates were set up for each transfection condition.

5.2.3 GLUT4 expressing construct and transfection
A construct expressing full length rat GLUT4 cDNA was purchased from Geneservice Ltd (Clone ID: IMAGE: 7192278) and sequence verified by DNA sequencing. COS7 cells were transfected with 300ng of cDNA construct per well using Lipofectamine 2000 (Invitrogen), and incubated at 37°C and 5% CO₂ for 4 hours. Cells were then infected with virus at 5MOI (Ad-miR.223 or control). Cells were harvested 24 hours after infection, and total protein lysates were collected as previously described (Section 2.6).

5.3 Results
5.3.1 Predicted targets of miR-223
In Chapter 4, miR-223 was shown to promote glucose uptake through increased Glut4 expression in cardiac myocytes. To identify the mechanism underlying this phenotype and to investigate the role of miR-223 in the heart, potential targets of miR-223 were prioritised using five independent in silico miRNA target prediction programs (Table 5.1). The number of predicted targets varied greatly between algorithms, with the least
Table 5.1. Number of miR-223 target genes identified by miRNA target prediction software. The human genome was used for all predictions.

<table>
<thead>
<tr>
<th>Prediction Program</th>
<th>Seed size (nt)</th>
<th>mismatch</th>
<th>Site conservation</th>
<th>Number of target genes predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target Scan†</td>
<td>7-8</td>
<td>✓</td>
<td>✓ (Mammals)</td>
<td>202</td>
</tr>
<tr>
<td>miRanda‡</td>
<td>Minimum 7</td>
<td>✓</td>
<td>✓ (Mammals)</td>
<td>1415</td>
</tr>
<tr>
<td>miRBase version 5*</td>
<td>Minimum 7</td>
<td>✓</td>
<td>✓ (minimum 2 species)</td>
<td>999</td>
</tr>
<tr>
<td>PicTar**</td>
<td>Minimum 7</td>
<td>x</td>
<td>✓ (vertebrates &amp; flies)</td>
<td>180</td>
</tr>
<tr>
<td>PITA§</td>
<td>Minimum 6</td>
<td>x</td>
<td>✓ (17 vertebrates &amp; flies)</td>
<td>285</td>
</tr>
</tbody>
</table>

†http://www.targetscan.org (Grimson et al., 2007)
‡http://www.microrna.org/microrna/home.do (Betel et al., 2008; John et al., 2004)
*http://microrna.sanger.ac.uk/targets/v5 (Griffiths-Jones et al., 2006; Griffiths-Jones et al., 2008)
**http://pictar.mdc-berlin.de (Krek et al., 2005)
§http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html (Kertesz et al., 2007)
amount of targets predicted by TargetScan and the most from Human miRNA Targets. Using the prior experimental knowledge of a role for miR-223 in glucose metabolism, only candidates previously known to be involved in glucose uptake and/or insulin signalling were prioritised for further investigation. In total, 10 genes were selected as candidates (Table 5.2).

5.3.2 Experimental validation of predicted candidates

Among the predicted target genes, two were selected for further analysis: myotrophin (Mtpn) and Rab10. Mtpn is a nuclear factor-κB interacting protein that was found to have increased expression in failing human heart (Gupta et al., 2002; Sil et al., 1993), and is targeted by miR-375 during the regulation of glucose-stimulated secretion of insulin (Poy et al., 2004). Rab10, on the other hand, is a Rab GTPase that promotes GLUT4 translocation to the cell membrane in adipocytes (Sano et al., 2007). To verify their authenticity as miR-223 targets, both miRNA/mRNA interaction (by luciferase assay) and protein expression (by immunoblotting) were analysed (Figure 5.1). As a positive control, previously confirmed miR-223 targets, NfIa and Igf1r, were also included in the analysis. For protein expression analysis, NRVMs were infected with Ad-miR-223 or control virus for 48 hours, cells were then harvested and whole cell protein lysates were analysed by immunoblotting. Both myotrophin and Rab10 exhibited no change in protein expression in miR-223 overexpressed NRVM. Surprisingly, neither of the positive controls (NfIa and Igf1r) showed a decrease in protein levels (Figure 5.1A). On the contrary, a two-fold increase was observed in nuclear factor IA level in cardiac myocytes overexpressing miR-223 (p<0.01).

The interaction between miR-223 and predicted mRNA 3’UTR was further examined by luciferase assay (Figure 5.1B). The 3’UTRs of the predicted mRNA transcripts were
Table 5.2. Predicted target genes of miR-223 involved in insulin signalling and glucose metabolism.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene ID</th>
<th>Gene Function</th>
<th>Identified by</th>
<th>Effects on insulin/glucose metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF1R</td>
<td>ENSG00000140443</td>
<td>tyrosine kinase</td>
<td>miRanda, TargetScan</td>
<td>Induces cardiac growth/hypertrophy, previously validated target of miR-223 (Johnnidis et al., 2008)</td>
</tr>
<tr>
<td>MEF2C</td>
<td>ENSG00000081189</td>
<td>Transcription factor</td>
<td>miRanda, TargetScan, PicTar</td>
<td>Regulates cardiac development and many metabolic effects, previously validated target of miR-223 (Johnnidis et al., 2008)</td>
</tr>
<tr>
<td>MTPN</td>
<td>ENSG00000105887</td>
<td>NF-κB interacting protein</td>
<td>TargetScan, PITA, PicTar</td>
<td>Increases in failing heart (Sil et al., 1993), involved in glucose-stimulated secretion of insulin (Poy et al., 2004)</td>
</tr>
<tr>
<td>NF1A</td>
<td>ENSG00000162599</td>
<td>Transcription factor</td>
<td>miRanda, TargetScan, PITA, PicTar</td>
<td>Transcriptional repression of GLUT4, previously validated target of miR-223 (Fazi et al., 2005)</td>
</tr>
<tr>
<td>PCK2</td>
<td>ENSG00000100889</td>
<td>carboxykinase</td>
<td>miRBase, Target Scan</td>
<td>Possible candidate for T2DM (Beale et al., 2007)</td>
</tr>
<tr>
<td>PKC-ε</td>
<td>ENSG00000171132</td>
<td>kinase</td>
<td>miRanda</td>
<td>Insulin exocytosis, negative regulation of insulin signalling (Mendez et al., 2003)</td>
</tr>
<tr>
<td>RAB10</td>
<td>ENSG00000084733</td>
<td>GTPase</td>
<td>miRanda, TargetScan, PicTar, PITA</td>
<td>Regulates GLUT4 translocation (Sano et al., 2007)</td>
</tr>
<tr>
<td>RPS6KB1</td>
<td>ENSG00000108443</td>
<td>kinase</td>
<td>miRanda, TargetScan</td>
<td>Component of Insulin signalling pathway (Chakraborty et al., 2007)</td>
</tr>
<tr>
<td>SP1</td>
<td>ENSG00000172845</td>
<td>Transcription factor</td>
<td>miRanda</td>
<td>Control glucose transporter expression (Copland et al., 2007; Hwang and Ismail-Beigi, 2006)</td>
</tr>
<tr>
<td>VAMP2</td>
<td>ENSG00000179036</td>
<td>Vesicle protein</td>
<td>miRanda</td>
<td>Required for GLUT4 translocation (Randhawa et al., 2000)</td>
</tr>
</tbody>
</table>
Figure 5.1. Experimental assessment of Rab10 and myotrophin as miR-223 targets. 

A. Immunoblot analysis of protein levels encoded by predicted target genes of miR-223. Upper panels, representative immunoblots of Rab10 and Mtpn in Ad-miR-223 and control virus infected NRVMs. Previously verified miR-223 targets Nuclear Factor IA and Igf1r are included in the analysis as positive controls. Lower panel, semi-quantitative densitometry of NFIA protein levels in Ad-miR-223 and control virus infected cells. Data are presented as mean ± SEM. ** p<0.01.

B. Analysis of miRNA/mRNA interaction by Luciferase assay. 3’UTR sequences flanking the predicted miR-223 target sites were cloned immediately downstream of the renilla luciferase gene in the psiCHECK-2 plasmid and transfected into COS7 cells. Cells were infected with Ad-miR-223 and control virus 5 hours after transfection, and luciferase activity was measured 24 hours after transfection. Renilla luciferase activity was normalized against firefly luciferase activity. 3’UTR of Nuclear factor IA was included in the analysis as a positive control. All data are presented as mean ± SEM, n=4; * p=0.012.
cloned downstream of the renilla luciferase gene using the psiCHECK-2 plasmid and transfected into COS7 cells. Cells were infected with Ad-miR-223 for 24 hours, and luciferase activity was determined as described in Section 5.2.2. Consistent with the immunoblot results neither Rab10 nor Mtpn 3’UTRs showed changes in renilla luciferase activity, thus suggesting no interaction between the 3’UTR and the miRNA. In contrast to previous studies, cells transfected with psiCHECK-2 plasmids that contain the 3’UTR of NfIa showed a slight, but significant increase in luciferase activity upon miR-223 overexpression (10% increase, \( p=0.012 \)). This result is in accordance with the apparent paradoxical increase in NfIa protein levels (Figure 5.1A).

### 5.3.3 Is Mef2c a target of miR-223 in cardiac myocytes?

Previous studies have shown that Mef2c is a target of miR-223 in neutrophils. Immunoblot analysis of human heart biopsy samples revealed that patients with either T2DM or LVD have significantly lower levels of MEF2C than that of the control group (Figure 5.2A). However, analysis of Ad-miR-223 infected NRVMs had no effect on Mef2c protein or transcript levels (Figure 5.2B), and transfection of NRVMs with a MEF2-Luciferase reporter construct confirmed no effect of miR-223 on Mef2c (experiment performed by collaborators at the Heart Failure Research Center, Academic Medical Center, Amsterdam, the Netherlands; data not shown). These results suggest that although miR-223 inhibits Mef2c in granulocytes and Mef2c is downregulated in the insulin resistant heart, it is not a direct target of miR-223 in cardiac myocytes in vitro.

### 5.3.4 Glut4 as a miR-223 target

As described in Chapter 4, overexpression of miR-223 caused an increase in glucose uptake in cardiac myocytes through an increase in total Glut4 protein level. In addition, in Section 5.3.2 miR-223 was found to increase the protein levels of NfIa (a known
Figure 5.2. Analysis of Mef2c by immunoblotting.  A. MEF2C expression in human LV. Biopsies were collected from LV of T2DM, LVD and control group patients as discussed in Chapter 2, 30µg of protein were resolved on SDS-PAGE. Blots were probed with MEF2C specific antibody. MEF2C is significantly lower in diabetic and LVD patients compared to the controls. B. Mef2c expression in Ad-miR-223 infected NRVMs by immunoblotting. C. Mef2c transcript level in miR-223 overexpressed NRVMs by TaqMan based qRT-PCR. Data are presented as mean ± SEM, n=3.
miR-223 target) in NRVMs. These data suggest that Glut4 could be a direct target of miR-223. In silico sequence analysis of the Glut4 3’UTR showed no predicted miR-223 target sites in this region of the transcript. However, whole cDNA sequence analysis using free energy based miRNA prediction program PITA (http://genie.weizmann.ac.il/pubs/mir07/index.html), revealed 38 potential target sites for rno-miR-223 in the rat Glut4 sequence with a combined energic score (ddG) of -9.39 (Table 5.3), and 47 potential target sites for mmu-miR-223 in mouse Glut4 cDNA with a combined ddG of -9.51 (Table 5.4). Analysis in the human GLUT4 cDNA also revealed 47 potential target sites for hsa-miR-223 with combined ddG of -11.04 (Table 5.5). These computational results suggest Glut4 could be a direct target of miR-223 across species. To test this experimentally, full-length rat Glut4 cDNA was co-expressed with miR-223 in COS7 cells (a cell line that does not express endogenous Glut4). Immunoblotting analysis of Glut4 showed a 1.6 fold increase ($p<0.05$) in total Glut4 level in cells infected with Ad-miR-223 as compared to control virus (Figure 5.3). This supports the hypothesis that miR-223 has a direct effect on Glut4.

5.4 Discussion

Identification of a bona fide miRNA target is a challenging task. In this chapter, predicted target genes of miR-223 from five miRNA target prediction algorithms were screened and targets with a functional role in glucose metabolism were prioritised (Table 5.1 and 5.2). As discussed in earlier sections, although most algorithms are designed on the six basic principles, various algorithms generate lists of disparate targets. Table 5.1 presents the number of mRNA targets of miR-223 predicted by each of the five algorithms. Among these, PicTar and TargetScan predicted the least number of targets as compared to microRNA.org and miRBase. This difference could be explained by the difference in filtering criteria used by the various algorithms.
Table 5.3. Predicted miR-223 binding sites in Glut4 cDNA in rat.

(http://genie.weizmann.ac.il/pubs/mir07/index.html)

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<sup>1</sup> Position of predicted miR-223 binding site on the Glut4 cDNA. Shown in base pairs.

<sup>2</sup> "X:Y:Z" seed notation: X= size of the seed; Y= number mismatches; Z= number of G:U wobble pairs.

<sup>3</sup> Energetic score, a score of ~ -10 is predicted to be functional, depending on miRNA concentration.

<sup>4</sup> Combined ddG from all predicted sites, indicates overall effect of all sites combined on Glut4.
Table 5.4. Predicted miR-223 binding sites in Glut4 cDNA in mouse.
(http://genie.weizmann.ac.il/pubs/mir07/index.html)

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1 Position of predicted miR-223 binding site on the Glut4 cDNA. Shown in base pairs.

2 "X:Y:Z" seed notation: X= size of the seed; Y= number mismatches; Z= number of G:U wobble pairs.

3 Energetic score, a score of ~ -10 is predicted to be functional, depending on microRNA concentration.

4 Combined ddG from all predicted sites, indicates overall effect of all sites combined on Glut4.
Table 5.5. Predicted miR-223 binding sites in Glut4 cDNA in man.  
(http://genie.weizmann.ac.il/pubs/mir07/index.html)

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¹ Position of predicted miR-223 binding site on the Glut4 cDNA. Shown in base pairs.

² "X:Y:Z" seed notation: X= size of the seed; Y= number mismatches; Z= number of G:U wobble pairs.

³ Energetic score, a score of ~ -10 is predicted to be functional, depending on microRNA concentration.

⁴ Combined ddG from all predicted sites, indicates overall effect of all sites combined on Glut4.
Figure 5.3. miR-223 expression directly affects total Glut4 level. COS7 cells overexpressing full-length rat Glut4 cDNA were infected with Ad-miR-223 or control virus. 24 hours after infection, total Glut4 expression was analysed by immunoblotting with Glut4 specific antibody. Upper panel, representative blot of total Glut4. Lower panel, semi-quantitative densitometry of Glut4 protein levels in COS7 cells. Cells overexpressed with miR-223 showed 1.6-fold increase in total Glut4 level as compared to cells expressed with Glut4 but treated with control virus. Data are presented as mean ± SEM. * p<0.05.
Predictions from TargetScan and PicTar preferentially select for transcripts with at least one conserved 7- or 8-mer site on the 3’UTR. Whereas algorithms such as miRanda and miRBase allow mismatch or wobble base pairing to the seed tend to generate large number of target sites/genes. However, a recent study of the impact of miRNAs on protein expression levels suggested inclusion of mismatch or wobble base pairing may compromise the proportion of true targets of the prediction (Baek et al., 2008).

Among the genes prioritised in this study, Rab10, Mtpn, NfIa, Igf1r and Mef2c were chosen for experimental validation in cardiac myocytes. Neither Rab10 nor Mtpn were confirmed as miR.223 targets by luciferase assay or immunoblotting analysis (Figure 5.1), although proteomic analysis in neutrophils cultured from miR.223−/− mice revealed 1.4 fold increase in Rab10 and Mtpn protein levels with similar fold changes in mRNA levels, suggesting Rab10 and Mtpn could be regulated by miR-223, at least in neutrophils (Baek et al., 2008). Surprisingly, NfIa, Igf1r and Mef2c, three previously verified miR-223 targets, were not downregulated by miR-223 in cardiac myocytes. Strikingly, protein levels of NfIa were actively increased by 2-fold following miR-223 overexpression (Figure 5.1A). These findings suggest that miRNA-mediated gene inhibition is tissue specific, and targets of miRNAs identified in one tissue type may not be relevant in another, particularly when one considers that miRNAs can ‘paradoxically’ increase protein levels of target mRNAs in some cell types (Vasudevan et al., 2007). Indeed, studies of the effect of cell cycle and cellular stress have revealed that miRNAs can actually activate translation (Vasudevan et al., 2007). This finding may be particularly important for cardiac myocytes that are terminally differentiated.
MEF2C is a transcription factor that regulates glucose metabolism and promotes Glut4 expression in the skeletal muscle (Moreno et al., 2003), and was found to be downregulated in failing hearts of diabetic patients (Razeghi et al., 2002). In the present study, immunoblotting analysis revealed MEF2C to be significantly downregulated in both diabetic and failing heart (Figure 5.2A). This finding confirms a potential role for MEF2C in the insulin resistant heart. MEF2C is a known target of miR-223 in myeloid cells, however, in vitro studies in cardiac myocytes detected no interaction between miR-223 and Mef2c (Figure 5.2B and C), indicating the decrease in MEF2C observed in T2DM and LVD patients is likely independent of miR-223.

In Chapter four, miR-223 was shown to increase total Glut4 protein in cardiac myocytes. Despite the fact that Glut4 3’UTR is not predicted to have miR-223 target sites, its protein levels were increased in COS7 cells overexpressing miR-223 (Figure 5.3). The mechanism underlying this effect is still unclear, however target prediction using full-length cDNA sequence of Glut4 revealed a number of potential target sites in rat, mouse and man, reflecting miR-223 binding to functional sites in coding, 5’ or 3’UTR mRNA sequence of Glut4 (Tables 5.3, 5.4 and 5.5). Although a large proportion of predicted targets consist 6-mer seed sequences, direct interaction between miR-223 and Glut4 could be enforced by multiple binding to target sites by the miRNA. This is supported by findings from the ENCODE (Encyclopedia of DNA Elements) project that identified multiple miRNA sites throughout the coding gene sequences (Birney et al., 2007).

What could be the mechanism behind miR-223 mediated upregulation of Glut4? Because Glut4 was upregulated in both dividing (COS7 cells) and non-dividing cells (NRVMs) upon miR-223 overexpression, the change in Glut4 protein level may not be
caused by cell-cycle specific miRNA-mediated translational activation (proposed by Vasudevan et al., 2007). Studies in human cell lines and C. elegans have shown interaction between the RISC complex and other mRNA binding proteins such as ribosomal proteins to suppress translation (Chendrimada et al., 2007), miR-223 associated RISC factor could be interacting with other RNA binding proteins that acts as translational enhancers. On the other hand, miR-223 might not be the direct regulator of Glut4, instead, it could be inhibiting the expression of a Glut4-specific repressor, thus when miR-223 is overexpressed, the repressor level decreases, and the inhibition of Glut4 is then removed.

Findings presented in this Chapter question the utility of miRNA target prediction programs, a point highlighted in a recent study that revealed only a third of predicted targets were verifiable experimentally, whereas numerous ‘non-targets’ were differentially regulated at the protein level following miRNA deletion in the mouse (Baek et al 2008). Furthermore, the striking observation of increased NfIa and Glut4 protein levels with miR-223 overexpression suggests miRNA-mediated gene regulation is cell-type specific, and the induced glucose uptake observed in cardiac myocytes could be a result of direct induction of Glut4 level by miR-223.
6.1 Introduction

In vivo study of gene function is a crucial step for determining a gene’s role in physiological processes. Loss- and gain-of-function studies in mouse have revealed profound functions of miRNA in many aspects of cardiac physiology, such as cardiac hypertrophy, cardiac contraction, arrhythmia and cardiac fibrosis. In this study, to examine the effect of miR-223 on Glut4 protein expression, two mouse models were developed: in vivo inhibition of miRNA using chemically modified oligonucleotides and cardiac-specific overexpression of miR-223.

By adopting the principles of RNAi, chemically synthesised antisense oligonucleotides (ASOs) can be used to silence endogenous miRNAs (Krutzfeldt et al., 2005). These ASOs contain sequences complementary to the mature sequences of the corresponding target miRNAs. To reduce RNase-mediated degradation and to facilitate cell uptake upon in vivo administration, these ASOs are often modified with 2’O-Methyl modified nucleotides on a phosphorothioate backbone, and contain hydroxyprolinol-linked cholesterol on the 3’ end. Krutzfeldt and colleagues (2005) showed that intravenous administration of ASOs to mice significantly decreased targeted miRNA levels. Carè and colleagues (Care et al., 2007) successfully inhibited miR-133 expression in the heart and induced cardiac hypertrophy with single infusion of anti-miR-133 ASO using an osmotic gradient pump. More recently, systemic inhibition of miR-29 by direct tail-vein injection lead to increased collagen mRNA expression in the heart (van Rooij et al., 2008b).
Gain-of-function transgenic (TG) animal models are a powerful tool for the study of the molecular and cellular mechanisms of diseases such as cardiac hypertrophy and heart failure. Transgenic mice can be generated by microinjection of DNA encoding the gene of interest directly into the pronucleus of fertilised mouse oocytes (Gordon and Ruddle, 1981; Gordon et al., 1980). Post injection, surviving oocytes are implanted into the oviducts of a pseudo-pregnant female and allowed to develop. Microinjection of DNA leads to the integration of 1 to 50 copies of the transgene at a single site on the chromosome. Rarely two sites of integration are found in a single animal. If the integration occurs before the embryo divides into the two cell stage, all cells will contain the transgene, thus allowing the transgene to be passed on to subsequent generations. Once founder mouse carrying the transgene is identified, it can be bred with inbred lines of wild-type mice to produce a line of heterozygous transgenic mice. Procedures for screening potential founders for the presence of the transgene, include PCR or Southern Blot using DNA isolated from tail tip or ear clip biopsies (Hogan, 1994). A wide range of mouse strains can be used for transgenic studies, and the well-characterised strain of choice for cardiovascular research, the C57/BL6, is often used. To increase transgene insertion, a founder transgenic mouse can be generated using a hybrid strain (e.g CBAB6/F1) and backcrossed to C57/BL6 mice (Auerbach et al., 2003).

Given that the integration of transgene is present in all nucleated cells of the transgenic animal, in order to express a gene in an organ-specific manner, a cardiac-specific approach relies on the use of a cardiac-specific promoter that is able to drive high levels of cardiac-restricted expression. The α-MHC promoter has been the promoter of choice to direct cardiac-specific overexpression and a wide range of transgenes have been
expressed using this construct (Cook et al., 2008; Molkentin and Robbins, 2008). In the heart, there are two isoforms of the myosin heavy chains, α and β-MHC. The genes expressing these proteins are located in tandem, separated by 4.5kb of intergenic sequence, with β-MHC gene located 5’ upstream of the α-MHC gene (Mahdavi et al., 1984). In the rodent, α-MHC is expressed continuously in the atria throughout the development whereas the expression of β-MHC is restricted to the ventricles during this period. While β-MHC is downregulated soon after birth, transcription of α-MHC is initiated and continues to be the predominant isoform of MHC in the adult (Lompre et al., 1984). The expression of α-MHC is highly specific to the cardiac myocytes (Subramaniam et al., 1991), this is enforced by a GATA motif 1.9kb upstream of the gene’s transcription start site, which only interact with cardiac myocyte specific GATA binding factors (Huang and Liew, 1997), thus the promoter activity of α-MHC is highly cardiac specific. Although the expression of α-MHC is at its highest in postnatal mouse heart, owing to the changes of α-MHC gene expression throughout development and the fact that the activity of the α-MHC promoter is suppressed during the development of many experimental forms of cardiac hypertrophy and heart failure, it is unlikely that the transgene expression can be maintained at a steady state over the prolonged period, which may be required for the development of any phenotype.

Magnetic resonance imaging (MRI) is a non-invasive, medical imaging technology used to assess the function and structure of the body. MRI technology uses a powerful magnetic field to align the nuclear magnetisation of hydrogen atoms in water in the body. Radiofrequency fields are used to systematically alter the alignment of this magnetization, causing the hydrogen nuclei to produce a rotating magnetic field detectable by the scanner. This signal can be manipulated by additional magnetic fields
to build up enough information to construct an image of the body. Cardiac MRI is based on the principles of whole body MRI with optimisations made in the use of ECG (electrocardiogram) gating and rapid imaging techniques, specific for the cardiovascular system. Cardiac MRI can provide highly accurate 3-dimensional representations of cardiac structure and function (Hoit, 2001). Apart from its clinical applications, cardiac MRI technology has also been developed to study phenotypic alterations in research animal models such as TG mice.

This chapter describes the molecular and physiological findings observed from both gain- and loss-of-function studies on miR-223 in the mouse with respect to earlier in vitro findings.

6.2 Materials and Methods

This section describes materials and methods used specifically for this chapter. For information on general materials and methods such as qRT-PCR and western blotting, please refer to Chapter 2.

6.2.1 miRNA inhibition in vitro by antisense oligonucleotides

Anti-miRNA oligonucleotides specific for miR-223 were commercially synthesised (Thermo Scientific). Each oligonucleotide consists of 2’O-methyl modified nucleotides, with phosphorothioate links between nucleotides 1, 2 and 3 and between the last four nucleotides. All inhibitors were tagged with a cholesterol molecule at the 3’ end. NRVMs were serum starved and transfected with 100nM of anti-miR-223 oligonucleotides or 100nM of non-targeting oligonucleotides comprising sequences antisense to cel-miR-67 (negative control) using Lipofectamine 2000 reagent (Invitrogen) following procedures described in Section 2.5.3. Transfections were done in triplicates. Cells were incubated at 37°C, 5% CO₂ for 48 hours before harvest.
Sequences of antisense oligonucleotides were:

Anti-miR-223: 5’UGUCAGUUUGUCAAAUACCCC3’

Anti-cel-miR-67 (negative control): 5’UCACAACCUCUAGAAAGAGUAGA3’

6.2.2 miR-223 silencing in vivo

All animal related procedures were carried out under the authority of the appropriate Home Office (UK) personal and project licences in accordance with the Home Office Animals (Scientific Procedures) Act 1986. Mice were maintained on a 12 h light/dark cycle and an *ad libitum* diet of standard mouse chow (RE3, Special Diet Service). Synthetic oligonucleotide inhibitors comprising complementary sequence to miR-223 and cel-miR-67 (negative control) were designed and supplied by Dharmacon Inc. (Thermo Scientific, Chicago, IL, USA). Ten-week old C57BL/6 male mice received either anti-mmu-miR-223 or anti-cel-miR-67 at a dose of 80mg/kg body weight or a comparable volume of buffer (used to solubilise the ASO, supplied by Dharmacon Inc.) through tail vein injection. Tissues were harvested 48 hours after treatment.

At the end of the experiment, tissues were harvested from adult animals which were humanly killed under the regulation of Schedule 1 of Animal (Scientific Procedures) Act 1986. Tissues were dissected, weighed, rinsed in cold PBS, and snap frozen in liquid nitrogen then stored at -80°C. Expression of miR-223 was determined by miR-223 specific qRT-PCR following procedures described in Sections 2.2.1. Blood samples were collected from the tail vein, clotted blood was centrifuged at 2000 × g for 15 min at 4°C, serum samples were then stored in separate tubes at -80°C. Blood glucose levels were measured from tail bleed using OneTouch® Glucose Monitor (LifeScan Canada Ltd, Burnaby, Canada) following manufacture’s instruction. Serum insulin level was measured by Enzyme-Linked Immuno Sorbent Assay (ELISA) using the Rat/Mouse
Insulin ELISA Kit (Linco Research, St Charles, MO, USA) following the manufacturer’s protocol.

6.2.3 Cardiac-specific transgenic mouse

Primary miR-223 sequence was amplified from BN rat genomic DNA using primers with restriction site linkers on the 5’end. Sall sites (GTCGAC) were added to the forward primers, and HindIII sites (AAGCTT) on the reverse primer. Primers used for the amplification were:

Forward: 5’cgtagtcgacGGTGTCTTAGCCAGTCATGT3’
Reverse: 5’cgtaaagcttCTCTGAAGGATTGCTATAGTT3’

PCR products were digested with Sall and HindIII, and inserted into the Sall/HindIII site of pBS2/α-MHC vector (gift from Rosenzweig Lab, Harvard Medical School, USA, with approval of J Robbins; Appendix B). Positive clones were selected by ampicillin and PCR; selected clones were sequenced. Once sequence verified, plasmids were amplified by maxi-prep as described in Section 2.4.8. For microinjection, the plasmid was digested with restriction enzyme NotI and digested DNA was resolved by agarose gel electrophoresis. The DNA band containing the α-MHC promoter and the pri-rno-miR-223 insert (~6.6kb) was excised out and purified using QIAquick Gel Extraction Kit (QIAGEN) according to manufacturer’s protocol. Purified DNA was eluted in Water for Embryo Transfer (Sigma), and applied onto a Spin-X 100 column (Sigma) and centrifuged at 13 000 × g for 5 min at room temperature. DNA was quantified by spectrophotometry (Nanodrop Technology), and diluted to 2ng/µl. Transgenic mice were generated by the Transgenics and Embryonic Stem Cell Research Libratory at the Clinical Sciences Centre, MRC by pronuclear microinjection of DNA into a F1 CBA/B6 × CBA/B6 hybrid mouse oocytes using standard technique (Hogan, 1994).
Chapter Six

Injected oocytes were implanted into a surrogate female. Positive offspring were screened by PCR and selected founders were used for backcrossing to C57/BL6 mice. All offspring from each generation were genotyped, and positive mice were used for backcross. Animals were maintained and bred in the Central Biological Services Unit at Hammersmith Hospital Campus, Imperial College. Mice were kept on a 12 hour light/dark cycle and an *ad libitum* diet of standard mouse chow. All procedures were carried out under the authority of the appropriate Home Office (UK) personal and project licences in accordance with the Home Office Animals (Scientific Procedures) Act 1986.

6.2.3.1 DNA extraction and genotyping

Tail tip or ear clip biopsies were collected from weaned pups. DNA was extracted following the HotSHOT method (Truett et al., 2000). Biopsies were incubated in 75µl of alkaline lysis buffer (Appendix A) for 5 min at 100°C, then mixed by vortexing for 20 seconds, and incubated again at 100°C for 5 min. The samples were then vortexed again and kept on ice to cool. Once cooled, 75µl of neutralising buffer (Appendix A) was added to each sample. For PCR, 1µl of the DNA sample was used for 10µl reaction. PCR was performed following the standard PCR protocol as described in Section 2.4.1.

Primers used for genotyping were:

Forward: 5’CCCACACCAGAAATGACAGACA3’ (binding to the α-MHC promoter),
Reverse: 5’cgtaagcttCTCTGAAGGATTGCTATAGTT3’ (binding to the miR-223 insert).

PCR products were resolved on agarose gel electrophoresis. Samples with a band of ~0.5kb are selected as positive.
6.2.3.2 Phenotypic Studies on the transgenic mice

Tissues were harvested from adult animals which were humanly killed under the regulation of Schedule 1 of Animal (Scientific Procedures) Act 1986. Tissues were dissected, weighed, rinsed in cold PBS and snap frozen in liquid nitrogen. Expression of the transgene was determined by miR-223 specific qRT-PCR and Northern Blotting following procedures described in Sections 2.2.1 and 2.3. For immunoblotting analyses, protein lysates were purified as described in Section 2.6.

6.2.3.3 Functional assessment of the heart by cardiac MRI

Cardiac function of mice at 10 months of age was assessed by cardiac MRI. Animals were anesthetised with 1.5–2% isoflurane-oxygen mixture, and ventilated using a small animal ventilation apparatus. Cardiovascular MRI was performed on a 9.4T horizontal MR scanner (Varian, Palo Alto, CA, USA) located in the Biological Imaging Centre (Clinical Sciences Centre, MRC, Imperial College London). Electrocardiogram was monitored with a monitoring and gating system (SA Instruments, Inc. Stony Brook, NY, USA). Scout Gradient echo images were acquired in order to obtain the short-axis plan (cross-sectional view) of the heart, transverse sectional view was obtained from the short-axis plan. The following parameters were used for image acquisition: RF coil = rapid 38; PW/length = 2msec; FLIP angle = 90°; amplification power ratio = 25.2–27.7dB; shim (line width) = 200–380Hz. All imaging were performed by facility staff at the Biological Imaging Centre (in collaboration with Dr W Gsell and Dr M Wylezinska-Arridge, Biological Imaging Centre, Clinical Sciences Centre, MRC).

Stroke volume was calculated by capturing consecutive cross section images of 1mm thickness from the atrium to the apex, the LV volume was calculated following the Simpson’s rule (Niwa et al., 1996) at the end of diastole and systole, and the stroke
volume was determined based on the difference between the end diastolic and systolic volumes.

6.3 Results

6.3.1 Loss-of-function studies

6.3.1.1 miR-223 inhibition in vitro

Loss-of-function studies were carried out using Anti-miR-223 ASOs. Prior to in vivo administration, the silencing effect of the ASOs on the miRNA in cardiac myocytes was tested in vitro. Cultured NRVMs were transfected with anti-rno-miR-223 or anti-cel-miR-67, a negative control that inhibits miR-67 in Caenorhabditis elegans. Cells were harvested 48 hours after transfection, and RNA extracted. Quantitative RT-PCR analysis revealed miR-223 level was reduced by 93% compared to the cells treated with negative control (Figure 6.1), demonstrating high efficiency of the ASO in silencing endogenous mature miR-223.

6.3.1.2 miR-223 inhibition in vivo

For in vivo loss-of-function studies of miR-223, chemically modified ASOs were administered by direct injection via the mouse tail vein. Ten-week-old C57/BL6 mice were treated with single dose (80mg/kg body weight) of Anti-mmu-miR-223, Anti-cel-miR-67 (negative control) or equal volumes of buffer control with three animals in each treatment group. Animals were sacrificed 48 hours after injection; body, heart and LV weight were recorded and tibial lengths were measured (Table 6.1). To assess the effect of ASOs on the heart, heart and LV weights were indexed to body weights and tibial length (Figure 6.2). As experiment was designed to observe acute molecular changes, no changes in either heart or LV weight were detected.
Figure 6.1. Inhibition of miR-223 in NRVMs by anti-sense oligonucleotides. Primary cultures of NRVMs were treated with 30nM of Anti-cel-miR-67 (negative control) or anti-rno-miR-223. Expression of miR-223 was measured by stem-loop qRT-PCR, and its levels in the anti-rno-miR-223 treated cells were compared with cells transfected with the non-targeting negative control. Percentage of knock down indicates the level of expression in antagomir treated cells in relation to the control cells (data presented as means ± SEM; * $p=0.0014$; n=3).
Table 6.1 Measurements of mice treated for loss-of-function studies.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Treatment</th>
<th>Body weight before injection (g)</th>
<th>Body weight 48 hrs after injection (g)</th>
<th>Heart Weight (mg)</th>
<th>LV weight (mg)</th>
<th>Tibial Length (mm)</th>
<th>LV weight/heart weight</th>
<th>LV weight/tibial length</th>
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<tbody>
<tr>
<td>1</td>
<td>Saline Control</td>
<td>23.0</td>
<td>24.5</td>
<td>131.2</td>
<td>81.3</td>
<td>18.0</td>
<td>0.62</td>
<td>4.52</td>
</tr>
<tr>
<td>2</td>
<td>Saline Control</td>
<td>25.0</td>
<td>22.1</td>
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<td>69.8</td>
<td>17.5</td>
<td>0.61</td>
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</tr>
<tr>
<td>3</td>
<td>Saline Control</td>
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<td>24.5</td>
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<td>79.7</td>
<td>17.0</td>
<td>0.64</td>
<td>4.69</td>
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<tr>
<td>4</td>
<td>Anti-cel-miR-67</td>
<td>22.3</td>
<td>21.4</td>
<td>108.0</td>
<td>70.5</td>
<td>17.0</td>
<td>0.65</td>
<td>4.15</td>
</tr>
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<td>5</td>
<td>Anti-cel-miR-67</td>
<td>23.8</td>
<td>22.8</td>
<td>110.1</td>
<td>73.8</td>
<td>17.0</td>
<td>0.67</td>
<td>4.34</td>
</tr>
<tr>
<td>6</td>
<td>Anti-cel-miR-67</td>
<td>23.1</td>
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<td>121.8</td>
<td>73.5</td>
<td>17.0</td>
<td>0.60</td>
<td>4.32</td>
</tr>
<tr>
<td>7</td>
<td>Anti-mmu-miR-223</td>
<td>23.6</td>
<td>21.6</td>
<td>106.0</td>
<td>74.3</td>
<td>16.5</td>
<td>0.70</td>
<td>4.50</td>
</tr>
<tr>
<td>8</td>
<td>Anti-mmu-miR-223</td>
<td>21.3</td>
<td>20.4</td>
<td>101.8</td>
<td>65.6</td>
<td>16.5</td>
<td>0.64</td>
<td>3.98</td>
</tr>
<tr>
<td>9</td>
<td>Anti-mmu-miR-223</td>
<td>24.9</td>
<td>23.6</td>
<td>125.6</td>
<td>80.4</td>
<td>18.0</td>
<td>0.64</td>
<td>4.47</td>
</tr>
</tbody>
</table>
Figure 6.2 Indexed heart and LV weight of ASO treated mice. Ten-week-old mice were administered with 80mg/kg of anti-mmu-miR-223, anti-cel-miR-67 or equal volumes of buffer. Animals were sacrificed 48 hours after treatment, and body, heart, LV weights and tibial length were measured before tissues were harvested. No difference in heart and LV weights were observed in mice treated with anti-mmu-miR-223. Data are presented as mean ±SEM, n=3.
Figure 6.3 Expression of miR-223 across tissues. Animals administered with ASOs were sacrificed 48 hours after treatment, tissues were harvested, and RNA was extracted. Expression of mmu-miR-223 were analysed by stem-loop qRT-PCR. Data are presented as mean ±SEM, n=3. *p< 0.05; **p<0.01.
6.3.1.3  
**Inhibition of miR-223 levels across tissues**

At end of the 48-hour treatment, mice were sacrificed and tissues were harvested. RNA samples were purified from LV, skeletal muscle (SM), liver, kidney, spleen, lung and brain. The expression of miR-223 was analysed by stem-loop qRT-PCR (Figure 6.3). In anti-mmu-miR-223 treated mice, the expression of miR-223 was decreased significantly by 48% in the LV and 30% in the SM. miR-223 level were dramatically decreased in liver and kidney (84% and 94% respectively), the route (hepatorenal) through which ASOs are excreted.

6.3.1.4  
**Blood glucose level is lower in anti-mmu-miR-223 treated mice**

In chapter 4, *in vitro* studies on miR-223 identified its involvement in regulation of glucose uptake. Therefore, non-fasted blood glucose were measured to determine the effect of systemic miR-223 inhibition. Glucose levels in anti-mmu-miR-223 treated animals were significantly reduced by 40% (*p*<0.001; Figure 6.4A), while serum insulin levels remained unchanged (Figure 6.4B). Hence the reduced blood glucose level is not a result of alteration in serum insulin level.

6.3.1.5  
**Alterations in Glut4 expression in LV and SM**

The expression of Glut4 levels were assessed in the LV and SM at the protein and mRNA levels. In the LV, immunoblotting analysis revealed Glut4 protein levels were significantly reduced (33%, *p*<0.05) compared to mice treated with buffer control and 21% compared to mice treated with anti-cel-miR-67. Surprisingly, total Glut4 levels in skeletal muscle were increased in anti-miR-223 treated mice (3 fold, *p*<0.001) as compared to buffer control or anti-cel-miR-67 treated animals (Figure 6.5). No significant changes in *Glut4* mRNA were observed in either LV or SM samples (Figure 6.6). Collectively, alterations observed in this study in the LV appear to complement the
Figure 6.4 Non-fasted blood glucose level and serum insulin level in ASO treated mice. Ten-week-old mice were administered with 80mg/kg of anti-mmu-miR-223, anti-cel-miR-67 or equal volumes of buffer. 48 hours after treatment, prior to sacrificing the animal, venous blood glucose level was measured using a glucometer (A), and blood serum were collected from the tail vein, and serum insulin levels were determined by ELISA (B). Data are presented as mean ± SEM, n=3. Statistical significance was analysed by one-way ANOVA; *** p<0.001.
Figure 6.5 Alterations in total Glut4 protein levels in LV and skeletal muscle. Ten-week-old mice were administered with ASOs as previously described. Whole cell protein lysates were harvested from LV and SM, total Glut4 protein level was determined by immunoblotting using Glut4 specific antibody. Upper panels, immunoblot of total Glut4 in LV. Gapdh are presented as loading control. Lower panels, semi-quantitative densitometry of Glut4 protein levels normalised against loading control. A=LV and B=SM. Data are presented as mean ± SEM, * p<0.05; *** p<0.001.
Figure 6.6 mRNA level of Glut4 by qRT-PCR in LV and SM. Total RNA were extracted from LV and SM of mice treated with Anti-mmu-miR-223 or controls. Glut4 mRNA levels were determined by SYBR Green based qRT-PCR. Fold change in expression was calculated using the ΔCt method, and the level of expression was normalised to total RNA concentration. Data are represented as means ± SEMs, n=3.
in vitro observations described in Chapter 4; however, the effects of miR-223 expression on Glut4 in SM are paradoxical.

6.3.2 Gain-of-function studies

To determine the effects of miR-223 overexpression in vivo, a cardiac-specific miR-223 gain-of-function mouse model was generated. As described in Section 6.2.3, pri-rno-miR-223 sequence was PCR amplified from the genomic DNA of BN rat and cloned into pBS2/α-MHC vector, downstream of the α-MHC promoter. The plasmid was then linearised and injected into CBA × C57/BL6 hybrid mouse oocytes. Injected oocytes were implanted into a surrogate female. Positive offspring were screened by PCR. Among the 10 offspring generated from the injected oocytes, two were positive for the transgene (Figure 6.7), and were selected for breeding. Founder mice were repeatedly back-crossed with C57/BL6 mice (Charles River) to obtain offspring on a C57/BL6 genetic background. Mice generated from each cross with the wildtype C57/BL6 mouse were genotyped by PCR, and positive animals were used for the next backcross. Two transgenic lines were established, and four backcrosses were achieved for each line at the time of submission of this dissertation.

6.3.2.1 Expression of miR-223 in the transgenic heart

To assess the specificity of transgene expression in the heart and the level of overexpression, tissues from TG mice at the second backcross were harvested, and the level of mature miR-223 was assessed by qRT-PCR and Northern Blotting. Figure 6.8 summarises miR-223 expression detected by qRT-PCR in the TG mice compared to the WT littermates, where expression of the miRNA is 64 folds higher in the TG compared to the WT (p<0.0001; Figure 6.8A and B). In addition, unvarying levels of miR-223 in the TG and WT spleen demonstrate the overexpression is heart specific (Figure 6.8C). Results from qRT-PCR analyses were further validated by northern blotting (Figure
Figure 6.7 Genotyping for founder animals. Tail tip biopsies were collected from founder animals. DNA was extracted using the HotSHot method. PCR were performed using forward primers binding to the MHC promoter and reverse primers binding to the transgene. PCR amplicons were resolved on 2% (w/v) agarose gel. Bands were visualised with ethidium bromide, and visualised under UV light. Expected band size: ~420bp. A. Initial genotyping. B. Confirmation of initial genotyping result. Animals 3 and 7 were identified as founder animals.
Figure 6.8 Analysis of miR-223 expression in transgenic mice by qRT-PCR. Heart and spleen were harvested from wildtype (WT) and transgenic (TG) littermates. Expression of mature miR-223 was analysed by qRT-PCR. A. Expression of miR-223 in heart and spleen of TG and WT presented in Ct values. B Expression of miR-223 in the heart of TG mice compared to WT ($p<0.0001$). C Expression of miR-223 in the spleen of TG mice compared to WT.
Figure 6.9 Northern Blot on miR-223 in TG mice. A. miR-223 expression in the LV of TG and WT mice. B. Expression of miR-223 across tissues in TG mice. 5S rRNAs are shown to indicate equal loading.
6.9). Comparing the expression of miR-223 across tissues in TG mice (Figure 6.9B), the expression was only elevated in the LV, as compared to the WT distribution (Figure 4.1, Chapter 4)

6.3.2.2 **Expression of miR-223 targets in transgenic heart**

As described in Chapter 5, in the attempt to identify targets of miR-223, the expression levels of several proteins were tested in NRVMs, however, none of the genes tested showed a decrease in expression upon miR-223 overexpression, this includes previously validated targets NFIA and IGF1R. To further assess this unexpected finding in vivo, the levels of Nfia, Igf1r and Rab10 were analysed in the TG heart (Figure 6.10). Similar to in vitro observations, no detectable changes were found in the genes tested. These findings further confirm that Nfia and Igf1r are not regulated by miR-223 in the heart, and supports the hypothesis that miRNA mediated gene regulation could be tissue-specific.

6.3.2.3 **Effects of miR-223 overexpression on Glut expression and translocation**

Expression of Glut was analysed by immunoblotting in the TG heart. Total Glut4 expression in the heart was not affected by miR-223 overexpression (Figure 6.11A). Although there is a trend of decreasing in Glut1 protein level in TG heart, this alteration was not statically significant (p=0.09, Student’s t-test). To assess the affect of miR-223 overexpression in Glut4 sub-cellular localisation in vivo, LVs were collected from TG and WT littermates, membrane and protein fractions were extracted, and membrane Glut4 content was analysed by immunoblotting (Figure 6.11B). Compared to whole cell protein lysates (30µg), the amount of Glut4 protein was 3-fold higher in the membrane fractions. No difference in membrane or cytosolic Glut4 expression levels was observed between TG and WT mouse heart.
Figure 6.10 Immunoblotting analysis of predicted miR-223 targets. Left ventricles of eight-week-old TG and WT littermates were collected. Total protein extracts were purified and immunoblotting analysis was performed using specific antibodies. Gapdh is presented to show equal loading.
Figure 6.11 Immunoblots of Glut4 and Glut1 expression. A. Levels of total Glut4 and Glut1 protein in the TG mouse heart compared to the WT. B. Levels of total and membrane Glut4 protein in the TG mouse heart compared to the WT. Upper panels, immunoblot of Glut1 and Glut4; lower panels, semi-quantitative densitometry on protein levels.
6.3.2.4 *Effects of miR-223 overexpression on insulin signalling*

In chapter 4, overexpression of miR-223 in cultured cardiac myocytes was shown to decrease Akt and Gsk3β phosphorylation upon insulin stimulation. To test the effect of miR-223 overexpression in the transgenic model, levels of phospho-Akt and Gsk3β at the resting state were determined by immunoblotting. Twelve 8-months-old animals were examined in total; 6 TG mice were compared with and 6 WT littermates, no significant difference in Akt and Gsk3β phosphorylation (Ser473 and Ser9, respectively) was observed ([Figure 6.12](#)).

6.3.2.5 *Effects of cardiac-specific expression of miR-223 on heart physiology*

No apparent structural cardiac abnormality was observed in the TG mice and there was no significant difference in heart and LV weight between the TG and WT mice. To assess the effects of aging on cardiac function of the transgenic mice, 8 TG and 6 WT non-bred male mice were maintained separately for aging. Animals were sacrificed at 10 months of age, tissues were collected, body, heart and LV weights were recorded and tibial lengths were measured. Indexed heart and LV weights were compared between TG and WT mice, and no difference was observed ([Figure 6.13](#)).

Among the aged mice, the cardiac function of 3 TG and 3 WT mice were assessed by cardiac MRI at 10 months of age. Animals were scanned with a 9.4T MR Scanner in collaboration with the Biological Imaging Centre (CSC, MRC, Imperial College London). Transverse and cross-sectional images of the heart were obtained ([Figure 6.14](#)). Compared to the WT controls, TG mice showed normal ventricular thickness and systolic function. No difference was observed in left ventricular stroke volume, ejection fraction, and percentage wall thickening between the TG and WT animals ([Table 6.2](#)).
Figure 6.12 Analysis of Akt and Gsk3β phosphorylation in the transgenic heart. Upper panel, immunoblot of phospho-Akt (Ser473) and phospho-Gsk3β (Ser9); lower panel, semi-quantitative densitometry on protein levels.
Figure 6.13 Indexed heart and LV weight of 10-month-old TG mice. Non-bred male mice were sacrificed at 10-months of age. Body, heart, LV weights and tibial length were measured during tissue harvesting. No difference in heart and LV weights were observed between TG and WT mice. Data are presented as mean ±SEM, TG n=8; WT n=6.
Figure 6.14 Representative cardiac MR images of TG and WT transgenic mice. Ten months old non-bred male were scanned with 9.4T scanner. A, C, E and G shows images of TG mouse (animal 3), and B, D, G and H are images from the WT mouse (animal 5). A and B, transverse sectional view captured at diastole. C and D, transverse sectional view captured at systole. E and F, cross-sectional view at the papillary muscle level captured at diastole; G and H, cross-sectional view at papillary muscle level captured at systole. Images showed normal systolic function of the transgenic mouse heart.
Table 6.2. Cardiac function of miR-223 TG mice compared to WT as assessed by **cardiac MRI**. Cross sectional images of 1mm thickness were captured along the short-axis to cover the entire ventricle. Stroke volume, ejection fraction and percentage wall thickening were calculated from images taken, as described in Section 6.2.3.3. Data are presented as mean ± SD.

<table>
<thead>
<tr>
<th></th>
<th>WT (n=3)</th>
<th>TG (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV stroke volume (mm³)</td>
<td>32.4 ± 2.94</td>
<td>34.02 ± 7.56</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>76.33 ± 2.05</td>
<td>73.9 ± 7.53</td>
</tr>
<tr>
<td>% wall thickening</td>
<td>32.04 ± 17.71</td>
<td>39.55 ± 20.81</td>
</tr>
</tbody>
</table>
These results indicate miR-223 transgenic mice have normal cardiac function, and miR-223 overexpression has no apparent effects on cardiac function in mouse in the absence of perturbation.

### 6.4 Discussion

*In vivo* functional studies are valuable tools for validation of *in vitro* observations. This chapter describes the application of both gain- and loss-of-function mouse models for miRNA studies. Anti-miR-223 ASOs were intravenously administered into wildtype C57/BL6 mice. Overall, significant reduction in miR-223 levels were observed in all tissues examined, with 48% knockdown observed in the heart, which was associated with a significant decrease in total Glut4 protein level in the heart (*Figure 6.5A*). This observation in the heart is in accordance with the findings described *in vitro*, where overexpression of miR-223 in cardiac myocytes induces total Glut4 protein levels. A significant reduction in blood glucose level was observed in mice treated with anti-mmu-miR-223 (*Figure 6.4*). This could be caused by unexpected increase in Glut4 protein level found in the SM (*Figure 6.5B*), although only 30% reduction of miR-223 level was achieved.

How can these apparently paradoxical observations be explained? Apart from conventional views that miRNA reduce the levels of its target protein, recent studies have shown miRNA can have different gene regulatory functions depending on the cell type (Vasudevan et al., 2008). Although similar in some ways, cardiac muscle is fundamentally different to skeletal muscle. The data presented in this chapter suggest, as a fine tuner of glucose metabolism, miR-223 could be controlling the same target in the heart and skeletal muscle, but regulating them in opposing ways. Alternatively, the *in vivo* observations could be caused by miRNA-silencing artefacts. Off-target effects in
siRNA studies have been noted to have observable phenotypic effects (Fedorov et al., 2006; Xu et al., 2007), and the silencing efficiency could result in different phenotypic outcome. In this study, only 30% silencing of miR-223 was achieved in the skeletal muscle, whereas in the heart, the miRNA was knocked down by almost 50%. Nonetheless, further proteomics or gene expression analyses on these tissues or other animals subjected to prolonged miR-223 inhibition could give insights on miR-223 targets in the heart and skeletal muscle, and possibly provide true explanation for these contradicting observations.

To study the effect of miR-223 overexpression in vivo, a heart specific gain-of-function model was generated. Although mature miR-223 level were increased in the heart (Figure 6.8), no detectable effects of miR-223 overexpression on cardiac function, metabolism or insulin signalling was observed. However, this does not imply miR-223 expression has no effects in the heart in vivo. Studies of systemic deletion of miRNA in C. elegans revealed, individually, only a few miRNAs are essential for development and viability, and few miRNA-target relationships result in an overt phenotype (Miska et al., 2007). This suggests, instead of gene switches that turn genes on or off completely, miRNA-mediated regulation acts as a buffering mechanism to increase the fidelity of processes (e.g. post-translational regulation). However, these subtle alterations can be challenging to detect using standard approaches. To detect the phenotypic effects in such cases, perhaps the best approach might be to subject miRNA loss- or gain-of-function models to environmental stresses (Flynt and Lai, 2008). For this study, based on the role of miR-223 in glucose metabolism, gain- or loss-of-function mouse models could be achieved using insulin stimulation or by subjecting the heart to alternative stresses, such as myocardial infarction or ischemia.
7.1 Summary of results

1. miRNAs are differentially expressed in insulin resistant human heart as compared to control heart samples. Hsa-miR-223 is upregulated in hearts of type 2 diabetic patients and patients with LVD.

2. In rodent heart, miR-223 is expressed at intermediate levels as compared to other organs and is expressed in both cardiac myocytes and fibroblasts with predominant peri-nuclear localisation.

3. Adenoviral mediated overexpression of miR-223 in rat neonatal cardiac myocytes increases glucose uptake independent of PI3K/Akt activity and AMPK signalling.

4. miR-223 overexpression inhibits insulin stimulated Akt/Gsk3β phosphorylation in neonatal cardiac myocytes.

5. Glut4 protein levels are increased by miR-223 overexpression in both cardiac myocytes and COS7 cells.

6. A previously proven miR-223 target in immune cells, Nfla, is upregulated by miR-223 in neonatal cardiac myocytes.
7. Systematic inhibition of miR-223 in vivo reduces blood glucose level, decreases Glut4 protein level in the heart and increases Glut4 protein levels in the skeletal muscle.

8. Transgenic mice overexpressing miR-223 specifically in cardiac myocytes have normal cardiac physiology and function at baseline.

7.2 General discussion and conclusion

The studies presented in this dissertation were designed to assess whether miRNAs have a role in the insulin resistant heart. The initial study determined the overall expression patterns of miRNA in the human heart, which is followed by in vitro gain-of-function studies in rat neonatal cardiac myocytes, and then these findings were investigated further in vivo using loss- and gain-of-function studies.

Chapter 3 presents the first quantitative miRNA expression analysis in the human heart using samples from patients with T2DM and LVD, diseases that are characterised by myocardial insulin resistance. Differences in miRNAs expression were observed in hearts from T2DM patients compared to previous end-stage LVD miRNA expression studies, suggesting a disease specific miRNA expression pattern in the heart. Among the dysregulated miRNAs identified, only one miRNA (miR-223) is co-ordinately upregulated in both T2DM and LVD patient hearts, suggesting a possible role for this miRNA in insulin resistance and glucose metabolism.

Previous studies of miR-223 have focused predominantly on its role in the myeloid system. The first evidence of the involvement of miR-223 in insulin signalling and glucose uptake in the heart is presented in Chapter 4, where the function of miR-223 in
glucose metabolism and insulin signalling was analysed \textit{in vitro}. It was found that, independent of AMPK activity, miR-223 increases cellular glucose uptake by upregulating Glut4 protein level. In parallel, miR-223 also inhibited insulin-stimulated Akt and GSK3β phosphorylation. Recent miRNA studies have shown miR-223 was increased post-MI in mouse (van Rooij et al., 2008b). It is known that MI and cardiac ischemia increases glucose uptake to the heart, and insulin-stimulated glucose uptake is beneficial during MI in humans (Bucciarelli-Ducci et al., 2006; Diaz et al., 1998; Krljanac et al., 2005). These findings indicate that glucose uptake induced by miR-223 could provide protective mechanism for the heart in ischemic conditions, as the usual insulin induced glucose uptake could be impaired due to acidosis produced by the ischemia (Beauloye et al., 2001; Hue et al., 2002).

In order to dissect the mechanism underlying miR-223 induced glucose uptake and Glut4 upregulation, predicted miR-223 mRNA targets were analysed in Chapter 5. Unexpectedly, none of the predicted targets tested showed direct interaction with miR-223 in cardiac myocytes, including previously confirmed targets. There could be a number of explanations for this observation. 1) miRNA induced gene regulation could be highly tissue/cell type specific, and targets that have been identified in one tissue may not be regulated by the same miRNA in another. 2) Messenger RNA encoding the same gene may be alternatively sliced in different cell types, as a result, miRNA binding site could be abolished (Sandberg et al., 2008). 3) Current understanding of miRNA-target interactions have been previously deduced from miRNAs that are highly expressed (Bartel, 2009). For miR-223, all previous target validation studies were performed in cells of myeloid lineage, where the miRNA is most highly expressed. In the heart, miR-223 is only expressed at moderate level, and moderately expressed
miRNA could interact with its mRNA target in a different manner which still needs to be elucidated. 4) Target-site accessibility may also be affected by cell type. RNA-binding cofactors that help miRNA binding to the target mRNA could be expressed only in certain tissue type, thus prevent miRNA-mRNA interaction when the cofactor’s expression is affected (Bhattacharyya et al., 2006). 5) miRNA may have opposite regulatory functions in dividing cells compared to cells at cell cycle arrest. Studies of the effect of cell cycle have revealed that miRNAs can actually activate translation in non-dividing cells (Vasudevan et al., 2007). This finding is particularly important for studying miRNA functions in the heart, as cardiac myocytes are terminally differentiated, thus miR-223 targets identified in other tissues (e.g. myeloid cells) may not be regulated in the same manner in cardiac myocytes.

In addition to gene repression, miRNAs can also increase protein levels of target mRNAs (Vasudevan et al., 2007, 2008). This raises the question, is the increase in Glut4 protein level (Chapter 4) a direct effect of miR-223? This was investigated in Chapter 5. Although Glut4 does not have a miR-223 target sites in its 3’UTR, site prediction using the full length Glut4 cDNA, including the 5’UTR and ORF, suggested a number of sites that could potentially be interacting with the miRNA. This is supported by findings from the ENCODE project that identified multiple miRNA sites throughout the coding gene sequences (Birney et al., 2007). *In situ* hybridisation and immunofluorescence analyses demonstrated that both miR-223 and Glut4 protein are localised predominantly in the peri-nuclear regions in cardiac myocytes, and studies have confirmed that in both NRVM and COS7 cells Glut4 protein increases with miR-223 overexpression, suggesting a direct interaction between the two. However, further
experimentations such as luciferase reporter analysis are required to confirm if miR-223 directly upregulates Glut4.

Two *in vivo* models were used to further investigate the biological function of miR-223. Chapter 6 presented physiological and molecular findings from both models. Both gain- and loss-of-function studies resulted in apparent healthy animals with normal cardiac physiology and function at baseline. Some differences were observed in the loss-of-function study where blood glucose levels were decreased. Glut4 protein level was downregulated in the heart, which complements the *in vitro* findings described in Chapter 4. However, no change in basal insulin signalling was observed. Surprisingly, Glut4 protein levels were upregulated in skeletal muscle, this could explain the dramatic decrease in blood glucose concentration, although the mechanism behind these robust observations needs to be studied further in skeletal muscle cells. Overall, the *in vivo* studies were less informative than initially anticipated. Indeed, studies in other model systems have shown that disruption of a single miRNA may produce subtle phenotypes that are difficult to detect *in vivo* (Li, 2005; Miska et al., 2007; Xiao et al., 2007a). This could be explained by the fact that most conserved miRNA target genes also contain conserved binding sites of other miRNAs, thus disruption of the interaction with only one miRNA might not result in marked changes of mRNA or protein expression. Another explanation is that targets of miR-223 or miR-223 itself could be part of a regulatory buffering network, where the effect of disruption at one point of the network (i.e. miR-223) is counterbalanced by bifurcating pathways and feedback mechanisms (Hornstein and Shomron, 2006). In this case, such a network would have to be perturbed before the disrupted miRNA has an effect on the phenotype. Thus, cardiac
stress such as myocardial infarction in heart specific miR-223 transgenic mice could yield more phenotypic evidence on the function of miR-223.

In conclusion, the work presented in this dissertation shows miR-223 is upregulated in the insulin resistant human heart and when overexpressed enhances PI3K/Akt-independent glucose uptake to cardiac myocytes through increased Glut4 protein expression. *In vivo* systemic inhibition of miR-223 lowered blood glucose concentration and decreased Glut4 protein level in the heart. The findings presented here implicate the potential role for miRNAs in cardiac glucose metabolism through the non-canonical pathway, and provide insights for future investigations on diabetic cardiomyopathy and insulin resistant heart.


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References


References


APPENDIX A

List of buffers and media

All chemicals were purchased from Sigma-Aldrich, unless otherwise stated. All buffers and media were prepared with double distilled water (ddH$_2$O).

**Buffers**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10× Orange G</td>
<td>40% (w/v) sucrose and 0.2% (w/v) Orange G, dissolve in ddH$_2$O.</td>
</tr>
<tr>
<td>10× TBE</td>
<td>1.1M Tris; 900mM Borate; 25mM EDTA; pH 8.3</td>
</tr>
<tr>
<td>10× TBS</td>
<td>0.1M Tris-base, 1.5M NaCl, pH 7.6</td>
</tr>
<tr>
<td>ADS buffer</td>
<td>116mM NaCl, 20mM HEPES, 0.8mM NaH$_2$PO$_4$, 5.6mM Glucose, 5.4mM KCl, 0.8mM MgSO$_4$, pH 7.35</td>
</tr>
<tr>
<td>Alkaline lysis buffer</td>
<td>25mM NaOH, 0.2mM EDTA, pH 12</td>
</tr>
<tr>
<td>DEPC treated water</td>
<td>1ml of diethyl pyrocarbonate (DEPC) per 500ml of ddH$_2$O, shake and leave overnight at 37°C before autoclaving.</td>
</tr>
<tr>
<td>Hybridisation buffer</td>
<td>1× TBS, 0.05% (v/v) Tween 20, 5% (w/v) non-fat milk</td>
</tr>
<tr>
<td>(For immunoblotting)</td>
<td></td>
</tr>
<tr>
<td>Neutralising buffer</td>
<td>40mM Tris-HCl, pH 5</td>
</tr>
<tr>
<td>PBS</td>
<td>3.2 mM Na$_2$HPO$_4$, 0.5 mM KH$_2$PO$_4$, 1.3 mM KCl, 135 mM NaCl, pH 7.4</td>
</tr>
<tr>
<td>SDS-PAGE running buffer</td>
<td>25mM Tris-HCl, 192mM glycine, 2% (w/v) SDS, pH 8.3</td>
</tr>
<tr>
<td>TE (T10E0.1, pH 7.5)</td>
<td>10mM Tris-HCl, 0.1mM EDTA (pH 7.5)</td>
</tr>
<tr>
<td>Transfer buffer</td>
<td>25mM Tris-HCl, 192mM glycine, 20% (v/v) methanol (Fisher Scientific)</td>
</tr>
</tbody>
</table>
### Media

#### For NRVM cultures

**Plating media**  
68% (v/v) DMEM (Invitrogen), 17% (v/v) M199 (Invitrogen), 10% (v/v) horse serum (Autogen Bioclear); 5% (v/v) FCS (Autogen Bioclear), 1 × Penicillin and streptomycin mix (Sigma; 100 units/ml penicillin, 100 µg/ml streptomycin)

**Maintenance media**  
80% (v/v) DMEM (Invitrogen), 20% (v/v) M199 (Invitrogen), 1 × Penicillin and streptomycin mix (Sigma; 100 units/ml penicillin, 100 µg/ml streptomycin)

**Glucose free media**  
80% (v/v) glucose free DMEM (Invitrogen), 20% (v/v) M199 (Invitrogen)

#### For COS7 cultures

**Maintenance media**  
90% (v/v) DMEM (Invitrogen), 10% (v/v) FCS (Autogen Bioclear), 1 × Penicillin and streptomycin mix (Sigma; 100 units/ml penicillin, 100 µg/ml streptomycin)

#### For bacteria cultures

**LB media**  
1.0% (w/v) Tryptone, 0.5% (w/v) Yeast Extract, 1.0% (w/v) NaCl, pH 7.0 Autoclave; cool to 55°C, add 50 µg/ml of either ampicillin or kanamycin

**LB agar plate**  
1.0% (w/v) Tryptone, 0.5% (w/v) Yeast Extract, 1.0% (w/v) NaCl, pH 7.0, 1.5% (w/v) agar. Autoclave; cool to 55°C, add 50 µg/ml of either ampicillin or kanamycin, pour into 10 cm plates
**APPENDIX B**

**Plasmid maps**

**pBS2/αMHC**

Map is not drawn to scale. Sequence information of insert fragment, pri-rno-miR-223, is presented below the plasmid map. For details of the αMHC promoter region, refer to (Subramaniam, Jones et al. 1991)

```
> pri-rno-miR-223
GGTGTCTTAGCCAGTCATGTTAGTGTCTGCCATTTGTCTCACTCCCAGAGTAAGGTATAATCAGCCC
ATTTTTTTTCTTTTCCAGTGCGATATCTCTTCCAGCTCTGAGGTAAAAGGTCTGAGTCGTTTGGTAT
GCCTTCTGCAGTGTTACGCTCCGTGTATTTGACAAGCTGAGTTGGACACTCTGTGTGGTAGAGTGTC
AGTTTGCTCAATACCCCAAGTTGCTGGCTATCGATCTTATAGAGAGCTGTGTTAGAGTGGTC
TGCTTCTTTGCTATGAGTTGCTTTGTTTCTTACCATGCTCGTGGATTTCAGTGATGGGG
AAGTGGTGGGCATTGCGATATTTTTCCTT
```

**Note:** Pre-rno-miR-223 sequence is shown in bold. Mature rno-miR-223 is shown in bold and underlined.
pCR2.1-TOPO® (Invitrogen, Paisley, UK)
psiCHECK-2 (Promega, Madison, WI, USA)

*3’UTRs of NfIa, Rab10 and Mtpn were sub-cloned into the NotI/XhoI sites.

> NfIa 3’UTR
TTGGTTCAGCAGTCAGCAGTTACATACATAACACACCGCAAGGACAGTGAAATCGCTCCTGTTGCAAG
GCAATTTCCAAAAAGACCGCAAAAGACTACTACTATCAGTTTGGTTGGGTTTTTGTTTTTTTGTTTTTT
TTTTTTTTTTTTTTTTTGGTTTTTTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTTAAGTTTTGAAATGCTGCACTTACATTTAAAAAAAAAC
ATTTTTCAACAATTTCAACAATGACACAAAAATTCACATGGAAATGGGGAAGATGGTCTGTTTTGACAGA
AACTGACA

> Rab10 3’UTR
TTAGGGTCCCCCTCCTGGACTCTATCATCATGTTTTTACTAGTAAAAGCAGCATTGCCAAAATATTCCTCGAT
TTCCACTATGAAAAATGCAATGTCTAAGCTTTTGAAGTTTGTAGGTTAAACCTACTGTTGTTAGTTAGA
TCTTTGCTTGTGTGTGGCTTCCCTCTACTGTTGTTGCGGTTGCCCTCTTATTTAACCTTTGTATA
ACCCTTAAAATCTGACCTAGTGTTTGGGAGATAACACTGAGATTGTTTGGCTAACAAGACTGACAAGTTTTGT
TGTTTGAATGATTATGATCGGACACTGTACAGAGAAAAGATATGGCCTC

> Mtpn 3’UTR
GGATTTTCTCTCCTCCTTCCCATCACAATTTTTGCTATGTTTTTCTTTTATATTAAGAAGGACAGATGCTTACAT
TGTATAATTCTAGGAGGCTAAGAGGAATATGCTTGGTGTTTCTTTTTTCTTTTTTTTTTCTTTAGC
TACGAGCATTATATAACATTTGAGAAACACTTTTGAAGCTCTCTGGTATGTTAAGAGGATTAAAAACACCA
TAGCCCTAAGAGGTGTATATCTATATATAAAGAGAAAGAAGGAGGCTTGTAGTTTTGGAGATAATATGAC
AGATTTTTGAGAGGCGCTCTCCTTGTGGTTGGAGGAGATGTTCCCTCTCTCAAAA

Note: miR-223 recognition sites are shown in bold.
pTarget™ (Promega, Madison, WI, USA).
pVQ Ad5CMV K-NpA (ViraQuest Inc, USA)
APPENDIX C

List of primers

**Genotyping primers for miR-223 TG mouse**

**Forward:** 5′CCCACACCAGAAATGACAGACA3’
Binds to αMHC (ENSMUST00000081857) intron 2, position 246-267

**Reverse:** 5′cgtaagcttCTCTGAAGGATTGCTATAGTT3’
Binds to Rat chromosome: RGSC3.4:X: 83869258 to 83869278
Amplicon size: 419bp

**Glut4** ENSRNOT00000023256 (for SYBR qRT-PCR):

**Forward:** 5′GATTCTGCTGCCCTTCTGTC3’
Binds to 792-811 of the transcript

**Reverse:** 5′CAGCTCAGCTAGTGCGTCAG3’
Binds to 917-936 of the transcript
Amplicon size: 145bp

**miR-223** ENSRNOT00000053628 (for generating adenovirus)

**Forward:** 5′actggaatctGGTGTCTTAGCCAGTCATGT3’
Binds to Rat chromosome: RGSC3.4:X: 83868923 to 83868942

**Reverse:** 5′actggcggccgcCTCTGAAGGATTGCTATAGTT3’
Binds to Rat chromosome: RGSC3.4:X: 83869258 to 83869278
Amplicon size: 356bp

**miR-223** ENSRNOT00000053628 (for generating cardiac-specific transgenic mouse)

**Forward:** 5′cgtagtcgacGGTGTCTTAGCCAGTCATGT3’
Binds to Rat chromosome: RGSC3.4:X: 83868923 to 83868942

**Reverse:** 5′cgttagctgtCTCTGAAGGATTGCTATAGTT3’
Binds to Rat chromosome: RGSC3.4:X: 83869258 to 83869278
Amplicon size: 356bp

**miR-296** ENSRNOG000000035619 (for generating adenovirus)

**Forward:** 5′actggaatctTTGTGTGAGGGACAGAGGG3’
Binds to Rat chromosome: RGSC3.4:3: 165193701 to 165193720

**Reverse:** 5′ctgcctgctgcACCCCAAGGCACTAAGTTG3’
Binds to Rat chromosome: RGSC3.4:3: 165193964 to 165193983
Amplicon size: 283bp

**Mtpn** ENSRNOT00000015808 3’UTR

**Forward:** 5′GGATTTTCTTCTCCCTTTCAC3’
Binds to Rat chromosome: RGSC3.4:4: 62896627 to 62896645

**Reverse:** 5′TGGGGTTTTGAGAGAGACATTTC3’
Binds to Rat chromosome: RGSC3.4:4: 62896961 to 62896980
Amplicon size: 354bp
**Nfla ENSRNOT00000004017 3’UTR**
Forward: 5’TTGGTTTCAGCAGTCAGCAG3’
Binds to Rat chromosome:RGSC3.4: 5 118507126 to 118507144
Reverse: 5’GGTGAGGACTTGAAC3’
Binds to Rat chromosome:RGSC3.4: 5 118507491 to 118507510
Amplicon size: 385bp

**Rab10 NM 017359.2 (RefSeq) 3’UTR**
Forward: 5’TTAGGGTCCTCCTCACTTG3’
Binds to Rat Chr6_random: 438050-438068 (UCSC)
Reverse: 5’GAGGCCATATCTTTTCTCTG3’
Binds to Rat Chr6_random: 438358-438377 (UCSC)
Amplicon size: 328

**T7 promoter**
5’TAATACGACTCACTATAGG3’
APPENDIX D

qRT-PCR data from individual patient samples (Table 3.3)

- **miR-367 - Ct**

- **miR-367 - FC**

- **miR-223 - Ct**

- **miR-223 - FC**

- **miR-342 - Ct**

- **miR-342 - FC**