

Regulation of calcium handling proteins by microRNA-1 in heart failure

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Abstract

Heart failure has a complex pathophysiology, a diverse aetiology, an increasing prevalence and only palliative treatment is currently available. The underlying molecular cause of cardiomyocyte malfunction is not fully understood. MicroRNA-1 is a post transcriptional genetic regulator that is highly expressed in the myocardium and has been found to influence cardiomyocyte proteins that are key for maintaining calcium homeostasis and contractility; the sarcoplasmic reticulum calcium transport ATPase (SERCA2a) and the sodium calcium exchanger (NCX). MicroRNA-1 expression is disrupted in both *in vivo* models and heart failure patients; this makes the impact of altered microRNA-1 expression on the development of heart failure pathophysiology an interesting target for investigation.

Using *in vitro* and *in vivo* models of heart failure, experiments were conducted to increase microRNA-1 expression by viral or non-viral transfection techniques. This produced a number of interesting results relating to cardiomyocyte function; impaired β -adrenoceptor mediated contractility, a hallmark of the heart failure phenotype, was reversed and can most likely be attributed to an increase in cyclic adenosine monophosphate (cAMP) production. SERCA2a driven sarcoplasmic reticulum (SR) calcium loading was improved and a greater release of calcium from the SR was recorded. Questions remain around the mechanism connecting microRNA-1 to these targets, particularly as gene expression was unaffected by increased microRNA-1. Future experiments would focus on bridging this understanding to expand upon these results.

The prognostic potential of circulating microRNAs in serum samples taken from a well characterised population of heart failure patients was also investigated. Two separate microarray platforms were conducted and the expression of a shortlist of 15 microRNAs validated further. Unfortunately no microRNA could be identified that could consistently distinguish between good and poor prognosis heart failure patients. This does not discount circulating microRNAs as possible prognostic biomarkers of heart failure, but a different approach would be required for future experiments.

Declaration of originality

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Induced downregulation of miR-1 expression in cultured adult rat cardiomyocytes replicates the elevated NCX function observed in heart failure

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List of abbreviations

- (AAV)** Adeno-associated virus
- (AC)** Adenylyl cyclase
- (ACE)** Angiotensin converting-enzyme
- (Acta1)** Skeletal muscle α -actin
- (Actc1)** Cardiac α -actinin
- (AKAP)** A-kinase anchoring proteins
- (ANP)** Atrial natriuretic peptide
- (Anti miR-1)** Antisense RNA oligonucleotide
- (AP)** Action potential
- (ASPA)** Animals Scientific Procedures Act
- (ATP)** Adenosine triphosphate
- (BAR)** Bin, Amphiphysin, Rvs
- (BARK)** β -adrenergic receptor kinase 1
- (BIN1)** Amphiphysin 2
- (BI%PeakH)** Baseline as a % of peak height
- (BNP)** Brain natriuretic peptide
- (BSA)** Bovine serum albumin
- (C.elegans)** Caenorhabditis elegans
- ([Ca]_i)** Calcium transient
- (CAD)** Coronary artery disease
- (CaMKK)** Calcium/ calmodulin dependent protein kinase kinase
- (cAMP)** Cyclic adenosine monophosphate
- (cDNA)** Complementary DNA
- (CFP)** Cyan fluorescent protein
- (CGP20712A)** ($\pm$)-2-Hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1H-imidazol-2-yl]phenoxy]propyl] amino]ethoxy]-benzamide methanesulfonate salt
- (CICR)** Calcium induced calcium release
- (CMO)** Cell mask orange
- (cMyBP-C)** Cardiac myosin binding protein C
- (Copernicus)** Carvedilol Prospective Randomized Cumulative Survival
- (CPAP)** Continuous positive airways pressure
- (CUPID)** Calcium Up-Regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease

(DCM) Dilated cardiomyopathy
(DMEM) Dulbecco's Modified Eagle Medium
(DMSO) Dimethyl sulfoxide
(ECC) Excitation contraction coupling
(EDTA) Ethylenediaminetetraacetic acid
(EDV) End diastolic volume
(EF) Ejection fraction
(E_m) Cardiomyocyte membrane potential
(E_{NCX}) NCX equilibrium potential
(EPAC1) exchange protein directly activated by cAMP
(ERK) Extracellular signal-regulated kinases
(ESV) End systolic volume
(FCS) Fetal calf serum
(FKBP) FK binding protein 12.6
(FRET) Förster resonance energy transfer
(FS) Fractional shortening
(G protein) Heterotrimeric guanine nucleotide-binding protein
(GFP) Green fluorescent protein
(GRK2) G protein coupled receptor kinase 2
(HAX-1) HS-1 associated protein X-1
(HBSS) Hanks balanced salt solution
(HCN) Hyperpolarisation-activated cyclic nucleotide-gated ion channels
(HDL) High-density lipoprotein
(HEPES) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
(HRC) Histidine rich calcium binding protein
(I_{Ca}) Calcium current
(ICM) Ischaemic cardiomyopathy
(IGF2) Insulin-like growth factor 2
(I_{NCX}) NCX ionic current
(JNKs) c-Jun N-terminal kinases
(JP2) Junctophilin 2
(K-H) Krebs-Henseleit
($K_{ir2.1}$) Inward-rectifier potassium ion channel 2.1
(LTCC) L-type calcium channel

(LVIDd) Left ventricle internal diameter end diastole

(LVIDs) Left ventricle internal diameter end systole

(M) Molar

(M2) M2-cholinoceptors

(MAPK) Mitogen-activated protein kinase

(MHC α -CN) Myosin heavy chain α calcineurin

(MI) Myocardial infarction

(MKKKs) MKK kinases

(MKKs) MAPK kinases

(MMPs) Matrix metalloproteinases

(MOI) Multiplicity of infection

(mRNA) Messenger RNA

(Myh6) α -myosin heavy chain

(Myh7) β -myosin heavy chain

(NCX) Cardiac sodium calcium exchanger

(NT-pro-BNP) N-terminal pro-brain natriuretic peptide

(NT) Normal Tyrode

(NYHA) New York Heart Association

(PBS) Phosphate buffered saline

(PDE) Phosphodiesterase

(PI3-Kinase) Phosphatidylinositol-4,5-bisphosphate 3-kinase

(PKA) Protein kinase A

(PLB) Phospholamban

(PMT) Photo multiplier tube

(PTX) Pertussis Toxin

(PV) Pressure-Volume

(RISC) RNA-induced silencing complexes

(RT-qPCR) Real time quantitative polymerase chain reaction

(RT) Reverse transcription

(RyR) Ryanodine receptor

(SC) Subcutaneously

(SERCA2a) Sarcoplasmic reticulum calcium transport ATPase

(SICM) Scanning ion conductance microscopy

(SR) Sarcoplasmic reticulum

(SRF) Serum-response factor

(SUMO1) Small ubiquitin-related modifier type 1

(TAC) Transverse aortic arch constricted

(TIMPs) Tissue inhibitors of the MMPs

(UTR) Untranslated region

(YFP) Yellow fluorescent protein

Chapter 1: Introduction

The heart, the myocardium and cardiomyocytes

The heart is a complex multi cellular organ whose function is to pump blood around the body for the duration of a human's lifetime. Located in the cardiothoracic cavity and made up of four chambers, the heart drives the cycle of returned oxygenated blood from the lungs being pumped out of the left ventricle through the aorta and distributed to the body via a network of arteries. A complimentary network of veins then returns deoxygenated blood to the heart, blood is then reoxygenated through the pulmonary circulation system and the whole process is repeated. To move blood in such a manner around a closed system of vessels the heart acts as the pump, allowing blood to fill its chambers during relaxation (diastole) before contracting (systole) to create pressure and expel the blood out thereby moving around the vessels. To be able to do this 70-80 times per minute during rest and up to ~200 during exercise the heart is an incredibly fatigue resilient muscle. The coronary circulatory system delivers an abundant supply of oxygen to the heart to ensure this continual functionality.

The heart is comprised of a range of cells types including smooth muscle cells, endothelial cells, fibroblasts, mast cells, immune system related cells and cardiomyocytes (Tirziu et al., 2010). Whilst all are important for the functioning of the heart, it is the last cell type that is responsible for the contractile capacity of the heart and make up the majority of the heart's mass. Cardiomyocytes are ~25x100 μm in size, with a single nucleus and a very high contractile strength and endurance. The muscle of the heart (myocardium) consists of many cardiomyocytes that are interlocked and supported by a framework of connective tissue to yield an intricate three dimensional network structure (Lunkenheimer et al., 2013). For contraction to occur, the cardiomyocytes require electrical stimulation.

The heart is able to generate an electrical signal known as the cardiac action potential (AP) because of the presence of specialist pacemaker cardiomyocytes. One group of these cells is located in the wall of the right atrium (sino-atrial node) and another at interface between the atria and ventricles (atrio-ventricular node). The sino-atrial node spontaneously generates an electrical signal which causes depolarisation of adjacent atrial cardiomyocytes, the AP is regenerated through the activation of voltage-gated sodium channels and spreads throughout the atrial walls to cause contraction of the atria. All cardiomyocytes are electrically coupled through gap junctions located primarily in the intercalated disks at the ends of the cells. This enables an AP that is generated in the pacemaker region to spread through the cardiomyocytes of the atria. At the surface of

cardiomyocytes are ~200 to 300 nm diameter invaginations in the cardiomyocyte cell membrane (sarcolemma) known as T-tubules. These are primarily located at the Z line and in greatest density in ventricular cardiomyocytes (Brette and Orchard, 2003). Their purpose is to conduct the AP down into the cardiomyocyte and encourage its' propagation.

As the electrical signal is spreading throughout the atria it is also conducted directly to the atrio-ventricular node by the internodal tracts. Contraction of the atria and ventricles does not occur at the same time; the two chambers of the heart are electrically isolated in order to prevent the AP spreading directly from the atria to the ventricles. From the atrio-ventricular node the signal is propagated down to the apex of heart and up through the ventricle walls by the Purkinje fibres before spreading throughout the ventricles (Pinnell et al., 2007). This results in the contraction of the cardiomyocytes that make up the ventricle muscle wall and blood being forced out of the ventricular chambers into the aorta and pulmonary artery.

Calcium is the key ion for excitation contraction coupling (ECC)

As described above it is the spreading of a cardiac AP from pacemaker cardiomyocytes across the myocardium that is the trigger for contraction of cardiomyocytes. The electrical excitation of the cardiomyocytes of the heart that result in contraction of the myocardium is known as excitation contraction coupling (ECC). When the AP comes into contact with a cardiomyocyte it results in a change in the membrane potential (E_m) across the membrane the cell. When the AP reaches a cardiomyocyte it triggers the opening of voltage sensitive ion channels; at first these are sodium channels, resulting a fast influx of sodium across the cell membrane and into the cell. As sodium is positively charged this causes the E_m to jump from the resting voltage of ~-90 mV to +25 mV, the sodium channels then inactivate and potassium is extruded from the cardiomyocyte to cause an initial repolarisation of the E_m . This is quickly followed by a the plateau phase of the AP; L-type calcium channels (LTCCs) are activated and increasing calcium entry into the cardiomyocyte (I_{Ca}) so prolonging the duration of AP and distinguishing the profile of the cardiac AP from skeletal muscle or nerve APs. Finally, the LTCCs inactivate and the entry of calcium into the cardiomyocyte is halted, combined with increased potassium conductance causes repolarisation of the E_m back to the resting voltage.

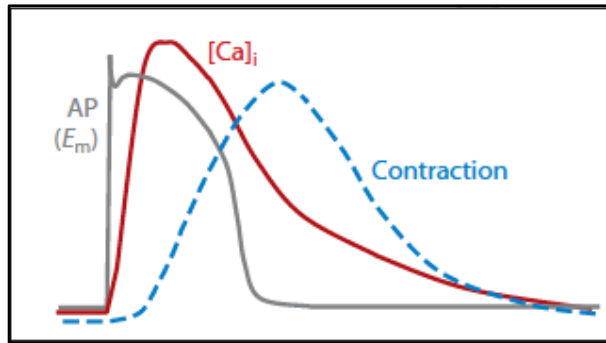


Figure 1A: A graph showing the time course of a cardiac AP, a calcium transient ($[Ca]_i$) and a contraction (Bers, 2014).

When the E_m of a cardiomyocyte depolarises this stimulates the entry of calcium into the cardiomyocyte via LTCCs. This acts as the stimulus for calcium mobilisation from the sarcoplasmic reticulum (SR) and the increased cytoplasmic calcium triggers a contraction by binding to cardiomyocyte myofilaments.

Calcium is the key ion for contractility and the regulation of its movement (homeostasis) throughout contraction and relaxation is tightly regulated. The calcium that crosses the sarcolemma through the LTCCs acts to change the conformation of the intracellular ryanodine receptor (RyR) from closed to open. When the RyR shifts to the open conformation this allows calcium to be mobilised from the sarcoplasmic reticulum (SR). With a disparity in calcium concentration in the SR compared to cardiomyocyte cytoplasm, calcium moves with the gradient and the cytoplasmic calcium concentration increases significantly (Bers, 2001). This is known as calcium induced calcium release (CICR). LTCCs and RyR form a dyad at the T-tubules and the area where the two components of CICR are localised is less than 12 nm in size (Franzini-Armstrong et al., 1999). When in such close spatial proximity it enables the calcium ions that cross the cell membrane through the LTCC to rapidly activate RyR; in effect it is a conduit between the intracellular RyR and the extracellular environment.

The T-tubule provides the structure for the tight electrophysiological relationship between the LTCCs and RyR. Immunolabelling reveals that the distribution of LTCCs across the entire sarcolemma is stacked in favour of the T-tubules (Scriven et al., 2000). This means that although the T-tubules possess only a third of the cardiomyocyte membrane capacitance, the majority of calcium ions that enter the cytoplasm pass through the sarcolemmal section of the T-tubules. This focused point of calcium entry is critical for ensuring there is a sufficient calcium concentration to stimulate the

intracellular mobilisation of calcium from the SR. Cardiomyocytes contain myofilaments, the primary constituents being actin and myosin and they are responsible for cardiomyocyte contraction and relaxation through a process known as the cross bridge cycle (de Tombe, 2003). The intracellular calcium mobilised from the SR binds to the myofilament protein troponin C. This releases the inhibitory influence of troponin I on the interaction between actin and myosin which enables the contractile machinery to be switched on. The net result is a shortening of the cardiomyocyte and on a global scale the contraction of the myocardium. It is the arrangement of actin and myosin myofilaments that gives cardiomyocytes a striated appearance at their surface.

Cardiomyocyte relaxation: calcium removal from the cytoplasm

For a cardiomyocyte to relax and the heart to enter diastole, first the cytoplasmic concentration of calcium must be reduced so that calcium dissociates from troponin C and the interaction between actin and myosin is reversed. The same amount of calcium that enters the cardiomyocytes to initiate mobilisation of calcium from the SR must also leave the cardiomyocyte. The LTCC supplies around 25-30% of the total calcium required to trigger a contraction (Bers et al., 1996) and this must be removed from the cardiomyocyte to maintain homeostasis. Removal of calcium from the cytoplasm occurs through multiple routes, the primary mechanism is through the cardiac specific subtype of sarcoplasmic reticulum calcium transport ATPase (SERCA2a). Operating in an adenosine triphosphate (ATP) dependent mechanism, with a high affinity but limited capacity for calcium, SERCA2a actively pumps calcium from the cytoplasm back into the SR loading it ready for the next contraction. The SR calcium loading performance of SERCA2a is managed by a number of factors. The key regulator of SERCA2a is phospholamban (PLB), by reducing the affinity of SERCA2a for calcium at its two calcium binding sites, the rate by which SERCA2a can load the SR with calcium is impaired. PLB consists of three domains; two cytosolic domains (IA and IB) and a transmembrane domain (II) (Simmerman et al., 1986). It is domain IA that is responsible for the physical association of SERCA2a and PLB whilst domain II is the inhibitory domain that influences SERCA2a's affinity for calcium (Kimura et al., 1996).

The crystal structure of the PLB-SERCA2a complex at 2.8-Å revealed that when PLB is associated with SERCA2a, the calcium ATPase adopts a conformation that is incompatible with calcium ions being able to bind to either of SERCA2a's binding sites (Akin et al., 2013). Therefore through interacting with SERCA2a, PLB is able to severely disrupt the two calcium binding sites of SERCA2a that are located between the transmembrane helices M4, M5, M6, and M8 near the cytoplasmic membrane

interface. PLB mediated inhibition of SERCA2a is dependent on the phosphorylation state of PLB. When dephosphorylated, PLB inhibition of SERCA2a comes into effect and this is reversed when PLB is phosphorylated. PLB has three known phosphorylation sites that are targeted by different kinases; Serine 10 (protein kinase C), Serine 16 (cyclic adenosine monophosphate (cAMP) dependent protein kinase) and Threonine 17 (calcium/calmodulin dependent protein kinase). Phosphorylation of each residue is associated with an increase in the rate of SR calcium loading (Kranias, 1985) and (Movsesian et al., 1984). PLB is dephosphorylated by cardiac SR-associated type 1 protein phosphatase, which in turn is regulated by two endogenous proteins, inhibitor-1 and inhibitor-2 in a cAMP-dependent phosphorylation manner (Carr et al., 2002).

Another important calcium handling protein is the cardiac sodium calcium exchanger (NCX) (Elton et al., 2011). Comprised of nine transmembrane segments and expressed at the sarcolemma, NCX exchanges one calcium ion for three sodium ions by secondary active transport (Philipson and Nicoll, 2000). Both SERCA2a and NCX remove calcium from the cytoplasm, but SERCA2a loads the intracellular store with calcium and does not actually remove any calcium from the cell. NCX transports calcium across the sarcolemma into the extracellular space, therefore it is NCX which drives the balance of calcium homeostasis across the sarcolemma. The transfer of ions is electrogenic and carries one net charge producing an ionic current (I_{NCX}). The direction of ionic transport is driven by both transmembrane ionic (sodium and calcium) and voltage gradients. The direction of NCS driven ionic transport is reversible as NCX can operate in two modes: forward (calcium-efflux) or reverse (calcium-influx).

During the contraction cycle NCX acts in both modes; it contributes to depolarisation during the AP upstroke by acting in reverse mode and permitting calcium entry. Initially when the E_m is more positive than local NCX membrane potential (E_{NCX}), calcium influx occurs together with an outward I_{NCX} . This is only transient as calcium is rapidly released from the SR increasing the local calcium concentration, this drives the E_{NCX} to a more positive voltage than the E_m , prompting NCX to switch to forward mode, resulting in calcium efflux and an inward I_{NCX} (Weber et al., 2003). NCX has a lower calcium affinity than SERCA2a, but suggested to have a greater transport capacity and extrudes the equivalent calcium to that which entered by the LTCCs, thereby maintaining the electrochemical balance across the membrane (Pogwizd et al., 2001). The time point at which NCX changes its function from reverse to forward mode and the net direction of I_{NCX} through the channel are key determinants on AP duration. Predominantly NCX operates in the forward mode during the contractile cycle, with only a very short period of calcium entry. If the calcium released from the SR

does not spread to a particular intracellular region of the cardiomyocyte then the E_{NCX} will not increase above cardiomyocyte E_m which would mean that NCX could maintain calcium entry throughout the contraction cycle.

The remaining mechanisms by which calcium can be removed from the cytoplasm are through the sarcolemmal calcium ATPase and mitochondrial calcium uniporter, together they are referred to as the 'the slow systems'. The contribution to the removal of calcium from the cytoplasm by the slower systems is small, only ~1% in a range of species. Therefore its influence on calcium homeostasis is not measured in experimental protocols. The same consistency is not true for SERCA2a and NCX across species, in the ventricular cardiomyocytes of human, ferret, dog, cat and guinea-pig the split is 70:28%, whereas in rats and mice the split diverges further to 92:7% (Bers, 2001). This represents a complication when extrapolating an observed behaviour or effect in rat or mouse to human cardiomyocytes. The experiments in this study were conducted almost exclusively in rat cardiomyocytes, adult and neonatal, therefore the data must be viewed with this caveat.

β -adrenoceptor influence on cardiomyocyte contractility

In order to alter cardiac performance, cardiomyocyte contractility must be regulated. This can be the heart rate (chronotropy), the force of contraction (inotropy) or the rate of relaxation (lusitropy). There are a number of mechanisms that can influence cardiomyocyte calcium homeostasis which results in a change to cardiomyocyte contractility. The mechanism that was primarily investigated experimentally in this study was the β -adrenoceptors. Part of the G protein coupled seven transmembrane adrenoceptor family that includes both α and β forms, there are three subtypes of the β -adrenoceptor; β_1 , β_2 and β_3 (Bylund et al., 1994). The distribution of β -adrenoceptors in the human heart is heavily weighted in favour of the β_1 over the β_2 subtype, with the β_3 showing minimal expression; in the atria the β_1 : β_2 ratio is 70:30 whilst in the ventricles it is 80:20 (Brodde and Michel, 1999). This is important because it means that the effect of β -adrenoceptor activation in the heart is driven principally through the β_1 subtype. Activation of β -adrenoceptors in the heart is triggered by the catecholamines adrenaline and noradrenaline with equal potency at the β_1 subtype, but at the β_2 subtype the potency of adrenaline is greater than noradrenaline.

β -adrenoceptor signalling through Protein Kinase A (PKA)

When activated by an agonist both β_1 and β_2 subtypes couple to the heterotrimeric guanine nucleotide-binding protein (G protein) $G_{\alpha s}$, this in turn activates adenylyl cyclase (AC). AC5 and AC6 isoforms are the cardiac isoforms and these catalyse the production of cAMP (Brodde et al., 2006). The increased levels of cytoplasmic cAMP stimulates the activation of PKA and this initiates a cascade of effects that influence calcium homeostasis and cardiomyocyte contractility. The targets of PKA are shown in figure 1B below:

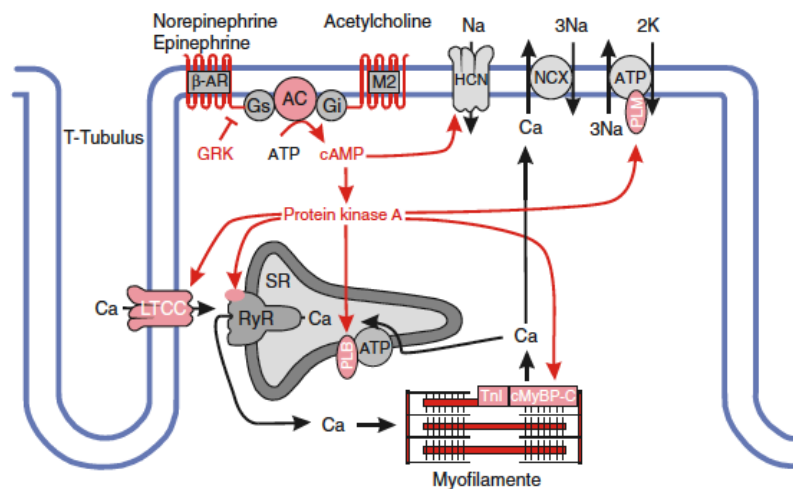


Figure 1B: The range of signalling pathways associated with β -adrenoceptor (β -AR) agonist activation and the resulting PKA activation (El-Armouche and Eschenhagen, 2009).

Phosphorylation targets of PKA: LTCCs, RyR, PLB, NCX, cardiac myosin binding protein C (cMyBP-C), and phospholamban (PLM).

PKA operates by phosphorylating its targets in order to alter their function and in cardiomyocytes PKA has a range of targets that affect cardiomyocyte contractility. The LTCCs expressed at the sarcolemma in the T-tubule are phosphorylated by PKA. This shifts the membrane potential at which the channel activates to a more negative voltage and changes the gating mode of the channel. Therefore, more LTCCs are activated by the action potential, resulting in a larger influx of calcium into the cytoplasm. The net result is that stimulation of CICR occurs more readily (Bers, 2008). PKA also phosphorylates PLB at the Serine 16 residue of the protein, this causes reduced PLB inhibition of SERCA2a and improved loading of the SR with calcium during diastole. The combined effect on LTCCs and PLB is a greater calcium release from the SR during systole and increased cardiomyocyte contractility (inotropy). $G_{\alpha s}$ is able to activate LTCCs directly but it is unknown if this occurs in human cardiomyocytes (Schneider et al., 1997). PKA is able to influence cardiomyocyte lusitropy by

phosphorylating troponin I which increases its inhibitory influence on cardiomyocyte contractility. Troponin I phosphorylation accelerates the dissociation of troponin C from calcium and results in relaxation of cardiomyocyte myofilaments. The extent to which the change in troponin I activity affects cardiomyocyte lusitropy is much less than the effect of increased SERCA2a activity after PLB inhibition is depressed by PKA phosphorylation.

Another target of PKA is RyR, the ion channel that gates the SR and allows calcium to flow out into the cytoplasm. The phosphorylation of Serine residues of RyR has been suggested to be important as it would appear to govern the open/ closed conformation state of the channel. The association of FK binding protein 12.6 (FKBP) has been suggested to impair RyR opening, with the phosphorylation of RYR by PKA triggering dissociation of FKBP12.6 and therefore increase the amount of calcium mobilised from the SR (Marx et al., 2000). Phosphorylation by the calcium/ calmodulin dependent protein kinase has also been found to increase the open probability of RyR (Ai et al., 2005). Dephosphorylation of the RyR Serine residues is achieved through the protein phosphatases, with type 1 regulating Serine 2808 and type 2a regulating Serine 2030 (Belevych et al., 2011). The importance of RyR phosphorylation by PKA in SR calcium mobilisation is disputed (Li, 2002) and the finer details of this uncertainty in the literature are beyond the scope of this study.

PKA also targets the protein phospholemman which interacts with the sarcolemmal sodium/ potassium-ATPase. When phospholemman is associated with the sarcolemmal sodium/ potassium-ATPase its affinity for intracellular sodium is decreased and so less sodium is extruded from the cytoplasm (Despa et al., 2005). β -adrenoceptor induced PKA activation results in a greater level of cytoplasmic calcium through increased I_{Ca} and SR calcium mobilisation. To ensure the cytoplasmic concentration of calcium is balanced, NCX exchanges greater numbers of calcium ions which in turn means an increased cytoplasmic sodium concentration. To compensate PKA phosphorylates phospholemman, which dissociates from the sarcolemmal sodium/ potassium-ATPase, whose affinity for sodium increases and more sodium is removed from the cytoplasm. Therefore this mechanism ensures that whilst cardiomyocyte contractility increases due to β -adrenoceptor activation an ionic balance across the sarcolemma is still maintained.

In order to ensure that when PKA is activated its targets are successfully phosphorylated the scaffolding protein A-kinase anchoring proteins (AKAP) holds PKA and its targets in close spatial proximity. In fact, studies have found that PKA must be co-localised with a target to ensure control over phosphorylation of the substrate in question (Wong and Scott, 2004). When this dynamic was disrupted by a purpose designed peptide that prevented the interaction between AKAP and PKA, it resulted in disrupted cardiomyocyte contractility kinetics due to the blockade of the phosphorylation of PKA targets (Fink et al., 2001).

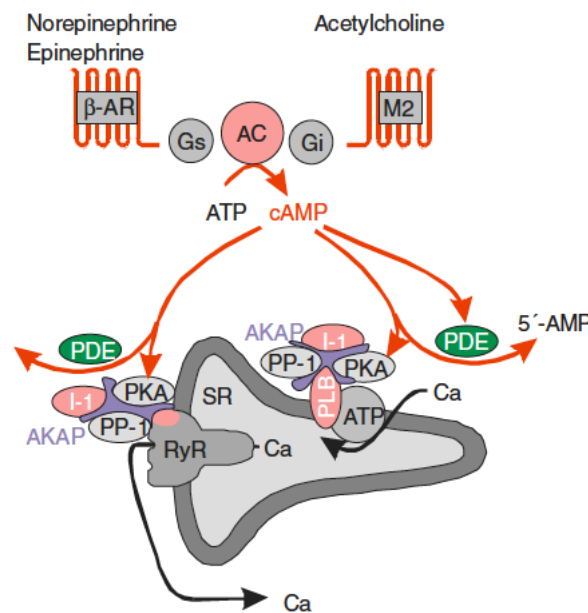


Figure 1C: Components of the PKA signalling complex and its targets that located at the sarcoplasmic reticulum (El-Armouche and Eschenhagen, 2009).

AC drives the production of cAMP, this simultaneously activates PKA whilst being broken down by phosphodiesterase (PDE). PKA targets such as PLB and the RyR are held in complex by the scaffolding protein AKAP. This confers both localisation and specificity of the cAMP driven PKA signalling β -adrenoceptor activation.

β_2 -adrenoceptor $G_{\alpha i}$ coupling

PKA activation and the phosphorylation of its targets is driven through β_1 and β_2 -adrenoceptor activation and coupling to $G_{\alpha s}$. In the atria this results in an increase in both inotropy and chronotropy whereas in ventricles the force of contraction is affected, with only the β_1 -adrenoceptor able to elicit a maximal increase in inotropy (El-Armouche and Eschenhagen, 2009). A further difference between the two β -adrenoceptors is the coupling to G proteins; the β_1 -adrenoceptor

couples exclusively to the $G_{\alpha s}$, the β_2 -adrenoceptor exhibits functional selectivity and is able to signal through the $G_{\alpha i}$ and non G-protein related pathways, as well as the $G_{\alpha s}$. When activated, $G_{\alpha i}$ signalling results in a depression of AC activity and reduced cAMP activity, in other words the opposite of $G_{\alpha s}$ signalling. In isolated cardiomyocytes from transgenic mice overexpressing the β_2 -adrenoceptor, higher concentrations of the non-selective β -adrenoceptor agonist isoprenaline resulted in a Pertussis Toxin (PTX) sensitive decrease in cardiomyocyte inotropy indicating the involvement of $G_{\alpha i}$. This effect was unchanged when the β_1 -adrenoceptor was blocked by pharmacological antagonism. The protein expression and activity of $G_{\alpha i}$ has been found to be increased in the cardiomyocytes from these transgenic mice (Gong et al., 2000). Therefore at the higher agonist concentrations the β_2 -adrenoceptor switches from $G_{\alpha s}$ to $G_{\alpha i}$, this reduces cAMP production and depresses cardiomyocyte inotropy (Heubach et al., 2003).

Spatial distribution of cardiomyocyte β -adrenoceptors

A further difference between the two cardiac β -adrenoceptors is the spatial distribution at the sarcolemma. When the cAMP signals of the β -adrenoceptors were measured separately by a combination of nanoscale live-cell scanning ion conductance and Förster resonance energy transfer (FRET) microscopy techniques. It was found that the β_1 -adrenoceptor cAMP signal is distributed across the entire cell cardiomyocyte surface, where the β_2 -adrenoceptor signal is focused at the T-tubules only (Nikolaev et al., 2010). As well as β -adrenoceptor distribution, the diffusion of cAMP signal attributed to the β -adrenoceptor subtypes is not uniform across the cardiomyocyte. In a separate study by Nikolaev et al the cAMP signal from β_1 -adrenoceptor activation in a localised area of freshly isolated cardiomyocytes by selective agonist exposure from a micropipette was recorded. The cAMP signal was detected more than 20 μm from the source and throughout large sections of the cell (Nikolaev et al., 2006). When the same protocol was repeated and the β_2 -adrenoceptor cAMP signal measured, only a localised increase in cAMP occurred that did not propagate across the cardiomyocyte and that had a cAMP signal amplitude of around half that of the β_1 -adrenoceptor signal. One factor that has been linked to localisation of the β_2 -adrenoceptor cAMP signal is caveolin-3. In one study where caveolin-3 was disrupted with expression of dominant-negative mutant version of the gene in adult rat cardiomyocytes, the β_2 -adrenoceptor cAMP signal spread from the T-tubules to larger area across the cardiomyocytes (Wright et al., 2014). This would suggest that caveolin-3 is important for retaining the cAMP signal derived from the β_2 -adrenoceptor in a local area at the T-tubule.

Ensuring that the cAMP signal resulting from β -adrenoceptor activation remains localised enables the cardiomyocyte to specify the effect of β -adrenoceptor activation. Cytoplasmic cAMP is a highly diffusible molecule that can spread across the entire cardiomyocyte extremely rapidly. Therefore without spatial control over the β -adrenoceptor cAMP signal, activation of a local area of β -adrenoceptors would result in a whole cell signal. As cAMP is so diffusible, control of its movement is tightly regulated by PDEs, a family of enzymes that breakdown cAMP and ensure compartmentalisation of cAMP in cardiomyocytes. In heart tissue expression of PDE1, PDE2, PDE3, PDE4 and PDE5 subtypes has been found, but with PDE1 and PDE5 expression in cardiomyocytes not always consistent (Maurice et al., 2003). PDEs also act as a negative feedback loop of cAMP level in cytoplasm.

The cAMP signal of the β -adrenoceptor subtypes are regulated by different subtypes of PDEs and the balance across PDE subtypes varies between species. For example, in mouse cardiomyocytes 60% of cAMP degradation occurs due to PDE4 whilst in rat PDE3 and PDE4 both play important roles in cAMP breakdown (Georget et al., 2003) (Leroy et al., 2008). In human cardiomyocytes the influence of PDE3 on cAMP breakdown appears to be equal to that of PDE4 (Hambleton et al., 2005). The cAMP signal derived from β_1 -adrenoceptors in rat cardiomyocytes appears to be regulated by the PDE4 subtype alone, with the β_2 -adrenoceptor signal controlled by three subtypes in the following descending order of influence; PDE4>PDE3>PDE2. As stated the cAMP signal of the β_2 -adrenoceptor is tightly controlled and more localised compared to the β_1 -adrenoceptor and it is this range of PDEs that has been suggested to be important for maintaining this (Nikolaev et al., 2006).

The scaffolding protein AKAP also contributes to the compartmentalisation of the β -adrenoceptor cAMP signal. By structuring PKA and its targets together in close proximity it ensures that the effect of PKA activation by cAMP is focused on the target that is part of the AKAP scaffolded complex. AKAP has targeting domains which enables AKAP to dictate the intracellular position that the PKA/target complex is located through protein/protein or protein/lipid interactions. (Dodge-Kafka et al., 2006). Together with bringing PKA into close proximity with other additional proteins such as PDEs in addition to its targets, AKAP plays an important role compartmentalising the cAMP signal of β -adrenoceptors in cardiomyocytes.

Heart failure

Heart failure can be described as an inability of the heart to pump blood efficiently around the body to meet the demands of the tissue. This is due to structural remodelling of the heart; the left ventricle muscular wall becomes much thinner which reduces the contractile force the myocardium can generate. Total cardiac mass increases and enlarges beyond the tolerated elastic limit of the cardiomyocyte contraction mechanism which can result in deformation of the myocardium. Ventricular geometry also changes from an ellipsis to a sphere. The myocardium becomes stiffer as fibrosis increases in the interstitium, this is due to greater activity of the endopeptidase enzymes matrix metalloproteinases (MMPs) which cleave the peptide bonds of collagen and reduced activity of the complimentary tissue inhibitors of the MMPs (TIMPs) (Martos et al., 2007). One consequence of these abnormalities is that the myocardium develops greater level of oxygen consumption, energy expenditure and wall stress. This shifts the balance towards ventricular dysfunction and reduced contractile efficiency (Cleland et al., 2001). The pathological components of the disease are not just confined to the heart, increased activation of the sympathetic nervous system due to dysfunctional baroreceptor stimulation results in increased noradrenaline release. With an increased sympathetic outflow the renin–angiotensin–aldosterone axis activity is also elevated; aldosterone promotes water retention which manifests itself as oedema in heart failure patients (Poole-Wilson et al., 2001).

Data obtained from cross sectional studies suggests the prevalence of symptomatic systolic cardiac failure is approximately 1% of the Western European population with a further 2% having asymptomatic systolic dysfunction or symptomatic heart failure without systolic dysfunction (Cleland et al. 2001). The predominant aetiology of heart failure is coronary artery disease (CAD) either in conjunction with hypertension or alone (McMurray and Stewart, 2000) but there are other potential causes for heart failure as well as CAD and hypertension such as diabetes and atrial fibrillation. In addition another cause of heart failure are cardiomyopathies which can be divided into primary and secondary cardiomyopathies. The former can be classified as dilated, hypertrophic and restrictive cardiomyopathies and mutations to genes encoding proteins involved in the cardiomyocyte contractile machinery have been attributed to development of the cardiomyopathy. Secondary cardiomyopathies are associated with an underlying condition such as infections and exposure to cardiotoxins.

The most common methodology used for diagnosis of heart failure is based on left ventricular function, specifically the volume of blood pumped out of the heart per beat; left ventricular ejection fraction (EF). When this metric drops below 40% it is considered dysfunctional, but this is not always associated with symptoms and many patients can go undiagnosed. In a study by Davies et al this was estimated to be ~3% from a normal sample population of nearly 4000 people over the age of 45 (Davies et al., 2001). Equally there are heart failure patients with a preserved left ventricular EF but have the same mortality risk as patients with reduced left ventricular EF. The New York Heart Association (NYHA) classification is used to stratify patients based on their functional capacity; the ability to carry out physical activity with or without the presence of fatigue, palpitation, dyspnea or anginal pain. The treatment regime is dependent on the stage of the patients' pathological development status and initially lipid lowering, hypotensive drugs and aspirin would be prescribed. When the condition becomes more severe, diuretics, angiotensin converting-enzyme (ACE) inhibitors and β -blockers would be used to combat the disease. All current heart failure treatments such as those listed above only have the capacity to slow the progression of disease development and not reverse the pathological remodelling that the heart undergoes. Ultimately, mortality occurs due to sudden death caused by an arrhythmic event or through contractile dysfunction. There is a substantial requirement for novel therapies that specifically target the myocardium and offset the course of the progression into failure, ideally correcting the pathophysiology.

Cardiomyocyte specific aspects of heart failure

As part of the pathophysiology of heart failure, the number of cardiomyocytes declines due to cellular apoptosis and necrosis which is often caused by a cardiac insult such as myocardial infarction (MI). Those remaining exhibit an increase in size (hypertrophy) due to exposure to increased mechanical stress and signalling molecules such as angiotensin-II and noradrenaline. Initially the hypertrophy may be able to compensate for the reduced contractile capacity of the myocardium and the heart maintains a normal output. Over time this deteriorates into decompensated hypertrophy whereby output does not meet demand as cardiomyocyte contractility progressively declines. Cardiomyocytes also become disorganised in their 3D spatial structure and by becoming misaligned the contractile efficiency of the myocardium is further reduced. The functioning of various intracellular components of cardiomyocytes that are involved in calcium homeostasis is disrupted as part of the pathological spectrum of changes that occur in heart failure. The current level of understanding of the nature of these changes varies, those that are relevant to this study are discussed in the following sections.

Impaired calcium homeostasis in systole

Earlier in this chapter the role that a number of intracellular cardiomyocyte components (e.g. SERCA2a and NCX) play in healthy cardiomyocyte contractility was described. Many have been found to malfunction in heart failure and contribute towards disrupted ECC that occurs as part of the disease pathophysiology. With calcium triggering its own release the movement of intracellular calcium is tightly regulated in healthy cardiomyocyte to maintain a stable contractile frequency and force. This process has inherent flexibility, if there is a contractile shortfall, more calcium is mobilised, inducing greater contractile force (Bers and Despa, 2006). In heart failure the ability of a cardiomyocyte to control the movement of calcium is impaired, this manifests as a reduced calcium transient and depressed contractility (Bers et al., 2003). When an AP reaches a cardiomyocyte it initiates activation of LTCCs, in heart failure although no overall downregulation in LTCC has been found (Bodi et al., 2005) there is a lower membrane density of LTCCs at the T-tubule (Bryant et al., 2015). Despite this the whole cell I_{Ca} is unchanged as the LTCCs have a greater open probability and this results in an enhanced single-channel current activity (Schroder et al., 1998). What is affected is the efficacy of LTCCs to elicit the mobilisation of calcium from the SR because of the change in membrane density (Gomez et al., 2001).

Across a range of heart failure aetiologies there is significant loss and disruption to the T-tubule network (Lyon et al., 2009); this represents an important aspect of the pathological phenotype. The change in the T-tubule network occurs at the start of the transition from hypertrophy to heart failure, suggesting that it is one of the early steps in the pathological development of heart failure (Wei et al., 2010). This disperses the closely structured components of CICR (LTCC and RyR) thereby reducing the amplitude and synchrony of calcium mobilisation from the SR which can contribute towards potentially fatal arrhythmic events. In conjunction with impaired coupling to LTCCs, the RyR also displays functional changes in heart failure. In simple terms the RyR displays an increased open probability, this allows calcium to leak out of the SR and into the cytoplasm.

Why RyR displays this behaviour in heart failure is disputed in the literature, with a study by Marx et al suggesting that hyperphosphorylation of the Serine 2808 residue of RyR by PKA resulting in a sustained dissociation of FKBP12.6 and increased open probability of RyR (Marx et al., 2000). When other groups have repeated this experiment, neither PKA phosphorylation of Serine 2808 or FKBP affects the gating of RyR (Xiao et al., 2004) (Houser, 2014). Furthermore, others have suggested that it is the decreases in protein phosphatase type 1 and type 2a expression that results in the

hyperphosphorylation of RyR (Belevych et al., 2011). What is not disputed is that RyR leaks calcium into the cytoplasm in heart failure cardiomyocytes. With calcium emptying from the SR during diastole there is a reduced amount of calcium to be released during systole and this results in the impairment of cardiomyocyte contractility observed in heart failure.

Dysfunctional calcium removal from the cytoplasm in diastole

The main component of restoring SR calcium concentration in diastole is SERCA2a and this too has been found to be defective in heart failure. Both messenger RNA (mRNA) and protein expression have been found to be reduced in myocardium of heart failure patients with dilated and ischaemic cardiomyopathies (Hasenfuss et al., 1994), which would suggest that SERCA2a is integral to development of the pathological end state regardless of the aetiological starting point. In addition, in those remaining SERCA2a proteins expressed at the SR the function is also depressed in heart failure, this has been shown in models of chronic heart failure and human myocardium samples (Lyon et al., 2011) (Flesch et al., 1996). This further increases the level of cytoplasmic diastolic calcium and decreases systolic SR calcium, adding to the disruption of cardiomyocyte contractility and propensity for arrhythmias.

SERCA2a represents an attractive target for therapeutic intervention because it has the capability to influence cardiomyocyte contractility and the stability of cardiomyocyte ionic balance. Professor Harding's laboratory has been involved for some time with targeting SERCA2a through gene therapy. Increased SERCA2a expression after gene therapy has resulted in a number of beneficial effects; improved contractility, increased peak systolic calcium amplitude (Del Monte et al., 1999) and reduced spontaneous calcium leak and the rate of arrhythmic events (Lyon et al., 2011).

This work has been taken further to a clinical trial, where SERCA2a was delivered to patients with advanced heart failure; the Calcium Up-Regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) trial was the first of its kind to utilise gene therapy as a delivery method of treatment for heart failure patients (Jaski et al., 2009). After three years the study reported improved symptoms, NYHA classification and left ventricular end systolic volumes. Importantly no adverse safety effects were detected and the improved expression of SERCA2a was maintained. A second trial (CUPID2) is ongoing and incorporates patients from over 50 sites across the world rather than just the USA with the results expected in 2015/16 (Hayward et al., 2014).

In addition to SERCA, NCX has also been found to contribute towards disrupted calcium homeostasis in heart failure. NCX contributes towards normalising cytoplasmic calcium levels by effluxing one calcium ion from cytoplasmic in exchange for three sodium ions. NCX typically dispels the same amount of calcium that enters the cardiomyocyte through LTCCs and thereby maintains the electrochemical balance across the membrane. The expression of NCX has been found to be upregulated in both heart failure patients (Studer et al. 1994) and animal models (Pogwizd et al., 2001). This upregulation in expression results in greater extrusion of calcium from the cytoplasm in exchange for sodium ions. This results in a greater inward sodium current which can cause delayed and early afterdepolarisations, these can result in cardiomyocyte arrhythmias and ultimately sudden death of a patient if the arrhythmia spreads across the myocardium (Pogwizd et al., 2001). In addition, fewer calcium ions remain for SERCA2a to pump back into the SR for systole and therefore cardiomyocyte contractility is reduced. Without the presence of the intracellular calcium mobilised from the SR or the calcium that has entered across the cell membrane through other transport mechanisms, the upregulated NCX expression or function would not increase the NCX mediated calcium flux and the effects described above would not occur.

Disrupted β -adrenoceptors signalling and distribution

In healthy patients the β_1 -adrenoceptor subtype comprises 70-80% of the total cardiomyocyte β -adrenoceptor population. In heart failure the expression of the receptor changes inversely to disease severity; expression falls to ~50% in NYHA class IV heart failure patients (Engelhardt et al., 1996). Interestingly the levels of the β_2 -adrenoceptor remain unchanged in heart failure. The remaining β_1 and β_2 -adrenoceptors are desensitised in heart failure and together with the depleted expression of β_1 -adrenoceptor a smaller response is elicited after activation by circulating catecholamines. This is a well established aspect of the pathological phenotype and is found across heart failure aetiologies and in animal models (Bristow et al., 1986) (Lefkowitz et al., 2000). The increased circulating catecholamine concentration found in heart failure is believed to stimulate desensitisation and can be replicated experimentally with *in vivo* with chronic catecholamine treatment (Osadchii, 2007) (El-Armouche et al., 2007).

Desensitisation of the β_1 -adrenoceptor is primarily due to the G protein coupled receptor kinase 2 (GRK2), also known as β -adrenergic receptor kinase 1 (BARK), in an agonist dependent manner. When activated by catecholamines the α and $\beta\gamma$ subunits separate and the γ element triggers localisation of the $\beta\gamma$ subunit to the sarcolemmal membrane. This enables the GRK2 to co-localise to

the β_1 -adrenoceptor by interacting with the $\beta\gamma$ subunit via a pleckstrin homology domain (Pitcher et al., 1995). It is the catecholamine induced activation of the β_1 -adrenoceptor that causes the chain of events that result in the GRK2 phosphorylation of the receptor and its desensitisation. Once phosphorylated the β_1 -adrenoceptor is unable to further activate the $G\alpha_s$ pathway, resulting in a reduction of AC activation and cAMP production (Rockman et al., 2002). In addition, phosphorylation by GRK2 renders the β_1 -adrenoceptor a target for binding of β -arrestin proteins (Freedman et al., 1995) and results in receptor internalisation. With reduced β -adrenoceptor sensitivity to catecholamines, myocardial performance is reduced and the ability of the heart to increase contractile force is impaired. There is uncertainty around β_1 -adrenoceptor downregulation in heart failure. There are studies that suggest the increased β_1 -adrenoceptor signalling due to elevated circulating catecholamines drives the heart failure pathophysiology (Engelhardt et al., 1999). Other evidence contradicts this conclusion, with the preservation of β_1 -adrenoceptor function suggested to delay the development of heart failure (White et al., 2000). What is undisputed is that β_1 -adrenoceptor function and expression decrease in heart failure. The catecholamine stimulation of the β -adrenoceptors appears to also promote hypertrophy through the induction of pro-hypertrophic genes which would suggest the cAMP from activated β -adrenoceptors spreads to the nucleus (Taimor et al., 2001).

GRK2 also causes desensitisation of the β_2 -adrenoceptor in heart failure but through an alternative mechanism. It has been found that in rat ventricular cardiomyocytes the β_2 -adrenoceptor exhibits functional selectivity in an agonist specific manner. The β_2 -adrenoceptor is able to activate either $G\alpha_s$ or $G\alpha_i$ G proteins, and in heart failure this dynamic arrangement becomes more heavily weighted in favour of $G\alpha_i$ activation (Brodde et al., 2006). $G\alpha_i$ acts as a handbrake on the $G\alpha_s$ mediated contractile response through limiting cAMP production and has been suggested to have the capacity to counter the pro-apoptotic activity of the β_1 -adrenoceptor in heart failure (Zaugg et al., 2000) (Zhu et al., 2001). The switch in coupling of the β_2 -adrenoceptor from $G\alpha_s$ to the $G\alpha_i$ protein (together with an increased $G\alpha_i$ expression) reduces the receptor's ability to elicit an increase in intracellular cAMP when activated by an agonist (Lohse et al., 2003). The change in G protein signalling has been suggested to be due to increased phosphorylation of the β_2 -adrenoceptor by GRK2 (Zhu et al., 2012), with induced overexpression of GRK2 resulting in a PTX sensitive depression of cardiomyocyte contractility in transgenic mice.

In conjunction with altered β_2 -adrenoceptor signalling in heart failure, the spatial organisation of the β_2 -adrenoceptor cAMP signal shifts from a concentrated area in the T-tubules to a more diffuse area

across the cell crest (Nikolaev et al., 2010). This may contribute towards reducing the ability of the β_2 -adrenoceptor to influence cardiomyocyte contractility. The β_2 -adrenoceptors undergo an uncoupling from pools of PKA that are in close spatial proximity at the T-tubule and are responsible for the cAMP response in healthy cardiomyocytes. Combined together this may decrease the ability of circulating catecholamines to influence contractility through the β_2 -adrenoceptors downstream PKA pathway.

MicroRNAs

MicroRNAs were first discovered in *Caenorhabditis elegans* (*C.elegans*) in the 1990s (Lee et al., 1993), the field quickly expanded and now several thousand microRNAs have been identified that are involved with the post transcriptional regulation of 74–92% of all mRNA (Miranda et al., 2006, Iwakawa and Tomari, 2015). The role of a microRNA is to act as the targeting component of the RNA-induced silencing complexes (RISC); a microRNA will anneal to a complementary section of the 3' untranslated region (UTR) of a mRNA sequence and this can result in a reduction in gene expression (Engels and Hutvagner, 2006). Around 22 nucleotides in length, microRNAs play a key post transcriptional regulatory role in a large range of biological processes. The ability to regulate gene expression in such a wide range of tissues highlights the importance of microRNAs and their potential for therapeutic exploitation. Conversely such a wide ranging influence also means that malfunction of their activity has been suggested to be a causative factor in a variety of disease states such as diabetes, cancer and heart failure.

Biogenesis of microRNAs begins when they are transcribed by RNA polymerase II as part of long primary transcripts (pri miRNAs) and the maturation of microRNAs requires a series of processing steps to reach the mature microRNA. Typically multiple microRNAs are produced from a single transcription unit rather than microRNAs being transcribed individually (Carthew and Sontheimer, 2009). The subsequent processing steps involved in the maturation of microRNAs are summarised in figure 1D.

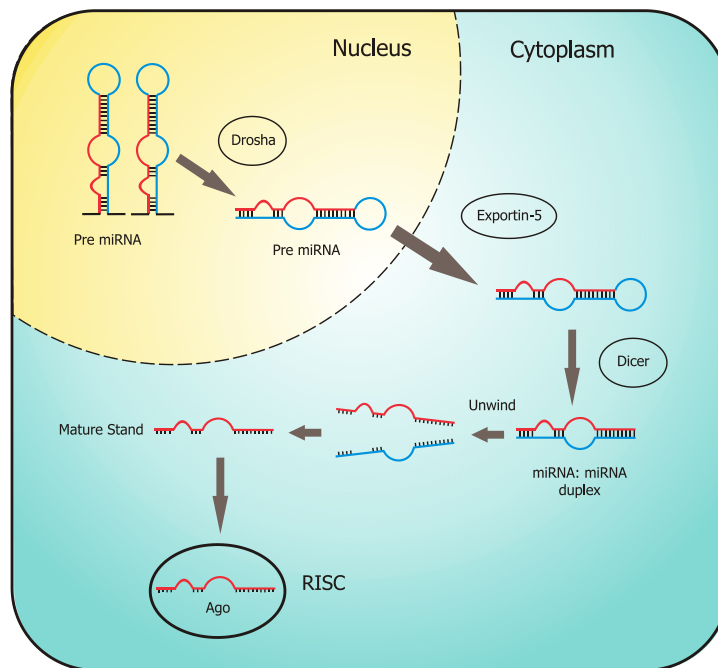


Figure 1D: The processing steps of microRNA biogenesis that occur in the nucleus and cytoplasm and result in the inclusion of a mature microRNA sequence as part of RISC.

In the first nuclear step (figure 1D), the ribonuclease enzyme Drosha processes pri miRNA into an ~70-nucleotide precursor hairpin. This is exported to the cytoplasm by Exportin-5 where it is cleaved by Dicer to yield a ~20 nucleotide miRNA/miRNA duplex. The complex is unwound, separating the mature/ guide strand (red) and passenger strand (blue). The mature strand is preferentially incorporated into RISC with other elements such as argonaute-2 (Ago) and the other strand (passenger or microRNA*) is released and degraded. Generally, the retained strand is the one that has a lower stability in the base pairings at the 5' end in the miRNA/miRNA duplex (Lee et al., 2003).

MicroRNA mechanism of action

Nucleotides 2-8 of the 5' end of mature microRNAs have the greatest influence on accuracy of annealing to a mRNA sequence. The microRNA seed region (nucleotides 2-7) forms a basic match and the strength of the microRNA-mRNA match can be improved by an A nucleotide after the 7th nucleotide. This is further improved by a 2-8 nucleotide match and again by a 2-8 nucleotide match followed by an A nucleotide (Filipowicz et al., 2008) but predicted microRNA-mRNA sequence annealments do not always result in repression of gene expression. The context of the annealing also is a determining factor in how effective the silencing of mRNA expression will be. Contextual elements include; the presence of a AU-rich nucleotide composition near the microRNA-mRNA

annealment site, whether co-expressed microRNAs are also able to anneal nearby as microRNAs can act cooperatively, the proximity that microRNA nucleotides 13–16 can also anneal to the mRNA sequence, the site of microRNA-mRNA annealment being a minimum of 15 nucleotides away from the stop codon and away from the centre of long UTRs (Grimson et al., 2007).

Most functional microRNA target sites are thought to be located in the 3' UTR of mRNA transcripts. Various web based tools (e.g. www.targetscan.org) are available that calculate potential microRNA-mRNA matches and the 'strength' of these matches using the context criteria such as the ones previously described. The availability of these tools has benefited the field in researching and understanding what the impact of altered microRNA expression could be in a pathological state as the potential mRNA targets of a microRNA can easily be ascertained. These tools are only pertinent if a microRNA is directly matched to a mRNA sequence of interest, or a known regulator of the mRNA of interest. The mRNA sequence target may be part of a regulatory cascade that is undiscovered or poorly understood and so it can be difficult to know which targets to study from the list of potentially hundreds of microRNA-mRNA sequence matches. Simply searching the literature for every sequence match may therefore not yield the responsible candidate gene to explain an observed result from a microRNA transfection experiment. Therefore it is important to combine a hypothesis driven approach with the microRNA-mRNA sequence matching tools.

The accuracy of microRNA-mRNA complementation determines the type of post transcriptional regulation as illustrated in figure 1D (Bartel, 2004); if sufficient, mRNA degradation occurs (figure 1D-A), with the cleavage performed by the catalytic PIWI domain of Argonaute-2, another RISC constituent (Liu et al., 2004). Partial interaction results in translational repression (figure 1D-B); examples have shown that repression has no effect on the translation of mRNA sequences that do not have a 5' cap. This implies that this form of repression affects translation initiation and it has been suggested that Argonaute-2 blocks translation by competing with eIF4 for the m7G (5') cap (Pillai et al., 2005). Repression may also occur post-initiation; mRNA sequences have been found in complex with polysomes during repression, which dissociate upon exposure to the protein synthesis inhibitor puromycin (Kong et al., 2008). This would imply that ribosomes are actively translating the mRNA sequence during the microRNA targeted repression, which would be after the initiation of translation has occurred. Evidence suggests that in these circumstances microRNA-mRNA interactions may result in the deceleration of translational elongation (Maroney et al., 2006).

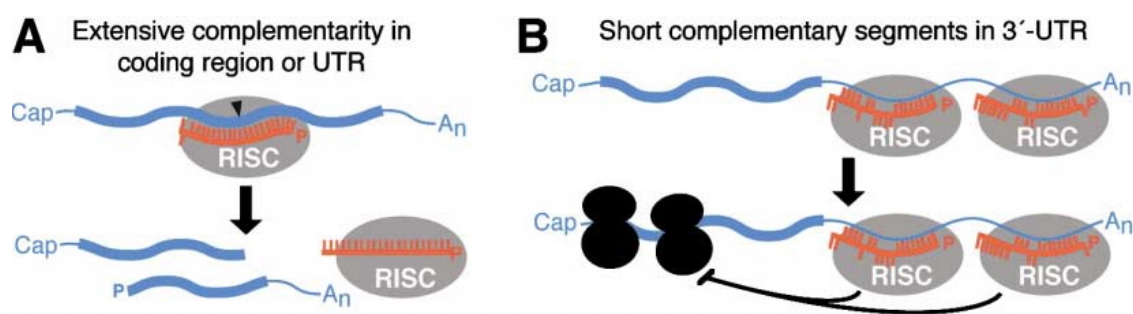


Figure 1E: The accuracy of microRNA binding to a complimentary section of the 3' UTR region of a mRNA sequence determines the form of post transcriptional inhibition that occurs (Bartel, 2004).

A) When a high level of annealment accuracy occurs the RISC complex degrades the mRNA sequence

B) Partial interaction between microRNA and mRNA results in translational repression by where the RISC competes for the 5' cap to prevent the initiation of translation

The functional relationship with between a microRNA and target mRNA sequence is not always a one-to-one ratio, multiple microRNAs can regulate an individual mRNA and a single microRNA can regulate multiple mRNA. The presence of multiple sites allow microRNAs to work synergistically, when working co-operatively it increases the potential for regulation of target gene expression. This highlights why microRNAs have extensive therapeutic potential, a single microRNA may concurrently regulate several important downstream disease related genes or pathways and thereby influencing multiple therapeutic targets. This also illustrates the significant complexity of microRNAs and how their full characterisation is required before any clinical application.

MicroRNA-1 expression in heart failure

Each cell type possesses a signature microRNA expression pattern that permits tight regulation of genes within the cell. The mapping of the healthy adult cardiomyocyte microRNA expression profile suggested microRNAs play an important role in adult cardiac physiology, with microRNA-1, microRNA-16, microRNA-27b, microRNA-30d, microRNA-126, microRNA-133, microRNA-143, and the let-7 family all with elevated expression relative to other microRNAs detected in adult cardiomyocytes (Thum et al., 2008). A genetic comparison revealed a striking similarity between the adult heart failure and fetal cardiac transcriptomes, with parallel expression patterns of 86.6% of upregulated and 83.7% of downregulated microRNAs (Thum et al., 2007). The study only considered mRNA changes, but because microRNAs can repress expression at the translational phase, any changes at this junction would therefore be missed which makes this not an absolute comparison. It has been hypothesised that the shift to a fetal profile is the stimulus for cardiomyocytes to develop a hypertrophic phenotype. Hypertrophy allows the heart to compensate for reduced contractile

capacity after an event like a MI, enlarging in size to maintain functional output. Initially compensation is sustained and controlled, but over time it develops into heart failure. With microRNAs positioned at a key juncture in the control over this process, changes in their expression may stimulate the progression into heart failure.

Because microRNA-1 has been found to be highly expressed in cardiomyocytes, there has been a focus in the literature to understand whether the expression changes in different cardiac diseases and if this was a causative factor in patients developing those diseases. Particular relevance for this study is the change of microRNA-1 expression in heart failure cardiomyocytes. *In vivo* models of heart failure suggest that microRNA-1 expression is downregulated; in the heart failure model used by Professor Harding's group (surgical proximal coronary ligation of adult rats in order to induce chronic MI followed by 16 weeks for heart failure to develop) microRNA-1 expression in failing left ventricular myocardium was decreased compared to control (Kumarswamy et al., 2012).

Other studies have also shown decreased left ventricular microRNA-1 expression in heart failure models (Care et al., 2007); transgenic mice overexpressing a constitutively active mutant of the serine-threonine kinase Akt, transverse aortic arch constricted (TAC) and exercised rats all produced the same change in microRNA-1 expression. In the myosin heavy chain α calcineurin (MHC α -CN) murine heart failure model, the animals express activated forms of calcineurin and develop hypertrophy one month after birth, then progress to severe systolic dysfunction after two months. Cardiomyocytes taken from MHC α -CN mice after two months show significant downregulation in microRNA-1 expression (Ikeda et al., 2009). Results from human samples are less consistent than their *in vivo* counterparts but more studies find microRNA-1 expression is reduced in patients than do not; in the explanted hearts of transplant recipients treated for ischaemic cardiomyopathy (ICM) and dilated cardiomyopathy (DCM) with an ejection fraction of <45%, microRNA-1 expression was downregulated compared to healthy controls (Ikeda et al., 2007). Conversely, another study found microRNA-1 expression to be increased by 2.87 fold in end stage heart failure DCM patients undergoing heart transplant (Thum et al., 2007).

This disparity in human heart failure patients compared to *in vivo* studies could be explained by the variation in patient demographic; cardiac disease (e.g. DCM, ICM, aortic stenosis, CAD), the stage of heart failure progression, previous and current pharmacological treatments, age and heterogeneity of myocardium samples. Sample population size is another important factor with many studies conducted confined to 25 samples at most. To achieve a more definite characterisation of

microRNA-1 expression in heart failure patients a larger sample population is required that takes into account the heterogeneity of heart failure patients. MicroRNA expression has also been found to change over time e.g. microRNA-21 (Tatsuguchi et al., 2007). Whether this occurs with microRNA-1 and the progression of heart failure is currently unknown. If experimental results could show this is the case for microRNA-1 it could offer an explanation for the variability in microRNA-1 expression in heart failure described in the literature; with the evolution in microRNA-1 expression over time specific to an individual patient's condition.

MicroRNA-1 and excitation contraction coupling

After establishing that microRNA-1 expression is altered in heart failure, the next step is to understand whether the mRNA sequence targets of microRNA-1 are affected by the change to microRNA-1 expression and what the impact this may have on cardiomyocyte function. Bioinformatic sequence analysis linked microRNA-1 to the mRNA sequence of NCX and as discussed earlier NCX expression is increased in heart failure. Greater NCX expression at the sarcolemma causes reduced myocardial contractility and a higher arrhythmogenic propensity (Pogwizd et al., 2001). Depressed microRNA-1 expression would mean weaker post transcriptional inhibition of NCX expression as there are fewer microRNA-1 sequences present to target and bind to the NCX mRNA sequences. As part of a previous Masters project, the functionality of relationship between microRNA-1 and NCX was tested; the expression of microRNA-1 was reduced in isolated healthy adult rat left ventricular cardiomyocytes by *in vitro* transfection of an antisense RNA oligonucleotide (Anti miR-1). In the experiment, the functionality of NCX was examined in cardiomyocytes loaded with a fluorescent calcium dye and spritzed with caffeine. The presence of caffeine nullified the influence of SERCA2a removal of calcium from the cytoplasm in order to isolate the calcium extrusion by NCX for investigation. This uncovered an elevation in NCX function; the rate of calcium extrusion across the sarcolemma was faster compared to the control group and this result was included as part of publication with Professor Thum's group (Kumarswamy et al., 2012).

This experiment would suggest that an alteration to microRNA-1 was able to elicit a functional effect on cardiomyocyte calcium handling thereby replicating a cellular aspect of heart failure pathophysiology. When the contractility of these healthy cardiomyocytes was examined, neither an increase nor decrease in microRNA-1 expression resulted in a change to inotropy at baseline or after exposure to a β -adrenoceptor agonist. Although NCX function was affected by the change to microRNA-1 expression, this was not great enough to affect cardiomyocyte contractility. Another

study by Tritsch et al found that the expression of microRNA-1 and in turn NCX was regulated upstream by the serum-response factor (SRF), a transcription factor of the MADS box family (Tritsch et al., 2013). Knock down of SRF expression resulted in an enhanced capability of microRNA-1 to inhibit NCX expression compared to the control group. This highlights the complexity of microRNA research, they are able to work in concert with a number of other components to regulate the expression of a single mRNA sequence.

A study by a collaborator of Professor Harding's group delivered increased microRNA-1 expression in a TAC banding model of heart failure through use of an adeno-associated virus (AAV). Two weeks after the banding surgery was conducted the single dose of AAV was administered and when the hearts were extracted 7 weeks later the expression of microRNA-1 had increased significantly compared to the control group (Karakikes et al., 2013). The primary finding of this work focused on the reduction of hypertrophy, reduced myocardial fibrosis and an improvement in overall haemodynamic cardiac performance as measured by echocardiography. In addition the AAV9 also resulted in an increase in SERCA2a mRNA and protein expression with a reduction in NCX expression. The ratio of SERCA2a/ PLB was also normalised. The authors hypothesised that by shifting the balance of SERCA2a/NCX more calcium was pumped into the SR and less was transported across the sarcolemma.

To substantiate this theory, in a separate experiment the authors took isolated cardiomyocytes from TAC rats and transfected them *in vitro* with an adenovirus to increase microRNA-1 expression. This resulted in a normalisation of cardiomyocyte calcium transients. As previously stated, NCX is a direct target of microRNA-1 for post transcriptional inhibition, but the authors did not attempt to investigate the mechanism by which microRNA-1 is able to affect SERCA2a expression which resulted in improved ECC. Increased microRNA-1 expression resulted in elevated SERCA2a expression, this means that the regulatory mechanism would need to involve one or more intermediaries. For example, an inhibitor of SERCA2a expression that was a target of microRNA-1, so that this inhibition would be removed (disinhibition) when microRNA-1 increases. This represents a gap in understanding that exists in relationship between microRNA-1 and SERCA2a that could be explored.

To complicate matters further, upregulation of SERCA2a by AAV9 treatment in isolated adult rat cardiomyocytes from a model of heart failure resulted in normalisation of microRNA-1 expression (Kumarswamy et al., 2012). The hypothesised mechanism for this dynamic was that the excess calcium present in the cytoplasm of failing cardiomyocytes leads to the calcium/ calmodulin

dependent protein kinase kinase (CaMKK)-dependent activation of Akt. Akt phosphorylates the transcription factor FoxO3A which triggers its translocation from the nucleus to the cytoplasm. FoxO3A is promoter for the intronic sequence encoding microRNA-1, therefore when it is located in the cytoplasm it can not promote transcription of the microRNA-1 sequence and expression is depressed. By increasing SERCA2a expression, the level of calcium in the cytoplasm is reduced and FoxO3A remains in the nucleus where it can promote microRNA-1 transcription (figure 1F). This result combined with the findings of Karakikes et al would suggest that microRNA-1 and SERCA2a expression are possibly linked in a reciprocal manner, with each one able to influence the expression of the other (Karakikes et al., 2013).

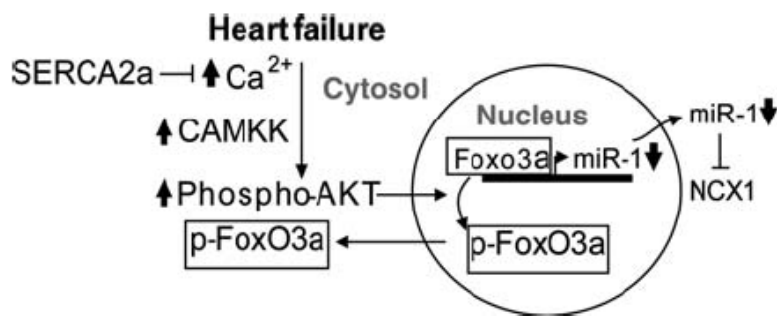


Figure 1F: The mechanism by which reduced cytoplasmic (cytosol) calcium drives a Akt-FOXO3A dependent reduction in microRNA-1 expression in heart failure. When SERCA2a expression is increased this cascade is altered and microRNA-1 expression is increased (Kumarswamy et al., 2012).

In addition to being linked to NCX and SERCA2a either directly or indirectly, microRNA-1 has been found to affect the expression of the regulatory subunits of protein phosphatase type 2a (Belevych et al., 2011). There are direct sequence matches between microRNA-1 and microRNA-133 and sections of the 3' UTRs of the B56 α and B56 δ subunits. The two subunits are key for the protein phosphatase type 2a mediated dephosphorylation of RyR, the channel which gates calcium release from the SR and has been found to leak calcium during heart failure. In a right ventricular pacemaker model of heart failure in dogs the authors found that microRNA-1 expression was increased which they suggest resulted in reduced protein expression of B56 α and B56 δ and increased phosphorylation at the Serine 2030 of RyR by calcium/ calmodulin dependent protein kinase. The end consequence of this cascade was an increased frequency of diastolic calcium waves and afterdepolarisations in isolated cardiomyocytes from the heart failure model. This would suggest microRNA-1 has a direct regulatory input on calcium release from the SR by influencing the phosphorylation state of RyR. As previously mentioned, the importance of the phosphorylation state of RyR on its open probability has been questioned, so this mechanism is dependent on that

paradigm being accurate. Interestingly the expression of microRNA-1 was increased in this model of heart failure rather than decreased, which may be due to the species or model of heart failure, but serves to highlight that the understanding of the role of microRNA-1 in heart failure is still at the early stages.

MicroRNA-1 has also been found to affect the expression of the mRNA sequences of GJA1 and KCNJ2. The former encodes connexin43 which forms the primary cardiac gap junction responsible for conduction of electrical signals between cardiomyocytes which acts as the stimulus for cardiomyocytes to contract. KCNJ2 encodes the subunit of the main inward-rectifier potassium ion channel 2.1 ($K_{ir2.1}$), this channel ensures that the E_m of cardiomyocytes during diastole is set and maintained at the correct voltage. Both are therefore important components of cardiomyocyte functionality and when disrupted result in abnormal cardiomyocyte behaviour. In a study of CAD patients the left ventricular expression of microRNA-1 was increased compared to healthy patients. It was shown that this resulted in an increased inhibition of the expression of two microRNA-1 gene targets; GJA1 and KCNJ2. Using a rat myocardial infarction model this change in GJA1 and KCNJ2 expression was linked to a greater depolarisation of the sarcolemmal membrane and an increased number of arrhythmic events such as premature ventricular beats and ventricular tachycardia (Yang et al., 2007). A separate study which used *in vivo* and *in vitro* experiments confirmed that microRNA-1 was able to regulate the expression of GJA1. Furthermore, normalisation of microRNA-1 expression in the trans aortic constriction *in vivo* model reduced the likelihood of ventricular tachycardia (Curcio et al., 2013). These studies show that a change to microRNA-1 results in a direct effect on the function of important cardiomyocyte gene targets.

MicroRNA-1 is an important cardiac microRNA, it is amongst a select group of microRNAs with a high expression in cardiomyocytes and it has been found to regulate a number of key cardiomyocyte calcium handling components of ECC. The expression of microRNA-1 has also been found to be different in heart failure compared to healthy, with microRNA-1 expression reduced in the majority of disease models and patient aetiologies. The downstream impact of this change in expression of microRNA-1 in heart failure on the spectrum of different proteins and regulatory factors of ECC is still being established, but with some interesting initial results in NCX, SERCA2a and RyR functionality already presented in the literature. With the direction of the change in microRNA-1 expression varying depending on the form of heart failure examined and why this diversity even occurs. Much work remains to understand the role of microRNA-1 in heart failure and what aspects of disease

pathophysiology it may be responsible for in order to determine its potential as a therapeutic target for intervention.

Circulating microRNAs

A rapidly expanding aspect to microRNA research is the investigation into the presence of microRNAs in a number of biofluids, such as urine, blood and breast milk (Reid et al., 2011). The discovery of circulating microRNAs came as a surprise as RNase enzymes rapidly break down any extracellular RNA that is found in the blood, however microRNAs are protected from degradation in circulation by one of a range of transportation vesicles. The mechanism of release from cells and the size of vesicles determines the nomenclature for these transport structures, with exosomes, microvesicles and apoptotic bodies have all been found to house microRNAs. The smallest to have been identified to date are exosomes, these are typically 50-100 nm in size and are released when a multivesicular body comes into contact and fuses with the plasma membrane of a cell. The next size up of vesicle are microvesicles (0.1-1 µm) and are formed through the blebbing of cell plasma membrane. Finally the largest vesicles are apoptotic bodies (0.5-2 µm), which as the name would suggest are released through a cell undergoing apoptosis (Creemers et al., 2012).

In addition to transportation by vesicle, Arroyo et al found that the majority of microRNAs circulating in human plasma and serum were protected from degradation by protein complexes. Through centrifugation and immunoprecipitation experiments the authors showed that the key component of the protein complex that transported microRNAs was argonaute-2 (Arroyo et al., 2011). There are two important offshoots from the observation that microRNA stability can be maintained outside of a cellular environment i) does functional microRNA activity occur outside of the cell and ii) can microRNAs present in biofluids be utilised as biomarkers of disease. Investigation into the second point has produced some interesting results, with changes in microRNA expression appearing to map a number of different pathological states such as cancer, kidney disease and MI.

Circulating microRNAs in heart failure

The diagnostic and/ or prognostic potential of circulating microRNAs as a biomarker heart failure is still being explored. To date, a selection of relatively small scale studies have been conducted, but the results of which have not yielded a consistent group of circulating microRNAs that can distinguish healthy from heart failure patients. This is not to say that individual studies have failed

to identify microRNAs in the plasma or serum that are significantly different in heart failure versus healthy patients, it is that there is little correlation in the results across studies. In one such example the expression of 6 microRNAs were found to be increased in 30 heart failure patients with dyspnea compared to 39 healthy or 20 dyspnea patients (Tijssen et al., 2010). Of the 6 candidates microRNA-423-5p proved to have the most significant difference between the groups and its expression increased with heart failure severity (NYHA classification). Patients were defined as having heart failure by using the Framingham criteria. Conversely, another study which used 10 heart failure patients (NYHA IV and III) found that the expression microRNA-423-5p was unchanged compared to 17 asymptomatic control patients (Fukushima et al., 2011). The authors instead suggested that microRNA-126 plasma expression was negatively correlated with heart failure severity and this pattern was reversible with improvement in a patient's condition from NYHA III to IV. Interestingly in the study by Tijssen et al, microRNA-126 expression did not have the same differential expression between healthy and heart failure groups.

A separate study focussed on the expression of circulating microRNAs that have a high expression in the myocardium and the muscle. In 24 patients with advanced heart failure (DCM and ICM) versus 13 healthy controls, the authors found that myocardium microRNAs-208b, -208a, -499 and the muscle microRNAs-1 and -133b showed a higher plasma expression. The size of the differential in expression was reduced after the patients were implanted with a left ventricular assist device to improve cardiac output, suggesting that the level of circulating microRNA was dynamic and reflected the severity of the patients' condition (Akat et al., 2014). Interestingly microRNA-423-5p showed no difference in expression between advanced heart failure and healthy groups but microRNA-126 expression was lower. In work conducted by Professor Thum's group, Watson et al identified 5 microRNA candidates (30c, -146a, -221, -328, and -375) that were able to distinguish between three groups of 90 patients each; between heart failure patients with preserved ejection fraction and those with reduced ejection fraction as well as between heart failure and healthy subjects (Watson et al., 2015).

Since the discovery of circulating microRNAs the number of studies that have investigated how the expression of circulating microRNAs changes in heart failure has grown hugely but as illustrated by the examples discussed, there is a lack in the cohesion of the findings to date. Which microRNAs are different in heart failure compared to healthy varies from study to study, as can the type of heart failure patient enrolled, the experimental approach and analytical methodology. This is not to say they should be disregarded, but a comprehensive and large-scale screening and validation study is

required to generate a more robust circulating microRNA expression profile. This could be used to identify one or more circulating microRNAs that can diagnose the disease more accurately than existing biomarker tests. Currently a full cardiac examination by echocardiography and electrocardiogram is required for a precise diagnosis, so there is a demand for a more straightforward, easy to administer diagnostic test. In addition, circulating microRNAs could also be used to act as prognostic markers for the disease, offering an insight into how a patient's condition may develop over time.

Overall study aims

The work presented in this study is divided into three results chapters. The first looks at the potential of circulating microRNAs to act as a prognostic biomarker for disease severity in heart failure patients. This work was made possible due to the availability of serial serum samples taken from a large, clinically well characterised cohort of heart failure patients enrolled in a clinical trial conducted around 10 years ago. It was an opportunity to study how circulating microRNA expression changed over time in these patients and if expression correlated with the severity disease. Therefore the aim for this work was as follows:

- Identify one or more circulating microRNAs that can distinguish heart failure patients that were enrolled in the Copernicus trial based on their clinical prognosis. A novel, predictive circulating biomarker that could match or better the selectivity and specificity than the current gold standard would be a powerful clinical tool.

The second and third chapters had related themes and focus on the role of microRNA-1 in cardiomyocyte function in heart failure. MicroRNA-1 expression has been found to be downregulated in the MI induced model of heart failure used by Professor Harding's group as well as other *in vivo* models and some human patients. It has also been found that microRNA-1 is able to influence both the expression and function of a number of cardiomyocyte components that play important roles in ECC and are known to malfunction in heart failure. The extent of this influence is still being established as well as the therapeutic potential if microRNA-1 expression is restored in heart failure. The work presented in the second results chapter is derived from experiments with transfecting isolated cardiomyocytes in order to change the expression of microRNA-1 and study the effect on cardiomyocyte function. The aims for this work were:

- Determine how increasing microRNA-1 expression in a range of cardiac cells types may be able to affect the expression of the key calcium handling protein SERCA2a.
- Investigate the effect of increasing microRNA-1 expression on heart failure cardiomyocytes calcium handling capability through measuring contractility.
- Determine if microRNA-1 can also affect other cardiomyocytes functions that are affected as part of the heart failure phenotype, e.g. T-tubular regularity and β -adrenoceptor function.

The results obtained and presented in the second results chapter prompted a continuation of the work and the effect of increasing microRNA-1 expression in an *in vivo* model of heart failure was studied. The aims for the third results chapter were:

- Explore the effect of β_2 -adrenoceptor mediated contractility after increased microRNA-1 expression in an *in vivo* model of heart failure.
- Determine whether increased microRNA-1 expression is able to affect the cardiac performance of adult heart failure rats over time by echocardiography.
- Take isolated cardiomyocytes from adult heart failure rats that have been treated with AAV9.miR-1 and measure SERCA2a function, T-tubular regularity and the expression of a range of cardiac relevant genes.

Chapter 2: Methods

Cardiomyocyte isolation and culture

Isolation of adult rat ventricular cardiomyocytes

The isolation of cardiomyocytes permits investigation into cardiomyocyte function on a single cell level. Isolation sacrifices some aspects of cardiac physiology such as physical and electrical cell to cell interactions or neural input and renders the cells in an unloaded state. There are many advantages to studying cardiomyocytes in this state; for example pharmacological substances such as isoprenaline and caffeine can be directly applied to a cell and washed off in a rapid fashion. Studying isolated cardiomyocytes is a method enabling a more detailed understanding of the individual contractile units that make up the heart.

Animals were anaesthetised using 5% Isoflurane and 2 ml/min O₂ before being killed by cervical dislocation. This provided a rapid method of sacrificing the animals (schedule 1) that is considered appropriate after a procedure by the Animals Scientific Procedures Act (ASPA) 1986. This method also minimised the effect of the surge in circulating catecholamines on the heart. After a trans abdominal incision the diaphragm was divided in order to expose the thoracic cavity. A median sternotomy allowed access to the heart, which was resected and quickly transferred to ~50ml ice-cold oxygenated Krebs-Henseleit (K-H) solution (119 mM NaCl, 4.7 mM KCl, 0.9 mM, 4 mM MgSO₄, 1 mM CaCl₂ 1.2 mM KH₂PO₄, 25 mM NaHCO₃ and 11.5 mM glucose) to arrest cardiac metabolism and limit ischemic injury. Heparin was added to the ice-cold K-H (4ml/L) to prevent blood clotting upon collection of the heart. 95% O₂/5% CO₂ was bubbled through the K-H to lower the pH to the physiological value of ~7.4. The presence of calcium and phosphate ions in the K-H solution can cause salt precipitation. The alkaline bicarbonate ions of CO₂ help prevent against this occurrence by maintaining pH of ~7.4. After beating arrested, any adherent fat or connective tissue was removed and the heart was hung from the aorta on the cannula of a Langendorff apparatus. To stimulate beating and flush any residual blood from the vasculature, the heart was retrogradely perfused with warmed K-H solution for 5 mins at a flow rate of 6-8 ml/min/g. To maintain the temperature as close to 37°C as possible, the heart was surrounded by a heated jacket. Next the heart was perfused with a preoxygenated 'low calcium solution' containing 12-15 µM CaCl₂ (120 mM NaCl, 5.4 mM KCl, 5 mM MgSO₄, 5 mM pyruvate, 20 mM glucose, 20 mM taurine, 10 mM HEPES, 5 mM nitrilotriacetic acid; 100% oxygen) for 5 mins. The solution was then switched to 'enzyme solution' for 10 mins and re-circulated, this contained 1g/l of type-2 collagenase (Cooper Biomedical), 0.8 g/l of hyaluronidase (Sigma) and 0.1% bovine serum albumin (BSA) (Sigma) to separate the muscle fibres. Retrograde

perfusion causes backward pressure and closes the aortic valve, forcing the solution to circulate the coronary vessels. This allows the enzyme solution greater penetration of the myocardium through diffusion out from the coronary vessels which results in a better separation of cardiomyocytes. The heart was removed from the cannula and the atria and right ventricle were removed to leave the left ventricle. For animals with a myocardial infarction (see section: Myocardial infarction (MI) surgery) the infarcted section of the left ventricle was removed and remaining tissue used for isolation of cardiomyocytes. This was cut into 2-4mm³ sections and transferred to a 50ml Falcon tube with 10ml of enzyme solution and shaken for 5 mins at 37°C (100% O₂). The tissue was filtered through a 300 µm gauze then added to 10ml of fresh pre-oxygenated (100% O₂) collagenase/ hyaluronidase enzyme solution and gently shaken at 37°C (100% O₂) for a further 30 mins. After this the solution was re-filtered through a fresh 300 µm nylon gauze and the supernatant centrifuged at low speed for 1 min. The resulting cell pellet was re-suspended in pre-oxygenated (100% O₂) 'suspension solution' (the same constituents as the low calcium solution but with 200 µM CaCl₂). The expected yield of viable cardiomyocytes from the enzymatic isolation of a healthy rat heart would be between 60-70% of the total cardiomyocytes in the heart.

Adult rat cardiomyocyte plating and culturing

After isolation, adult rat cardiomyocytes can survive in culture for around 48 hours. This period can be utilised for a range of purposes, such as transfection of cardiomyocytes in order to change gene expression or long term incubation with pharmacological agents. After isolation the cardiomyocytes were prepared for culture; the cardiomyocytes were centrifuged at 700x g for 1 min to form a pellet, the supernatant was aspirated and discarded. The pellet was resuspended in 5ml of warmed Dulbecco's Modified Eagle Medium (DMEM) (4mM Glutamine) (Gibco BRL, Life Technologies). This was repeated twice, before the pellet was resuspended in 5ml warmed M199 media (Gibco BRL, Life Technologies) that contained the following additions (0.5 mg/ml BSA, 0.66 mg/ml Creatine, 0.66 mg/ml Taurine, 0.32 mg/ml Carnitine, 50 units/ml Penicillin, 50 µg/ml Streptomycin (all Sigma)). Depending on the size of the pellet, more M199 was used to resuspend the cells so that the pellet was completely dissolved and no clumps of cardiomyocytes remained. Using a pipette 1 µl of cell suspension was transferred onto a glass coverslip, the number of cardiomyocytes were counted and that number was used to calculate the total available. The volume of M199 containing the number of cardiomyocytes required for a particular plating density was calculated, and this volume added to cell culture plates (Thermo Scientific). The plating density of cardiomyocytes and well size was dependent on the experiment and number of cardiomyocytes available. As a standard, the

cardiomyocytes were plated at around 60-70% confluency to minimise overlap. If the cardiomyocytes were to be cultured in suspension then an uncoated cell culture plate was used. If the experiment required the cardiomyocytes to be adhered to the plate surface, the well was coated before plating with mouse laminin protein (Gibco BRL, Life Technologies). A 10x dilution of the stock (1mg/ml) was made, and depending on the well size, between 5-20 µl pipetted onto the surface of the well. This was evenly distributed across the surface of the well and allowed to dry at room temperature for ~1 hour before cardiomyocyte plating. During culture the cardiomyocytes were kept in an incubator at 37°C and 5% CO₂.

Fixation of isolated adult rat cardiomyocytes

Fixation was used to maintain the structure of adult cardiomyocytes in order to study extracellular structures such as T-tubules at a later date. Cardiomyocytes in suspension solution were plated onto Laminin coated glass bottomed Matek dishes and allowed to adhere over ~1 hour. Afterwards the suspension solution was carefully aspirated and 4% paraformaldehyde (Sigma) in phosphate buffered saline (PBS) added to the cardiomyocytes for 15 mins at room temperature. To remove the paraformaldehyde, the solution was aspirated and PBS added and left for 5 mins, two further washes were then done. The plates were stored at 4°C for future experiments.

Adult rat cardiomyocyte culturing with PTX

Culturing the cardiomyocytes for up to 48 hours permits exposure to pharmacological agents over a longer time period. This can reveal the 'chronic' effect of an intervention, which may be different to exposing the cardiomyocytes to the same intervention at a higher concentration for a shorter period. After adult rat cardiomyocytes were isolated and plated in cell culture multi well dishes, they were incubated at 37°C and 5% CO₂. After 24 hours, PTX (Sigma) was added directly to the well, then mixed with the solution using a Pasteur pipette to reach a final concentration of 250 ng/ml. The dish was returned to the incubator for another 24 hours before any experiments were conducted.

Neonatal mouse and rat cardiomyocyte isolation, plating and culture

Before starting, all dissection instruments, beakers and media bottles were autoclaved. Neonates were anaesthetised using 5% Isoflurane and 2ml/min O₂ then sacrificed by cervical dislocation (schedule 1). The chests were opened with scissors and the hearts were dissected out. These were

transferred into a culture dish that contained Hanks balanced salt solution (HBSS) (Gibco BRL, Life Technologies) and then cut into 1mm³ cubes. Enzyme A and P were prepared in the buffer (constituents not disclosed by the manufacturer, all Miltenyi) and warmed to 37°C. The heart tissue was transferred to Miltenyi tubes and the HBSS removed, Enzyme A and P solutions were added and the tubes inverted to mix with the tissue. This was incubated at 37°C for 15 mins before being spun on the GentleMACs (Miltenyi) with the neonate specific protocol. This process of incubation at 37°C and centrifugation was repeated twice more, afterwards 7.5ml of M199 10% (100 ml M199: 10 ml neonate serum, 50 units/ml Penicillin, 50 µg/ml Streptomycin, 340 µl 200mM/l Glutamine, 500 µl 0.4 mg/ml Vitamin B12) was added to resuspend the pellet. Then the cell suspension was passed through a Pre-Separation Filter (70 µm) that was placed on a 50 ml Falcon tube. The filter was washed with 3ml of M199 10%, and the cell suspension centrifuged at 1000x g for 5 mins, the supernatant was aspirated to leave a cell pellet. This was resuspended in 20 ml 10% M199 per 12 hearts and plated at appropriate confluency in multi well cell culture plates or individual Matek dishes that had been coated with Laminin (see section: Adult rat cardiomyocyte plating and culturing) and incubated at 37°C and 1% CO₂. After 24 hours the culture media was removed and replaced with M199 5% (100 ml M199: 5ml neonate serum, 50 units/ml Penicillin, 50 µg/ml Streptomycin, 340 µl 200 mM l Glutamine, 500 µl 0.4 mg/ml Vitamin B12) and incubated at 37°C and 5% CO₂.

In vitro cardiomyocyte transfection

In vitro transfection enables the transfer of a gene of interest to a population of cells in culture. This method can be used to over express or silence the expression of a gene of interest. After exposing the cells in culture to a transfection agent that facilitates the transfer of the gene of interest into the cells, a period of incubation is required for the change in expression of the gene of interest to occur. In this study, the duration of this transfection period varied depending on the specific cell type and experiment, the specific time period for an experiment is noted in each results chapter.

MicroRNA-1 adenovirus

The microRNA-1 (adeno.miR-1) and green fluorescent protein (GFP) (adeno.GFP) adenoviruses used for the *in vitro* transfection experiments were kindly provided by Professor Roger Hajjar and Dr Ioannis Karakikes, The Mount Sinai School of Medicine, New York. The stock concentration of the adeno.GFP was 1.2x10¹² virus particles /ml (vp/ ml), whilst the adeno.miR-1 was 1.4x10¹² vp/ ml.

The production process of the adenoviruses does not have a 100% success rate, resulting in some virus particles being included that were 'empty', meaning some viral particles were generated that did not contain the microRNA-1 or GFP constructs. The result of this is that the actual number of virus particles that contained microRNA-1 or GFP sequences was probably lower than 1.2×10^{12} vp/ml and 1.4×10^{12} vp/ml respectively, but these were the viral concentrations used to determine the final concentrations to which the cells were exposed.

To allow the adult rat cardiomyocytes to recover after isolation and washing, the adenovirus was added to the well 2 hours after plating. The appropriate volume of adenovirus solution was pipetted directly into the well and this was carefully mixed with the M199 in the well using a Pasteur pipette to ensure uniform distribution across all the cardiomyocytes in the well. When the neonatal mouse cardiomyocytes were used the adenovirus was added 48 hours after plating by the same method. This was found to be the optimum time for transfection post plating. After 48 hours the neonatal cardiomyocytes had recovered fully from isolation and this time point of adenovirus addition yielded higher transfection efficiencies.

Lipofection

Transfection of adult rat cardiomyocytes by lipofection offers an alternative to viral methods. Lipofection has a low toxicity rate, this characteristic makes it suitable for adult cardiomyocytes as they are easily damaged in culture. The trade off is that the transfection efficiency is not comparable to viral methods (Djurovic et al., 2004). For transfection of adult rat cardiomyocytes two solutions were prepared; the first contained OptiMEM-I (Gibco BRL, Life Technologies) and Lipofectamine2000 reagent (Invitrogen). The second was formed of OptiMEM-I and a microRNA-1 construct; mature microRNA oligonucleotide (Pre miR), Anti miR (100 nM) or random sequence oligonucleotide (Scrambled control) (Ambion). The optimum ratio of Lipofectamine2000 reagent to microRNA construct varies depending on the cell type, for adult rat cardiomyocytes the manufacturers recommendations were followed. The two were mixed and stored at room temperature for 20 mins to allow the nucleic acid-lipofectamine complexes to form. After isolation and washing, a pellet of adult cardiomyocytes was carefully resuspended in this solution and added to a culture plate and kept at 5% CO₂ and 37°C for 4 hours before M199 culture media was added to the transfection solution. The adult rat cardiomyocytes were then cultured for 48 hours to allow the change in gene expression to come to fruition.

H9c2 cell culture

When in culture, H9c2 cells were maintained in 150 cm² flasks and plated in cell culture plates for experiments. DMEM (Gibco BRL, Life Technologies) (4mM Glutamine, 50 units/ml Penicillin, 50 µg/ml Streptomycin, 10% fetal calf serum (FCS)) was used as a culture media and the cells were kept at 5% CO₂ and 37°C. When 80% confluency was reached in the flasks the H9c2 cells were passaged. Growth medium was aspirated and one wash with warm PBS Solution (Gibco BRL, Life Technologies) was performed. Cells were then detached using Ethylenediaminetetraacetic acid (EDTA) trypsin (0.25%, Phenol Red) (Gibco BRL, Life Technologies) for 5 mins at 37°C. Prolonged exposure to Trypsin can damage the cells, so to inactivate it warmed DMEM 10% was added. Using a haemocytometer the cells in suspension were counted and plated in uncoated flasks or dishes. Cells were allowed to attach overnight prior to performing any experiments. To preserve a stock of H9c2, cell pellets from low passages were resuspended in freezing medium; FCS with 10% Dimethyl sulfoxide (DMSO) (Sigma) and stored in cryovials in liquid N₂.

Molecular biology techniques

RNA isolation from cells

The first stage of gene expression analysis is to extract the RNA from the cells/ tissue and measure the concentration. For extraction of RNA from adherent cells in culture, the medium was aspirated and two PBS (Gibco BRL, Life Technologies) washes were performed. Cells/tissue were then lysed in 1ml of Trizol reagent (Invitrogen) in Eppendorf tubes. For cardiomyocytes cultured in suspension, the medium containing the cardiomyocytes was pipetted into a 1ml Eppendorf and centrifuged for 5 mins at 1000x g. The supernatant was aspirated and 1ml of Trizol added to resuspend the pellet. The cells were frozen at -80°C for later use then thawed at room temperature when required. After thawing the cells were incubated at room temperature for 5 mins then 200 µl of chloroform (Invitrogen) added to each Eppendorf and vortexed vigorously. The Eppendorfs were centrifuged at 4°C at 12000x g for 15 mins. This formed a clear aqueous phase at the top of the solution that was carefully removed by pipette and transferred to a clean Eppendorf. 500 µl of Isopropanol (Sigma) was added and after incubation at room temperature for 10 mins the Eppendorfs were centrifuged at 4°C at 12000x g for 10 mins. Typically a white pellet was formed after this centrifugation, the supernatant was discarded and the pellet washed in 1ml of 75% ethanol (Sigma). Following a 5 min centrifugation at 7500x g at 4°C, the ethanol was removed and pellets were allowed to air dry at

room temperature. It was essential to remove all ethanol at this stage before resuspending the pellet in 20 µl RNase free H₂O. The NanoDrop (Wilmington) machine was then used to quantify the concentration of RNA in each sample.

RNA isolation from serum

Isolation of RNA from serum samples for microRNA expression analysis was conducted using a different method. The miRNeasy 96 Serum/Plasma Kit (Qiagen) was used to isolate cell-free total RNA (including microRNA) from human serum samples. In principle, the 96 well format of the kit utilised a phenol/guanidine-based lysis of samples and silica-membrane-based purification of total RNA. Each sample was added to an RNeasy MinElute spin column, during centrifugation the total RNA binds to the membrane and phenol and other contaminants are efficiently washed away. The high-quality RNA was then eluted in a small volume of RNase-free H₂O. The manufacturers protocol was followed with a starting volume of 200 µl serum. After adding the Lysis Reagent (Qiagen) to each sample and vortexing, 1 fmol/ µl of spike in C.elegans microRNA-39, a microRNA mimic (Qiagen) was added to each sample. The spike in C.elegans RNA provided a measure of the success of RNA isolation and a housekeeping gene to which the expression of microRNAs of interest could be compared. The amount of RNA yielded from patient serum samples was low and the concentration was not measured.

TaqMan reverse transcription (RT)

To generate complementary DNA (cDNA) of mature microRNAs from the isolated RNA a different type of RT was required. A specific RT primer for the microRNA sequence was used rather than a generic primer that would result in the amplification of all RNA. For microRNA, the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) was used to reverse transcribe 10 ng of total RNA. This method permitted two microRNA sequences to be reverse transcribed in the same reaction, thereby improving efficiency. For every sample analysed the house-keeping small RNA RNU6B was also reverse transcribed with its own specific primer:

	Amount (μ l)
RNase-free H ₂ O	0.58
10x RT buffer	0.75
5x miRNA specific RT primer A	1.5
5x miRNA specific RT primer B	1.5
Multiscribe RT enzyme (50 U/ μ l)	0.5
dNTP mix (100 mM total)	0.075
RNase inhibitor (20 U/ μ l)	0.095
Total volume	5
RNA	2.5

Table 2A: The constituents of the TaqMan MicroRNA Reverse Transcription kit used for TaqMan RT of standard genes of interest and the amount (μ L) required for 1 reaction.

For TaqMan RT of standard genes the iScript select cDNA synthesis kit (Bio-Rad) was used, which utilised a random primer designed to expand an exon-exon region. Depending on the yield of RNA from the isolation, between 100-2000ng of total RNA was reverse transcribed:

	Amount (μ l)
5x iScript reaction mix	5
Oligo (dT)20 primer	2
RNase-free H ₂ O	Variable
Template RNA	Variable
iScript reverse transcriptase	1
Total volume	20

Table 2B: The constituents of the iScript select cDNA synthesis kit used for TaqMan RT of standard genes of interest and the amount (μ L) required for 1 reaction.

Reactions were performed by incubating samples in a 7900Ht Thermal Cycler (Applied Biosystems), following the protocol specified by the manufacturer for each kit. Once cDNA synthesis was completed, samples were diluted in the appropriate volume of RNase-free H₂O ready for RT-qPCR.

TaqMan real time quantitative polymerase chain reaction (RT-qPCR)

Following RT to generate cDNA, RT-qPCR was used to determine the gene and microRNA expression levels. For microRNA RT-qPCR, specific microRNA 20x RT-qPCR primers were mixed with TaqMan Gene Expression PCR Master Mix (both Applied Biosystems). This was pipetted into a Fast Optical 384 well plate followed by diluted microRNA cDNA to reach a final volume of 10 µl/well. Each sample was assayed in triplicate and the RNU6B amplified as a control. For all RT-qPCR experiments, three wells of the plate were included per sample as technical replicates, plates were sealed with Optical Adhesive Covers (Applied Biosystems) and briefly centrifuged to ensure all the solution was at the bottom of the well before amplification. All experiments were performed using an ABI Prism 7900HT sequence detection system (Applied Biosystems).

For microRNA RT-qPCR, the following temperature protocol was used:

95°C	15 min (heat activation of polymerase)	

95°C	15 sec	
60°C	1 min	40x cycles

15°C	hold	

For analysis of standard genes, the transcripts of interest were amplified using either SYBR Green or TaqMan methods. For SYBR Green, iQ SYBR Green Supermix (Bio-Rad) was used, whereas for TaqMan, the TaqMan Gene Expression Master Mix (Applied Biosystems) was used. For both TaqMan and SYBR Green protocols, after pipetting all wells had a final volume of 10 µl/well and GAPDH was included on all plates in separate wells for normalisation in data analysis. TaqMan RT-qPCR analysis of the expression of standard genes used the same temperature protocol as microRNA RT-qPCR, but for SYBR Green RT-qPCR the following temperature protocol was used:

95°C	3 min (heat activation of polymerase)	

95°C	15 sec	
XX°C	30 sec (temperature varies due to primer annealing temperature, this was optimised for each primer)	

72°C 40 sec

40x Cycles

Settings for melt curve:

95°C 1 min

55°C 1 min

80 repeats at 10 seconds each (temperature + 0.5°C)

The threshold of amplification was manually determined using the Applied Biosystems software (RQ Manager), the Ct values were then exported for analysis.

SYBR Green microarray

After RNA was isolated from serum samples (see section: RNA isolation from serum) the Human miRNome microRNA Profilers QuantiMir™ kit (SBI Biosystems) was used to tag and convert microRNAs into detectable and quantifiable cDNAs. Then the expression of 1, 113 microRNAs in each sample was assessed by RT-qPCR. The manufacturers protocol was followed, with the exception of the endogenous controls for normalisation. The three wells in each RT-qPCR plate were replaced with the primers for spike in C.elegans. RT experiments were run using a 7900Ht Thermal Cycler and RT-qPCR conducted using an ABI Prism 7900HT sequence detection system (both Applied Biosystems).

TaqMan microarray

The Megaplex™ Pools (Applied Biosystems) kit was another method that was used to screen a large population of microRNAs in serum after RNA isolation. This kit used a TaqMan based method of RT-qPCR and in total, the expression of 667 microRNAs were analysed. The manufacturers protocol was followed and the optional pre-amplification step included due to the low concentration of microRNA in each sample. Again the spike in C.elegans was used as a normalisation control rather than those included in the kit. RT experiments were run using a 7900Ht Thermal Cycler and RT-qPCR conducted using an ABI Prism 7900HT sequence detection system (both Applied Biosystems).

TaqMan validation of circulating microRNA expression

After determining the expression of a large range of microRNAs by microarray, the expression of a selected group of microRNA were validated by TaqMan RT-qPCR. The same method was used as detailed in section: TaqMan real time quantitative polymerase chain reaction (RT-qPCR).

Investigation of cardiomyocyte contractility

The IonOptix system (IonOptix corp.) was used to measure contractility of individual cardiomyocytes. This technique utilises a video edge detection device methodology and produces a quantitative measure of cell length. When the cell contracts, it shortens in length and this is recorded by the IonOptix system. By controlling the solution the cardiomyocytes are exposed to and the frequency and voltage of electrical stimulation, it is possible to examine specific aspects of the contractile process of an individual cardiomyocyte in a range of conditions.

Apparatus setup

The IonOptix MyoCam camera (IonOptix Corp.) was connected to a Nikon TE-200 inverted microscope and a PC running the Ion Wizard software (IonOptix Corp.). A Perspex chamber with two platinum electrodes mounted on either side was placed on top of the microscope stage, cardiomyocytes in ~200 μ l solution were added to the chamber for an experiment. The electrodes were connected to an electrical stimulator that allowed the frequency, amplitude and width of the stimulating pulse to be adjusted. Usually stimulation was set at 0.5 Hz, 50 V and 0.5 milliseconds (ms) pulse width for all adult rat cardiomyocytes experiments. The cardiomyocytes were superfused with K-H solution 1 mM CaCl_2 at a rate of 2 ml/min by a peristaltic pump (Marlow-Watson). The K-H solution was heated to 37°C and bubbled with 95% O_2 / 5% CO_2 to maintain a pH of 7.4. A single cardiomyocyte was positioned in the centre of the field of view produced by a 20x objective lens, brought into focus and the IonOptix camera was rotated to obtain a clear image along its horizontal axis. Using the Ion Wizard software, the edges of the cardiomyocyte were matched to a pair of markers, the relative distance between the markers was recorded and the resulting length calculated by the software and displayed on the screen, figure 2A is an example of this graphical display.

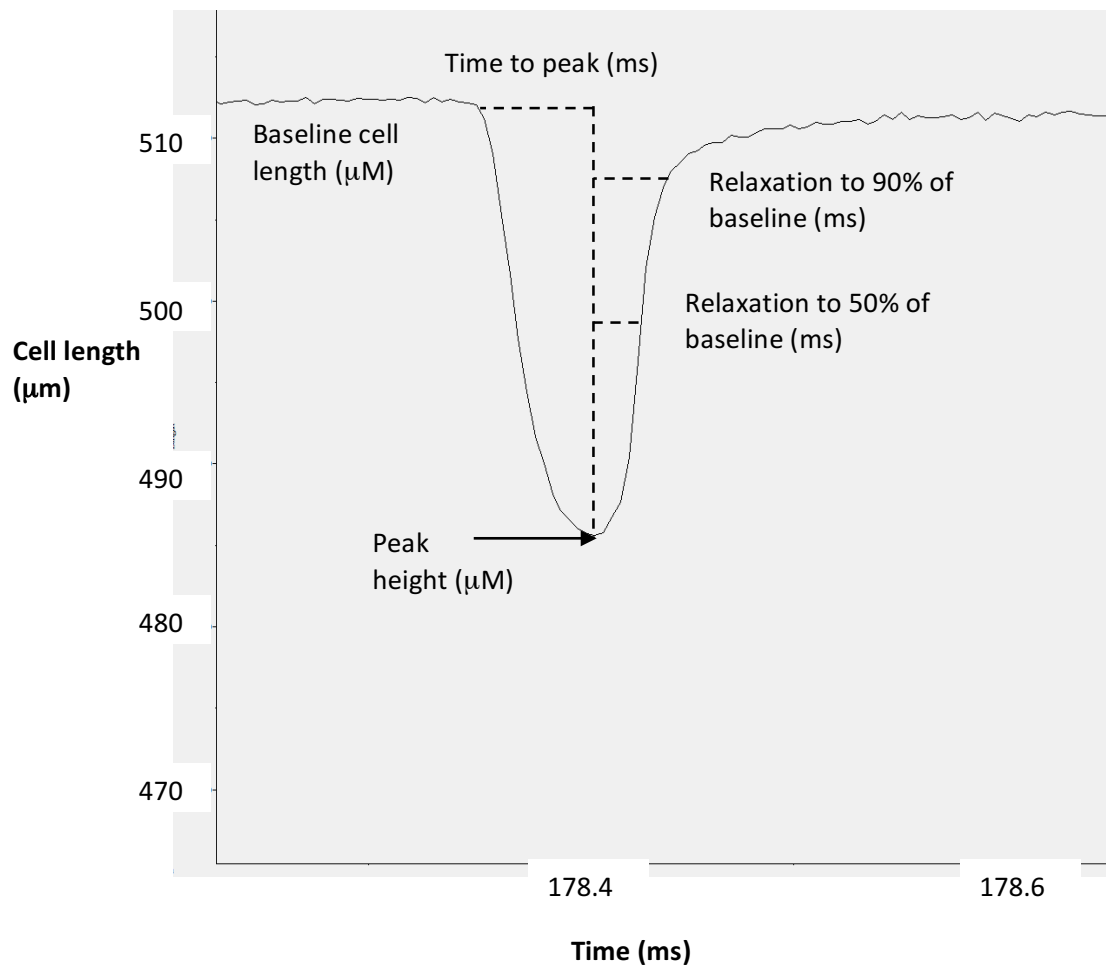


Figure 2A: An example of a single cardiomyocyte contraction after electrical stimulation as detected by the IonOptix system and the parameters measured by the Ion Wizard software.

The trace represents absolute cell length (y axis) plotted over time (x axis). Before the downward deflection is diastole, where the cardiomyocyte is in the relaxed state and the cell length is stable. When the electrical stimulation passes between the platinum electrodes this stimulate a contraction and replicates systole. This causes the cardiomyocyte to shorten in length to a peak contractile amplitude (peak height) before relaxing back to its baseline length.

Upon electrical stimulation a cardiomyocyte will contract and the cell length decreases. The Ion Wizard software displays cell length in real time and a contraction appears as a downward deflection. Electrical stimulation of the cardiomyocyte replicates systole, after which the cells relaxes and cell length increases and returns to a baseline resting length (diastole). A single contraction is broken down into the different phases by the Ion Wizard software and used for analysis of different aspects of cardiomyocyte contractility:

- Baseline cell length: the resting length (μm) of the cardiomyocyte.
- Time to peak: the time (ms) taken for the cardiomyocyte to shorten from baseline cell length to the peak shortening length.
- Peak Height: this is the maximum contractile amplitude calculated by subtracting the shortest cardiomyocyte length from the baseline length.
- Baseline as a % of peak height (Bl%PeakH): the difference between the cell length at baseline and peak height as a percentage of the baseline cell length.
- Relaxation to 50% of baseline: the time (ms) taken for the cardiomyocyte length to decrease by 50% from the peak shortening value.
- Relaxation to 90% of baseline: the time (ms) taken for the cardiomyocyte length to decrease by 90% from the peak shortening value.

Therefore, from a single contraction a range of contractile parameters can be determined, but because the cardiomyocyte is unloaded it is not a true representation of inotropy. Around 10 transients from a period of stable contractility were analysed as a group and the average parameters from a group taken for results. The selection of cardiomyocytes by the operator was a key element of the protocol. Many cardiomyocytes cannot be used because of their morphology or behaviour, particularly after a period of culturing. The criteria used for cell selection was as follows:

- Rod shaped cells with the clearest striation pattern and squarest edges possible. Over time in culture cardiomyocytes round up, the more round the cardiomyocyte the less likely it is to survive a contraction protocol.
- A baseline as a % of peak height (Bl%PeakH) between 2-6%, outside of this range and a cardiomyocyte may not respond to pharmacological agents that affect contractility because it is in a hyper- or hypo-contractile state.
- Exhibited a period of quiescence in K-H solution (1mM CaCl_2) then upon stimulation, stable contractions without any periods of arrhythmia for approximately 20 mins.
- Not overlapping other cardiomyocytes; if any cell in contact starts to contract arrhythmically it can trigger the cardiomyocyte being studied to also contract arrhythmically.

Isoprenaline concentration response curve

The non-selective β -adrenoceptor agonist isoprenaline (Sigma) was used to generate an increase in contractile amplitude. A stock solution of isoprenaline was made up in distilled H₂O, then diluted in oxygenated K-H solution and superfused over the cardiomyocytes. The first concentration was 1×10^{-10} molar (M) and isoprenaline was added incrementally by half log increases to 1×10^{-6} M, or until the cardiomyocyte displayed sustained arrhythmic contractions and/or rounded up and died. To maintain the stability of isoprenaline in solution ascorbic acid (1.7mM) (Sigma) was added to the oxygenated K-H solution and the isoprenaline was kept on ice until required. After 10 mins of stable baseline contractions the superfused solution was switched to the lowest dose of isoprenaline, the next dose was then applied after the contractile response had plateaued or a period of 10 mins had elapsed.

β_2 -adrenoceptor specific studies

To investigate the contractile response of the β_2 -adrenoceptor alone the β_1 -adrenoceptor antagonist ($\pm$)-2-Hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1H-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamide methanesulfonate salt (CGP20712A) (Sigma) was used in conjunction with isoprenaline. By blocking the activation of the β_1 -adrenoceptor, isoprenaline effectively becomes a selective agonist for the β_2 -adrenoceptor and any contractile response recorded in the presence of CGP20712A and isoprenaline can be attributed to the β_2 -adrenoceptor. After a suitable cardiomyocyte was identified and 10 mins of stable basal contractions were observed, a single dose of 3×10^{-8} M isoprenaline was applied and the contractile response allowed to plateau. This was followed by a period of around 30 mins of superfusion with oxygenated K-H (1mM CaCl₂) solution to wash out the isoprenaline from the bath and allow the cardiomyocyte to recover. A stock solution of CGP20712A was prepared in K-H solution to a yield a final concentration of 300 nM CGP20712A, this was superfused over the cardiomyocytes for 20 mins before the cardiomyocytes were exposed to 3×10^{-8} M isoprenaline and the contractile response allowed to plateau.

Recording intracellular calcium transients

To monitor changes in intracellular calcium concentration within cardiomyocytes a fluorescent dye, Fura-2, was used. Fura-2 is a ratiometric indicator that shifts its excitation spectrum when it binds calcium. Evaluation of changes in intracellular calcium is achieved by using two excitation

wavelengths and calculating the ratio of the two fluorescence intensities recorded when the dye is excited at each wavelength. Ratiometric dyes correct for unequal dye loading into cells, leakage and bleaching and variable cell thickness because the ratio does not depend on the absolute intensity of the two signals but is a comparison of the emission intensity.

When dissociated from calcium, Fura-2 has a peak excitation wavelength of about 380 nm. This changes to around 340nm when it binds to calcium. When the concentration of calcium increases, the intensity of fluorescence recorded when Fura-2 is excited by light at 340 nm will also increase, whilst the intensity recorded after 380 nm exposure decreases. It is the ratio of the emitted fluorescence after exposure to the two wavelengths that is used to determine the level of calcium. At 360 nm the absorption is the same for all calcium concentrations. This middle point between 340 and 380 nm is the isosbestic point of Fura-2 and the fluorescence emitted at after exposure to 360 nm is independent of calcium concentration. UV light is poorly transmitted by most objective lenses unless they are composed of quartz glass so in practice the excitation wavelength of 360 nm is used rather than 340 nm. At 380 nm, light is less affected by transmission through glass but the absorption is lower. In order to calculate the ratio, fluorescence from Fura-2 is recorded at 520 nm when the dye is excited at 360 nm and this represents the numerator value of the ratio. Almost simultaneously the fluorescence is recorded from Fura-2 at 520 nm when the dye is excited with light at 380nm, this second measurement is the denominator.

Apparatus setup

Excitation with 360 nm and 380 nm UV light at a ratio sampling rate of 1000 Hz was achieved with the IonOptix μ Step light source. The light from a 75W Xenon arc bulb was focused through a filter switcher. This allowed light at either 360 nm or 380 nm to be passed via a fibre optic light guide to the cell chamber on the microscope. The emitted fluorescence from the cell on the stage of the microscope was reflected by a dichroic mirror onto a photo multiplier tube (PMT). The Cell Framing Adapter was used to optimise the cell fluorescence recording. This adjustable iris framed a rectangular area of the microscope field of view and allowed the selected cell to be isolated from other fluorescent objects by masking the background. The analogue output signals from the PMT were digitised and recorded by the IonOptix Fluorescence System Interface connected to a PC. Light of longer wavelengths passed the dichroic mirror and was directed to the camera. A red filter in the microscope condenser allowed the transmitted image to be recorded simultaneously with the cell fluorescence. The IonWizard software calculated the ratio of the fluorescence intensities at 360 and

380nm excitation and this ratio was used for comparison between groups. All components of this setup were from IonOptix Corp.

Loading adult rat cardiomyocytes with Fura-2 dye

Cardiomyocytes suspended in ~4ml of suspension solution were added to a round-bottomed Falcon™ tube. To this, the Fura-2AM dye (5 μ M) (Molecular Probes) in its membrane permeant acetoxymethyl ester form was added. The tube was wrapped in aluminium foil to protect the dye from light exposure and very gently rocked at room temperature for 15 mins. Thereafter the tube was centrifuged for 1 min at 300x g to form a cell pellet, the supernatant was aspirated and discarded. The pellet was resuspended in fresh suspension solution and allowed to sit at room temperature for another 20 mins. When Fura-2 crosses the membrane of cells the ester groups hydrolysed (de-esterification), this prevents the dye from crossing back, trapping the dye in the cytoplasm of the cell.

Recording calcium transients

Cardiomyocytes were loaded onto a glass coverslip fixed in a plastic mould on the microscope stage using a Pasteur pipette. Two platinum electrodes provided field stimulation and the cardiomyocytes were superfused with normal Tyrode (NT) (140 mM NaCl, 6 mM KCl, 10 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) at 37°C. Cardiomyocytes were paced for 2 mins at 1Hz to establish steady state contractions then the fluorescence was recorded for 1 min. The average of 5-10 transients were taken for each Fura-2 measurement and analysed using the Ion Wizard software. After capturing and analysing the selected group of calcium transients the following parameters were calculated:

- Baseline calcium: The baseline fluorescence ratio (360/380 nm) prior to the cardiomyocyte being stimulated by the platinum electrodes, a measure of diastolic calcium concentration.
- Peak Height: The maximum amplitude of fluorescence ratio recorded, the peak amplitude of the calcium transient. After a caffeine spritz this represents the total amount of calcium loaded in the SR.
- Time to peak: The time (ms) taken for the calcium transient to reach peak fluorescence ratio during the rising phase of the transient.

- Time to baseline 50%: The time (ms) for the calcium transient to return to 50% of the baseline fluorescence ratio during the decay phase of the transient. This is a measure of calcium clearance from the cytosol through different routes, more active/ efficient these mechanisms are the faster the calcium is cleared.
- Time to baseline 90%: The time (ms) for the calcium transient to return to 90% of the baseline fluorescence ratio during the decay phase of the transient.
- Tau: Derived from fitting a single exponential curve to the second phase of the transient decay. When there is a two-part decay from a calcium peak the IonOptix wizard is unable to fit an R50 value to the second decay. Tau therefore provides a method of quantifying the second part of the decay.

Measuring SERCA2a function

Cardiomyocytes were paced for 2 mins at 1Hz to establish a period of consistent contractions and fill the SR with calcium to a steady state. The pacing was stopped and the fluorescence recording started, after 5 seconds the solution superfusing the cardiomyocyte was switched from NT to a zero sodium zero calcium solution containing 10mM caffeine (140 mM LiCl, 6 mM KCl, 10 mM glucose, 10 mM HEPES, 1 mM MgCl₂, 1 mM EGTA). After another 5 seconds the solution was switched again to zero sodium zero calcium solution without caffeine. This was continued until the calcium measurement returned to pre-stimulation levels (basal). Caffeine exposure triggers an emptying of calcium from the SR by activating the RyR and the zero sodium zero calcium solution prevents NCX from effluxing calcium from the cell. By removing the caffeine the RyR is no longer continually activated, which in turn means calcium can be restored to the SR by SERCA2a. To determine the function of SERCA2a, tau was fitted to the second phase of the decay to give an indication of the rate at which reuptake of calcium into the SR occurs through SERCA2a. The calcium release caused by 10 mM caffeine was measured as Peak Height which indicates total SR calcium content. An example of a transient after a caffeine spritz is shown in figure 2B.

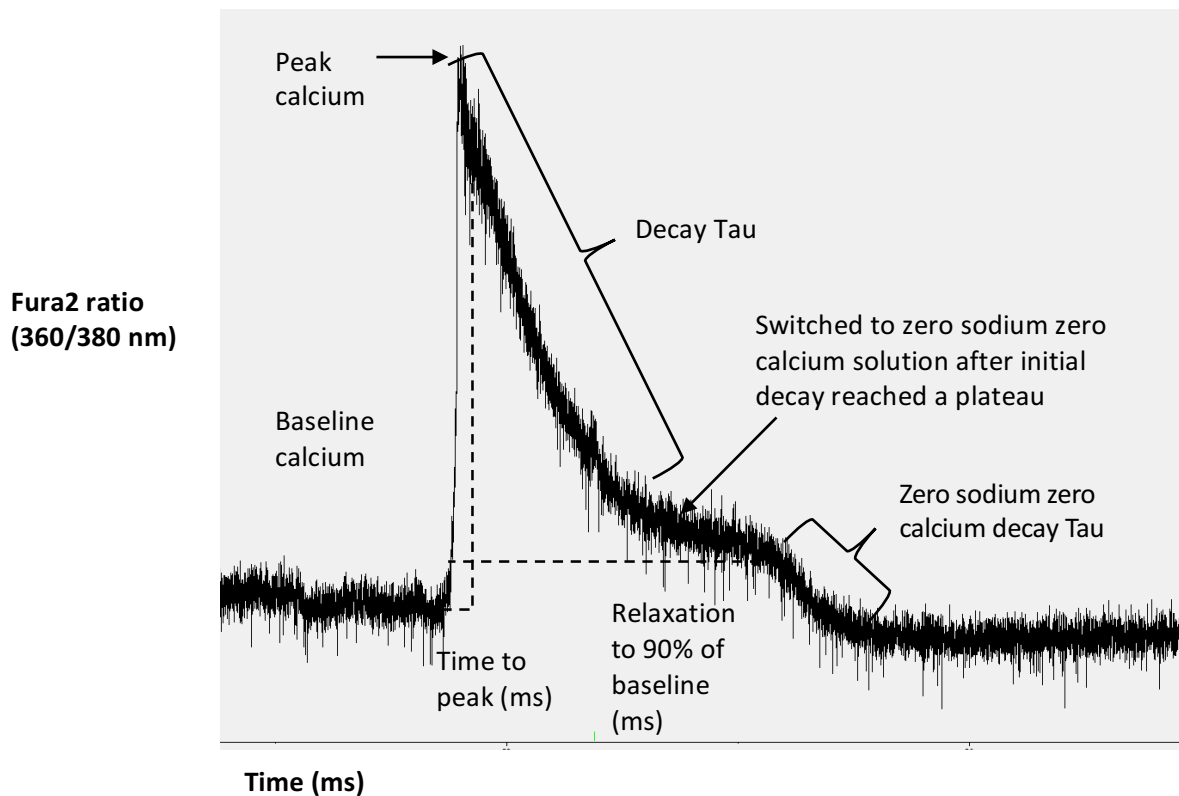


Figure 2B: An example of a Fura2 calcium transient recording from a single cardiomyocyte after a caffeine spritz and the parameters obtained from the recording.

The trace represents Fura2 ratio (y axis) plotted over time (x axis). A stable baseline recording was taken before the caffeine in zero sodium zero calcium solution is spritzed onto the cardiomyocyte. This caused the calcium in the SR to empty into the cytoplasm which is detected as an increase in Fura2 ratio. The calcium is then removed from the cytoplasm and the decay Tau for the first decay section was fitted and calculated. The caffeine was removed and the decay Tau for zero sodium zero calcium solution was then calculated before the Fura2 levels returned to baseline.

Förster resonance energy transfer (FRET)

FRET is based on the principle of energy transfer between two fluorophores in close proximity and with overlapping excitation and transmission wavelengths (Stryer and Haugland, 1967). If the higher energy fluorophore is excited at its particular wavelength of excitation the energy emitted will in turn excite the lower energy fluorophore via a non-radiative transfer of energy. This principle has been applied to measuring the level of intracellular cAMP. The FRET sensor for cAMP used in this study was an exchange protein directly activated by cAMP (EPAC1). Consisting of a guanine nucleotide exchange factor for Rap1 that becomes activated after direct binding of cAMP that is

fused to cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) in a single molecule (Nikolaev et al., 2004). When cAMP binds to the EPAC1 FRET sensor the fluorescence will decrease as the energy transfer from one fluorophore to the other is reduced as the two fluorophores move further apart.

This gives the molecule the capacity to be highly sensitive to cAMP levels. DNA encoding this sensor was successfully transferred into mouse embryos to produce transgenic EPAC1 mice that express the construct. This removes the need for maintenance of cells in culture for periods after transfection with a cAMP sensor as part of a viral particle. Cardiomyocytes derived from the transgenic EPAC1 mice gives rise to a molecule that has both yellow and blue fluorescence when excited at 488nm. This appears as a faint green fluorescence in an unfiltered image.

Apparatus setup

The FRET microscopy system consisted of an ORCA-ER CCD camera (Hamamatsu Photonics) attached to a Nikon TE2000 inverted microscope equipped with 30WDi halogen lamp illuminator pillar. The system had an EX436/20 excitation filter combined with DM455 dichroic mirror. The fluorescence emitted from a cell was split into YFP and cyan fluorescent protein (CFP) channels using a Dual View (Optical Insights, equipped with 535/40 and 480/30 emission filters) beam splitter. Images recorded onto a PC were used to quantify the relative ratio of YFP to CFP and hence monitor FRET output. Analysis was performed using Micro-Manager Software; subsequently bleed through from the CFP channel into the YFP channel was corrected for. Bleed-through was calculated by establishing the relative intensity of the fluorescence in the yellow channel when imaging CFP expressing HEK293 cells. Solutions were applied to cells directly via a gravity-driven perfusion system. Reagents and all FRET microscopy studies were performed utilising a HEPES based FRET buffer (144 mM NaCl, 10 mM HEPES, 1 mM $MgCl_2$, 5 mM KCl and buffered to pH 7.4). Mouse neonatal cardiomyocytes plated on Matek dishes were superfused at 1 ml/min. The bath solution was evacuated using a peristaltic pump as desired.

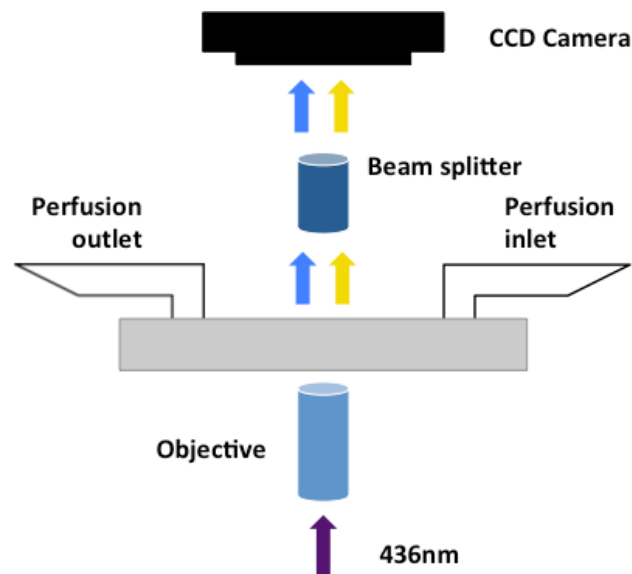


Figure 2C: Schematic of the FRET Microscopy system including the micro-perfusion system

Determining the amplitude of FRET shift caused by β_2 -adrenoceptor cAMP production

This protocol was used to determine whether neonatal EPAC1 cardiomyocytes transfected with adeno.miR-1 had a greater β_2 -adrenoceptor cAMP response compared to cells transfected with adeno.GFP. FRET buffer with CGP20712A (100 nM) was applied (1-2 ml/min) to provide β_1 -adrenoceptor blockade. After a baseline period of ~5 mins had elapsed isoprenaline (100 nM) was applied to stimulate the β_2 -adrenoceptors. Following the plateau of the isoprenaline-induced FRET response, the cardiomyocytes were exposed to a solution containing 5 mM of a forskolin analogue (NKH477) (Tocris), NKH477 activates adenylyl cyclase and was used to establish the maximal FRET response of each cell. The protocol used in this study compared the increase in cAMP levels after isoprenaline relative to the cAMP increase after full activation of AC by NKH477. Rather the use raw cAMP FRET responses which can be variable between cells, this corrected measure meant that the β_2 -adrenoceptor mediated cAMP response could be compared between individual cardiomyocytes and between separate preparations.

Confocal microscopy to measure T-tubule density

Confocal microscopy offers a number of advantages compared to optical microscopy, such as improved resolution by the elimination of out-of-focus light and including shallow depth of field. Adult cardiomyocytes have a striated surface that is visible under a microscope and this is the Z line structure of the cardiomyocyte myofilaments. To visualise the T-tubules the fluorescent dye Cell

Mask Orange (CMO) (Invitrogen) was used to stain the T-tubules so that the regularity could be quantified. The most common dye for staining T-tubules is Di-8-ANEPPS, but this cannot be used in conjunction with GFP as the emission wavelengths overlap. Therefore it would be impossible to distinguish the T-tubules from the rest of the cell that has been transfected with GFP.

Cell mask orange (CMO) staining

CMO (5 $\mu\text{g/ml}$) was added to isolated cardiomyocytes in suspension solution for 5 mins at 37°C. The solution was mixed using a rotary mixer and protected from light during the incubation period. Cells were then washed twice in fresh suspension solution and resuspended in a low CaCl_2 (150 μM) form of NT and kept at room temperature until imaging.

Apparatus setup

A laser was used as an excitation light source in order to achieve the high intensities of light required by the system and laser light also remains in a narrow beam over long distances. The beam of light was reflected off a dichroic mirror and from there it was directed to reflect off two mirrors mounted on motors which scan the light beam across the cell. If a cardiomyocyte is stained with the CMO dye, it will fluoresce and the emitted light passes back across the mirrors and through the dichroic. The light was then focused to pass through a pinhole and the light which reaches the other side of the hole was measured by the PMT. This method allows one point of the cell to be observed at a given time, and the image is built up one pixel at a time. For the experiments in this study an inverted Nikon Eclipse TE-300 microscope with a 'Bio-Rad Radiance 2000' confocal attachment was used with a Nikon 40x oil immersion lens (numerical aperture =1.4).

CMO was excited using exposure to light set at a wavelength of 561 nm from an argon laser and the corresponding excitation power at the back of the aperture was $\sim 280\mu\text{W}$. The spatial resolution of the optical system was calculated to be $0.41 \pm 0.05 \mu\text{m}$ and the effective pixel size at the sample was $0.24 \mu\text{m}$. Each acquisition consisted of 15,000 frames of 960×200 pixels. The camera integration time was set at 1.46 ms and the time between frames was 1.5 ms, resulting in an image acquisition rate of 667 frames per second for a total duration of 22.5 seconds. Therefore the line imaging rate achieved was $\sim 130,000$ per second. The two image stacks representing the different channels were acquired simultaneously then co-registered using a USAF test chart (Edmund Optics, Barrington, USA).

Cardiomyocytes were plated using a Pasteur pipette onto a glass coverslip fixed on the microscope stage and scanned by eye for suitability:

- Rod shaped cells with clear striation pattern and squarest edges as possible. Over time in culture cardiomyocytes round up, the more round the cardiomyocyte the less likely it is to survive a contraction protocol.

When a cell was found it was positioned so that it was the only cell in the field of view. Several images of each cardiomyocyte taken and saved for analysis.

Fourier transform analysis

Images were opened in ImageJ (NIH, USA) and a small area (40x5 microns) was selected, automatically thresholded by the software to the default binary threshold and transformed into binary black and white image. White areas were quantified as a proportion of cell area providing an assessment of T-tubule density. These binary images were turned into power-frequency peaks via a 1D Fourier transform in MatLab (The MathWorks, Inc.) (figure 2D) using a custom written macro (Ibrahim et al., 2010) and this produced a peak at the most common frequency of 0.5 Hz. The output value from MatLab has an arbitrary unit; T-tubules are typically present at the membrane every 2 microns and the product of the calculation by MatLab is a measure of how regularly T-tubules appear every 2 microns. The amplitude of this peak increases as the regularity of the fluorescent stain increases; the relative regularity of the fluorescent stain can be compared between cells and cell types.

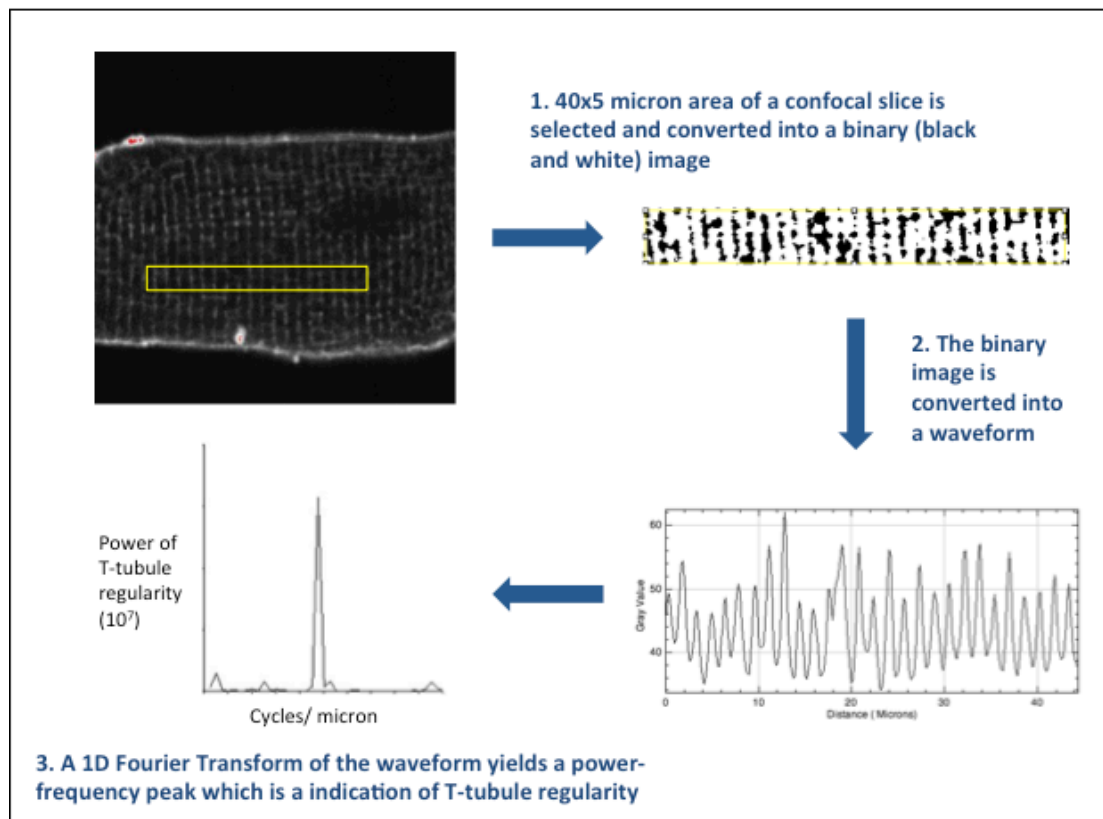


Figure 2D: Representative images showing the process used to convert an image of a CMO stained adult cardiomyocyte into a power-frequency peak which allows the regularity of the T-tubules at the cell surface to be determined.

In vivo experiments according to ASPA

Myocardial infarction (MI) surgery

All studies were performed on adult male Sprague Dawley rats weighing 200-270 g. Animals were anaesthetised using isoflurane 5%, reducing to 2% once intubated and ventilated. Pre-operative medication administered was as follows: 0.015-0.03 mg buprenorphine (0.05-0.1 mg/kg), 1.25-1.5 mg enrofloxacin (5 mg/kg), and 2.5 ml 0.9% saline subcutaneously (SC). The rat was connected via the endotracheal tube (16 G intravenous cannula) to the Harvard Ventilator (Harvard Apparatus), and ventilated using volume-controlled ventilation at a rate of 90/ min, with a tidal volume of 2-2.5 ml, giving a min volume of 180-225 ml/min. A shaved area over the left side of the precordium was sterilised with 1% Chlorhexidine (Hibiscrub™) and a sterile field was established using a drape. Surgery was performed with the rat secured in the left lateral position using micropore tape (3M) on an electrical heat-pad to maintain body temperature during surgery.

A left parasternal incision was made with subsequent separation of two heads of pectoralis major to reveal underlying ribs. These were separated in the 4th interspace by blunt dissection. The thymus gland was retracted and held to expose and stabilise the heart. The pericardium was removed from the anterior surface of the heart using blunt dissection. The left anterior descending coronary artery was ligated by passing a 6-0 Prolene suture (Johnson and Johnson Medical Ltd) through the left ventricular wall 2 mm inferior to the infero-medial corner of the left atrial appendage and tying it firmly. The path of the needle ideally remained intramyocardial to avoid bleeding. Puncture and exit sites were separated by approximately 2 mm. The left anterior descending coronary artery is not visible in the intact rat heart (even using an operating microscope), so a judgement was made on whether sufficient MI had been produced on the basis of pallor or cyanosis of the ventricular wall including the left ventricular apex and also by ensuring hypokinesis of the anterior wall of the left ventricle prior to closure. Inadequate initial infarction necessitated placement of further sutures.

Before closure of the ribs space with a 4-0 Ethibond mattress suture (Johnson and Johnson Medical), continuous positive airways pressure was delivered to reinflate the lungs and expel any air from the chest space. Finally the thoracotomy and skin were closed using a 2-0 Ethibond discontinuous suture (Johnson and Johnson Medical). Anaesthesia was stopped and 100% oxygen was continued. Animals were ventilated until they showed signs of spontaneous movement at which time they were extubated and placed in an oxygen-enriched, temperature-controlled recovery chamber for at least 30 mins. Surviving rats were housed in single cages and were checked daily for any signs of deterioration or distress. Animals were initially housed singly for 1 week to reduce suture breakdown complications, before being returned to group housing. After 16-20 weeks animals had progressed into a state of heart failure as confirmed by echocardiographic analysis of ventricular function.

Echocardiography

Echocardiography involves the use of high frequency 'ultrasonic' waves and the differences in sound penetration between tissues of variable densities to produce a real-time 2D image of the contracting heart. An ultrasound transducer converts electrical energy into sound waves at frequencies over 20,000 cycles per second that propagate through the body at a transmission velocity of ~1540 m/s. When a wave hits an obstruction it is reflected or scattered. The reflected sound is detected and the time required for it to return to the transducer is measured. This is used to calculate the distance the

obstruction is away from the origin of the sound wave. The intensity of reflected sound waves is a measure of the relative amount of energy propagating or reflecting through an area of acoustic resistance (or impedance). Acoustic impedance is the product of sound velocity and physical density. At the boundary between different tissue densities there is an acoustic mismatch which results in some sound waves being reflected. The energy of the reflected components directly relates to the acoustic impedance. The greater the density mismatch, the more sound waves are reflected back and thus a brighter image is seen. The transducer detects these waves or 'echoes' and the scan converter on the ultrasonography forms an image.

Male adult Sprague Dawley rats were anaesthetised in an induction chamber with 5% Isoflurane. Following the loss of the righting reflex, the fur was shaved from the pectoral region and the animals were placed in a supine position on a heated Vevo 770 stage. Anaesthesia was maintained by placing the rat's nose and muzzle into a Bain coaxial circuit face mask through which a mixture of 1.5% Isoflurane was passed at 1.5L/min. Pedal and palpebral reflexes were tested at regular intervals to ensure that the animals were sufficiently sedated. At this point hair removal gel (Nair) was applied to the animal's chest to ensure the removal of all hairs, this was removed and the area promptly rinsed after no more than 5 mins. Warm ultrasound scanning gel which has the same conductivity as the human body and couples the transducer to the skin was spread over the bare shaven chest of the animal.

The position and angling of both the Vevo 770 stage and the probe was altered to obtain a clear B-mode image of the heart. For optimum penetration, the probe was positioned perpendicularly to the structure of the heart. Acoustic impedance is very high between air and bone and as insufficient energy can cross the interface no information can be collected from the organs underneath. This is a limitation for using ultrasound to monitor cardiac function as the position of the ribs can reduce the ultrasound penetration to the heart. When a stable mid left ventricle B mode image was obtained and recorded. The Vevo 770 was switched to the acquisition of M-mode images; the motion of tissue detected by one line of the B-mode image over time. This yielded an image whereby the LV chamber dimensions at both end-systole and end-diastole during the cardiac cycle could be measured. The Vevo 770 was then switched back to B-mode and the position of imaging was altered to focus on the pulmonary artery. When in a suitable position a Pulse-Wave Doppler measurement of the pulmonary artery was made.

AAV9 jugular vein injections

For cardiac gene therapy trials the adenovirus and the AAV are the most common choices (Vinge et al., 2008). AAVs can provide long-term transgene expression and low immunogenicity, making them the most suitable viral vector for clinical applications (Williams and Koch, 2004). To ensure that there are no side effects relating to the inherent viral characteristics, the viral genes essential for replication and encapsidation are removed and replaced by the gene of interest. This means that whilst the gene of interest induced in the AAV is expressed in the transfected cells the genes required for new viral particles are not. To target the heart with a therapeutic viral vector is technically challenging as the majority of the systemically administered vector dose is cleared by the liver. This results in a significant dilution in the final concentration of virus that reaches the heart. An alternative to this is to deliver the virus in a more direct fashion; intra-myocardial, intra-vascular via the coronaries and intra-pericardial. These routes have produced more promising results in terms of transfection efficiency. AAVs have also been engineered so that they selectively target cardiomyocytes, this is known as tropism and the serotype AAV9 has been designed with the greatest cardiac tropism (Bish et al., 2008). In addition to this, inclusion of tissue specific promoters to drive transgene expression has also improved tissue selectivity of viral vectors. The use of AAV in delivering therapeutic cardiac effects has been recorded in both human clinical trials (Jaski et al., 2009) and *in vivo* model of heart disease (Karakikes et al., 2013).

The AAV9.LacZ and AAV.miR-1 viruses used in this research were kindly manufactured by Prof. Giacca's team at the AAV Vector Unit of the International Centre for Genetic Engineering and Biotechnology Trieste, as described previously (Arsic et al., 2004). For the AAV9.miR-1 vector, the precursor sequence of the human miR-1 was cloned into the multi-cloning site of an engineered the pAAV-MCS (Agilent Technologies) plasmid. AAV particles were generated in HEK293T cultures by cross packaging of the transgene-containing genome into serotype 9 AAV capsids. The produced AAV titers in dialysed fractions were determined by copy number measurement via PCR; stocks typically ranging between 1×10^{12} and 1×10^{13} vp/ ml of solution.

For *in vivo* delivery of the AAV9 vectors, jugular vein injections were chosen due to the close proximity of the vein to the heart and the technical expertise already existing in the group. Two types of AAV9 were used; AAV9.LacZ and AAV9.miR-1, the first acting as a control against the mirR-1 treatment. The AAV9 were stored as stock solutions at -80°C at concentrations of 1.8×10^{13} viral genomes/ml and 3.3×10^{13} vp/ ml respectively. Animals were randomly divided into two groups and

received either AAV9.LacZ or AAV9.miR-1. Both stock solutions were diluted in RNase free H₂O so that animals were administered with a final AAV9 dose of 1.45×10^{12} viral genomes in 250 μ l of solution. Delivery of such a small volume required a precision glass syringe (Hamilton). The tip of a 30 G needle was bent in a 90° angle for ease of injection. Male Sprague Dawley rats that had undergone MI surgery 16-20 weeks previously (heart failure) were anaesthetised in an induction chamber with 5% Isoflurane. Anaesthesia was maintained by placing the rat's nose and muzzle into a Bain coaxial circuit facemask with 2% Isoflurane. The area around the left clavicle was shaved and was sterilised with 1% Chlorhexidine (Hibiscrub™) and a sterile field was established using a drape. Surgery was performed with the rat secured in the supin position using micropore tape (3M) on an electrical heat-pad. Pre-operative medication administered was as follows: 0.015-0.03 mg buprenorphine (0.05-0.1 mg/kg), 1.25-1.5 mg enrofloxacin (5 mg/kg), and 2.5 ml 0.9% saline SC.

A 1cm incision was made in a 45° angle on top of the left clavicle. The fat and fascia surrounding the jugular vein was carefully removed by blunt dissection. Excessive tampering of the jugular vein can cause it to contract, making it impossible to inject, or worse to open and the animal would suffer significant bleeding. When the jugular was sufficiently exposed, tension was applied and the injection made, with the AAV9 solution applied through the syringe over the course of a minute. Afterwards the solution was allowed to clear, the needle removed and gentle pressure applied to stop any bleeding. The skin was then closed using a 5-0 Ethibond discontinuous suture (Johnson and Johnson Medical Ltd). Animals were kept single-caged after recovery from this date onwards. A period of 9 weeks was permitted before sacrifice to allow maximum time for the therapeutic intervention to take effect.

Statistical analysis

The statistical software Graph Pad Prism was used for all statistical analysis. The number of replicates for each group is indicated by 'n', in some of the single cell experiments this represents the number of cells (non-hierarchical grouping) whereas in others it is the number of animals (hierarchical grouping), this distinction is made in the text. Where the P value of the statistical test was significant, this is indicated on the figures by asterisks; *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. P<0.05 was considered significant.

For a comparison of the individual means from two groups where the data did not have a normal distribution, the non-parametric Mann-Whitney test was used to determine statistical significance.

As many of the experiments had data sets with low n numbers this was the most common test used. In the instances where the data sets from an experiment passed the D'Agostino & Pearson omnibus normality test a t-test was used. For the data that was fitted to a curve by non-linear regression analysis, a F-test was used to determine if the non-linear model used to fit to one data set was different to another. The F-test gave a P value for the likelihood that the two models were different by chance. Where more than two unmatched group were present a one-way ANOVA was used with a with Tukeys post-hoc test which assumes that the data has a normal distribution or a Kruskal Wallace post-hoc test which assumes the distribution is not normal.

Chapter 3: Can circulating microRNAs act as reliable prognostic biomarkers of heart failure?

Introduction

Biomarkers of heart failure

Biomarker is a broad term and encompasses a variety of biological entities, including hormones, enzymes, electrolytes and genetic sequences. Such is the multifaceted nature of heart failure that some of the biomarkers already identified are circulating in the blood whilst others are found in different types of bodily tissue. Regardless of their physiological origin or make up, there are a number of hallmarks that a biomarker should ideally possess to be clinically beneficial and practical for large scale use:

- The process of validating a biomarker must be comprehensive, with testing conducted in a large patient population that accurately represents the heterogeneity of the disease.
- Measurement of a biomarker should be technically straightforward, both in terms of obtaining the biological material for testing and the assay which is used to determine the biomarker's concentration. Heart failure research has focused on circulating biomarkers due to the ease in which blood can be serially taken from patients.
- The test used to assess a biomarker should be robust, with well-defined variation and minimal analytical imprecision.
- A biomarker should reflect a physiological process that has been affected by the disease, ideally both the presence and development of the disease. If a biomarker is not directly linked to disease pathophysiology there is a risk a change in the biomarker concentration could be attributed to another cause, because of this reason heart failure biomarker research has typically targeted biomarkers that originate from the myocardium.
- The information acquired after quantifying a biomarker must be clinically relevant and beneficial to the healthcare professional as well as providing additional information beyond what is already known. This enables a more accurate, reliable and faster diagnosis and potentially a more successful treatment strategy.

To date a range of different circulating biomarkers of heart failure have been discovered, but none meet all of the criteria listed above to a high enough standard (Braunwald, 2008). Of the options currently available brain natriuretic peptide (BNP) and the N-terminal pro-brain natriuretic peptide (NT-pro-BNP) are regularly used as clinical diagnostic tools (Felker et al., 2009). The precursor of both peptides, a pre-pro-hormone BNP is synthesised intracellularly by cardiomyocytes then cleaved

into BNP and NT-pro-BNP, and the release of the precursor is triggered by hemodynamic stress; ventricular dilatation, hypertrophy and elevated wall tension (Daniels and Maisel, 2007). The first study to show that pro-BNP-NT was able to predict mortality in patients with severe heart failure also found that treatment with the β -adrenoceptor antagonist carvedilol was associated with a significant decrease in NT-pro-BNP concentration which suggests that NT-pro-BNP correlated with disease severity (Hartmann et al., 2004).

BNP is also a valuable metric for reaching a more accurate diagnosis of patients presenting with dyspnea; in one study a BNP threshold of 100pg/ml was 90% sensitive (correctly identified positive results) and 76% specific (correctly identified negative results) for diagnosing heart failure as the cause of dyspnea (McCullough, 2002). Incorporating serial natriuretic peptide measurements in order to titrate heart failure patient therapy has resulted in a significantly reduced rate of mortality across a number of studies (Felker et al., 2009). BNP also has been found to have prognostic capacity as a biomarker for predicting disease outcome; a meta-analysis showed that compared to other biomarkers, BNP can predict the outcome of both acute and chronic heart failure patients; where a 100pg/ml increase in BNP concentration was an associated 35% increase in the risk of death (Doust et al., 2005). There is room for improvement in the prognostic capacity of BNP and NT-pro-BNP; of the 3916 chronic heart failure patients enrolled in the Valsartan heart failure trial the sensitivity and specificity measurements of 0.590 to 0.696 were recorded respectively (Masson et al., 2006). The circulating concentration of natriuretic peptides can also be elevated in other medical conditions (table 3A) besides heart failure which could potentially lead to a misdiagnosis. Heart failure is more common in patients with an advanced age, but natriuretic peptides can be elevated in older individuals despite them not suffering from heart failure. Therefore, although BNP and NT-pro-BNP are the best predictive indicators of heart failure available their accuracy could be increased and there is a requirement for biomarkers of heart failure with improved diagnostic and prognostic capabilities.

Causes of elevated Natriuretic Peptide levels other than chronic heart failure	
Left ventricular dysfunction	Advanced age
Atrial fibrillation	Renal dysfunction
Pulmonary disease (acute respiratory distress syndrome and lung disease with right heart failure)	High output states (sepsis, cirrhosis and hyperthyroidism)
Pulmonary embolism	Acute coronary syndromes

Table 3A: Examples of physiological conditions where natriuretic peptide levels are higher compared to healthy patients but the cause is unrelated to chronic heart failure (Daniels and Maisel, 2007).

The Carvedilol Prospective Randomized Cumulative Survival (Copernicus) trial

The Copernicus trial was a multi-centre clinical trial looking at the effect of the β -adrenoceptor antagonist carvedilol on moderate to severe heart failure patients. The trial found that carvedilol reduced the combined risk of death or hospitalisation for heart failure by 31% which resulted in the trial ending prematurely so the treatment could be put in practice (Packer, 2002). 2289 patients in total were enrolled and were studied for an average period of 10.4 months, with some patients studied as long as 14 months. The clinical phenotype of the patients was highly characterised; for example if a patient was hospitalised or died during the trial, the time from that patient beginning the trial to the event was recorded. The cause of hospitalisation was recorded along with any treatment and the duration of the stay. At set time points throughout the trial blood samples were taken with the serum isolated and screened for a standard panel of factors with patient physiological and cardiovascular measurements also recorded.

Of the 2289 total patients enrolled in the Copernicus trial, 3088 serum samples from 1277 patients were stored at -80°C at the Royal Brompton Hospital. The large cohort of serial serum samples coupled with a highly characterised patient population offered a unique opportunity to search for a novel biomarker of heart failure that stratified patients based on their prognosis. Many studies only have access to a small patient population which makes identifying biomarkers that are consistently different between good and poor prognosis disease groups difficult. This issue is further amplified by the heterogeneity of heart failure pathophysiology and impact this may have on a circulating biomarker. Furthermore, any new biomarker would have to be compared against NT-pro-BNP and show that it can be a more consistent and more accurate diagnostic tool.

Circulating microRNAs are an emerging field of biomarker research in cardiovascular disease. Although some interesting results have been presented in the literature, their capacity as biomarkers of heart failure is inconclusive as contradictory results have been found by different groups. This is discussed in more detail in the Introduction section: Circulating microRNAs in heart failure. The prognostic value of circulating microRNAs as a biomarkers for heart failure is currently unknown and represents a novel area of research to explore. This formed the basis of the scientific rationale and combined with the availability of the large number of serum samples from the Copernicus trial created a viable opportunity for a study to be conducted. The typical approach to identifying a circulating microRNA biomarker is to compare two or more groups, e.g. healthy versus disease and find one or more microRNAs that is consistently different in the two groups. In the experiments presented in this chapter, the circulating microRNA in serum from heart failure patients with a good disease prognosis was compared to those with a poor disease prognosis.

Because there was a large population of patients and serum samples available from the Copernicus trial, a small pilot group of patients was selected and their baseline serum sample tested. The patients were part of the placebo group only so as to avoid any positive therapeutic effect of carvedilol on circulating microRNA expression. The first step was to screen the pilot patient baseline serum samples with a microRNA microarray. There are a selection of commercially available microRNA microarrays which enable the expression of around 1000 microRNAs to be determined. Two were used in this study, a SYBR Green and a TaqMan RT-qPCR based microRNA microarray. For microarrays to measure such a large number of microRNAs there is a sacrifice in their quantitative accuracy and in reality they only provide qualitative output on microRNA expression. This information was used to filter down to a shortlist of microRNAs candidates for further testing. These were the microRNAs that had the most consistent and greatest difference in expression between two prognosis groups. The expression of the microRNA candidates was validated by TaqMan RT-qPCR; the current gold standard in terms of quantifying gene expression. If successful this process would aim to yield 1-4 microRNAs whose expression translates from a microarray through validation. These would be taken forward and tested in the all of the available Copernicus trial patients to confirm if the expression would correlate with disease prognosis.

The experiments conducted in this study were performed at the Hannover Medical School in the laboratory of Professor Thomas Thum. This was because of capacity of the laboratory to handle such a large microRNA expression study and the microRNA research experience and expertise of Professor Thum.

Aim

- Identify one or more circulating microRNAs that can distinguish heart failure patients that were enrolled in the Copernicus trial based on their clinical prognosis. A novel, predictive circulating biomarker that could match or better the selectivity and specificity than the current gold standard would be a powerful clinical tool.

Methods

Quantifying microRNA expression in Copernicus patient serum samples

RNA Isolation

Because the samples to examine were serum rather than cells, a different protocol was required instead of the standard method. It was also essential to isolate total RNA, so that it included microRNA. A 96 well format was used rather than the standard single column method to make the experiment for time efficient. A full method of RNA isolation from serum is described in the Methods section: RNA isolation from serum.

RT-qPCR Technologies

Two forms of RT-qPCR based microRNA microarrays were conducted in this study; a SYBR Green microarray and a TaqMan microarray. Both produce quantitative data on expression but have distinct technological mechanisms for quantifying expression. SYBR Green uses a dye that emits fluorescent light when bound to any double stranded DNA and represents the signal that is detected by a RT-qPCR machine. The more double stranded DNA present, the greater the fluorescent signal and this equates to a higher expression of the sequence of interest. The advantage of this technique is that a specific primer is not required meaning cheaper setup and running costs, but this feature of SYBR Green is also linked to its primary disadvantage; the dye binds to any double stranded DNA so it is liable to false positives by binding to non-specific double stranded DNA sequences. Multiple molecules of the fluorescent dye may bind to a single sequence, again creating a result where expression of a gene of interest is higher than the reality.

SYBR® GREEN I DYE ASSAY CHEMISTRY

1. Reaction setup: The SYBR® Green I Dye fluoresces when bound to double-stranded DNA.



Figure 3A: Graphical visualisation of SYBR Green fluorescent dye molecules bound to a double stranded gene.

<http://www.lifetechnologies.com/uk/en/home/life-science/pcr/real-time-pcr/qpcr-education/taqman-assays-vs-sybr-green-dye-for-qpcr.html>

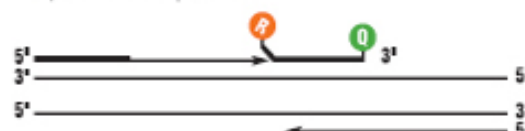
TaqMan is a newer technology and is based on fluorogenic labelled probes that use the 5' nuclease activity of Taq DNA polymerase. TaqMan oligonucleotide probes have a fluorescent reporter dye on the 5' end and a quencher dye on the 3' end and the quencher blocks the fluorescent signal from the reporter. A TaqMan primer binds to the 3' end of the DNA sequence of the gene of interest whilst the TaqMan probe is attached to the primer and anneals downstream of the primer. As the primer is extended the quencher of the TaqMan probe is cleaved by the 5' nuclease activity of Taq DNA polymerase and this allows the fluorescent signal of the reporter to be unquenched. As polymerisation continues all of the probe is removed and the target gene is replicated. Cleavage of the probe and subsequent Taq DNA polymerase activity only occurs if the primer is annealed to the gene of interest, this means that detection of a fluorescence signal can only be attributed to the presence of the specific DNA and not non-specific binding of the primer (false positive). This results in TaqMan providing greater specificity and selectivity compared to SYBR Green (Ginzinger, 2002). The drawback of the technique is that a specific primer must be generated for each gene to be tested, which increases cost and reduces the throughput of experiments.

TAQMAN® PROBE-BASED ASSAY CHEMISTRY

1. **Polymerization:** A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan® probe, respectively.



2. **Strand displacement:** When the probe is intact, the reporter dye emission is quenched.



3. **Cleavage:** During each extension cycle, the DNA polymerase cleaves the reporter dye from the probe.



4. **Polymerization completed:** Once separated from the quencher, the reporter dye emits its characteristic fluorescence.



Figure 3B: The stages of a TaqMan primer binding to a double stranded gene of interest and the fluorescent probe being cleaved by the 5' nuclease activity of Taq DNA polymerase. This results in a fluorescent signal being detected, as the quencher is no longer blocking the signal from the reporter.

<http://www.lifetechnologies.com/uk/en/home/life-science/pcr/real-time-pcr/qpcr-education/taqman-assays-vs-sybr-green-dye-for-qpcr.html>

Patient Selection

To determine the circulating microRNA expression of all 2289 available patients at the first step was not possible due to financial and time constraints. Therefore the baseline serum samples of a pilot cohort of 80 patients from the placebo group only were selected for screening by microRNA microarray. This encompassed 40 who were deemed to have had a good disease prognosis and 40 with a poor disease prognosis. Of the patient parameters that were characterised in the Copernicus study it was the time to event that was used to stratify patients based on their prognosis.

Throughout the course of the Copernicus trial patients were censored due to death or hospitalisation (or ultimately due to the trial ending). The numerical value for time to event corresponds to the number of days for which a patient was observed before an event. Therefore the greater the value for time to event, the longer the patient survived during the trial and the better their prognosis. Patients that died during the trial were allocated to the poor prognosis group, whilst the 40 with the greatest value for time to event were selected for the good prognosis group with attempts made to equalise the distribution of patient sex as much as possible in each group. All patients selected for the poor prognosis group were enrolled at least 6 months before the trial ended to ensure their low time to event value was not due to the unscheduled ending of the trial due to the positive results on carvedilol on patient outcome.

SYBR Green microRNA microarray

The SYBR Green microRNA microarray enabled a high throughput screen of the expression of 1, 113 microRNAs, but sacrifices quantitative accuracy in order to achieve a quantification of such a large catalogue of microRNAs. The baseline serum samples from the 80 pilot cohort patients were split into good and poor disease prognosis and the RNA isolated from these serum samples. The RNA from the 40 samples selected for each prognosis group were then split to form 5 pools per prognosis (8 patients per pool) this is represented by figure 3C. The main reason behind pooling samples was to reduce costs; rather than conducting 80 microRNA microarrays the pooling reduced the number to 10, pooling also reduced the volume of serum required for a microarray. Conversely there are also a number of disadvantages of pooling samples; there is a loss of information on the variation between patients, any subtle changes in differences between patients can be lost as only the major differences can be detected, any results from pooled samples must be validated to ensure that the differences recorded are real and an in depth understanding of the patients clinical history is required to ensure that the samples originate from as close to a homogenous source as possible.

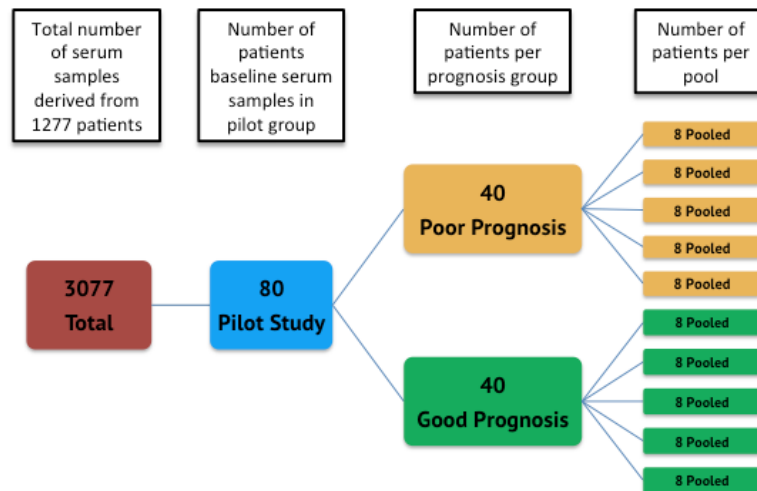


Figure 3C: The selection method pathway for reducing the large number of patients in the Copernicus trial to a pilot group for initial investigation of circulating microRNA expression by SYBR Green RT-qPCR microRNA microarray.

The total number of 3077 serum samples derived from 1277 patients was reduced to a pilot group of 80 samples. This was made up of baseline serum sample taken from 40 patients with a good prognosis and 40 with a poor prognosis. The RNA isolated from these samples was randomly divided into 5 groups per prognosis and pooled, resulting in 8 patient's baseline samples per pool used for the SYBR Green microRNA microarray.

From the 1, 113 microRNAs screened in the pools of RNA, a short list was selected for further validation, the following criteria was used to sort and select candidates:

- Amplification of microRNA expression by RT-qPCR (raw Ct value) was detected in all 5 pools of a prognosis group.
- The mean raw Ct value of all 5 pools was ≤ 40 (the microRNA microarray kit manufacturer recommended ≤ 35 for cellular samples, but as circulating microRNA expression is known to lower than cellular the threshold was adjusted).
- Each RT-qPCR amplification plot was visually checked to ensure the apparent difference in Ct value between prognosis groups was 'real' and not an anomaly due to the position of the Ct threshold value on the amplification curve.

The expression of the microRNAs that passed these criteria were then sorted by statistical analysis:

- The raw Ct values and delta Ct values were statistically compared by unpaired non-parametric T-test (Mann Whitney) in order to assign a P value of statistical significance

which was used to rank the microRNAs so that a shortlist of microRNAs could be taken further for validation.

TaqMan microRNA microarray

The patients used for TaqMan microRNA microarray were different to those used for the SYBR green microRNA microarray. Due to a higher cost per sample only 1 pool per prognosis comprised of RNA from 5 patients were tested rather than 5 pools per prognosis. From the 40 good prognosis patients the 5 with the highest time to event value were selected; the 'best' good prognosis patients. The 5 patients with the lowest time to event values from the 40 poor prognosis patients were selected; the 'worst' poor prognosis patients. The purpose was to select the patients at the extremes of each group and there increase the likelihood of detecting larger and more reliable differences in microRNA expression that was linked to disease prognosis. This process is represented by figure 3D:

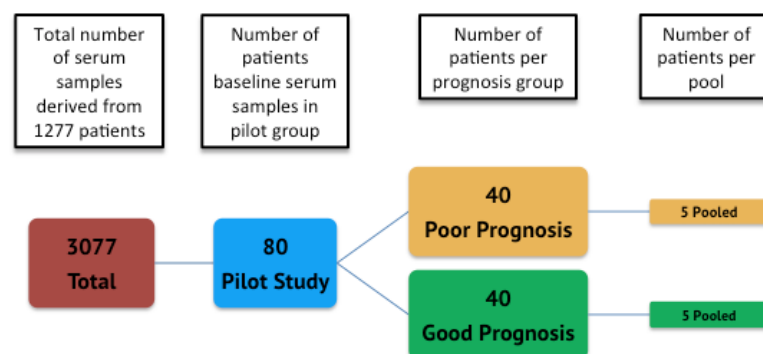


Figure 3D: The selection method for reducing the large number of patients in the Copernicus trial to a pilot group for initial investigation of circulating microRNA expression by TaqMan RT-qPCR microRNA microarray.

The total number of 3077 serum samples derived from 1277 patients was reduced to a pilot group of 80 samples. This was made up of baseline serum sample taken from 40 patients with a good prognosis and 40 with a poor prognosis. The RNA isolated from 5 'best' good prognosis and the 5 'worse' prognosis were selected and pooled to form the two groups used in the TaqMan microRNA microarray.

The criteria for validation was the same as the SYBR Green microRNA microarray except that only mean Ct values of microRNAs that were ≤ 35 rather than ≤ 40 were considered for validation. The TaqMan microRNA microarray protocol included a pre-amplification step which significantly

enhanced the capacity to detect low expressed microRNAs without introducing amplification bias to the sample. Typically the expression of genes of interest are normalised to a housekeeping gene whose expression remains stable but for both SYBR Green and TaqMan microRNA microarrays the expression of the microRNAs tested was normalised to spike in *C.elegans*. Global normalisation is an alternate method to spike in *C.elegans*; where the Ct value of all the wells in a plate is used as the normalisation value. Both methods of analysis were calculated and but only the spike in *C.elegans* normalisation is shown in the result as the global normalisation showed no difference compared to the spike in *C.elegans* method.

TaqMan RT-qPCR

The expression of the microRNAs shortlisted from the two microRNA microarrays were then tested again by TaqMan RT-qPCR to validate the observations of the microarrays. This was done in all 80 baseline serum samples from the patients selected for the pilot group, rather than pooled RNA that was used in the microarrays. The protocol followed is detailed in Methods section: TaqMan real time quantitative polymerase chain reaction (RT-qPCR).

Results

SYBR Green microRNA microarray

The RT-qPCR experiments of the 1, 113 microRNAs tested for the two prognosis groups in the SYBR Green microarray was split over three 384 wells plates. In addition to the microRNAs, the expression of spike in *C.elegans* expression was also tested. The Geometric mean of the expression of the spike in *C.elegans* for each RT-qPCR plate (plate no. 1-3) across the 5 pools in a prognosis group was calculated (table 3B) and was used for the normalisation of the expression of the microRNAs tested on that particular RT-qPCR plate.

Plate No.	Good Prognosis		Poor Prognosis		ΔCt	Fold Change
	Geometric Mean	$\pm SEM$	Geometric Mean	$\pm SEM$		
1	24.54	0.02	24.97	0.02	0.74	1.00
2	24.62	0.09	25.24	0.04	0.65	1.00
3	24.87	0.05	25.38	0.07	0.70	1.00

Table 3B: Spike in C.elegans expression included as part of the SYBR Green microRNA microarray in the two prognosis pools used in the pilot study.

The expression of spike in C.elegans was measured in the RNA from both prognosis groups in triplicate on each of the three RT-qPCR plates. The Geometric mean from each triplicate is shown and the variation ($\pm SEM$). The difference between prognosis groups (ΔCt) and fold change was also calculated to give an indication of the stability of C.elegans between prognosis groups.

There was minimal variability ($\pm SEM$) in the spike in *C.elegans* expression for each RT-qPCR plate of each disease prognosis group and between the two disease prognosis groups (fold change). This suggested a stable RNA isolation, cDNA amplification and RT-qPCR process and a reliable method for normalisation. From the 1,113 microRNAs screened in the SYBR Green microRNA microarray there were 48 downregulated and 15 upregulated candidates in the good versus the poor prognosis cohorts that met the inclusion criteria for validation. After sorting by statistical power, 7 downregulated and 3 upregulated microRNA candidates were selected for further validation by TaqMan RT-qPCR. MicroRNA-30e was upregulated in good versus poor prognosis and also included despite expression amplification in the SYBR Green microRNA microarray only detected in 9 of the 10 pools. MicroRNA-30e has previously shown changes in expression and to affect important cardiac

gene targets in studies using an *in vivo* heart failure model of Professor Harding's group (Roca-Alonso et al., 2015).

Table 3C shows the SYBR Green microRNA microarray results of the selected 7 downregulated and 4 upregulated candidates. The mean Ct values across the five pools of each prognosis cohort and variability across the five pools ($\pm$ SEM) are shown. The Δ Ct expresses the difference between the mean raw Ct values (good – poor prognosis) and was used to calculate the fold difference between the prognosis groups ($2^{\text{POWER} - (\Delta\text{Ct} \times \text{fold difference of Spike In C.elegans between good and poor progression groups for the RT-qPCR plate})}$). This equation included the fold difference between Spike in C.elegans, the purpose was to account for variation in C.elegans expression across the 3 RT-qPCR plates for each two prognosis group. The fold difference of C.elegans between good and poor prognosis is 1.00 (table 3B) so this effectively means this equation becomes ($2^{\text{POWER} - (\Delta\text{Ct})}$). A Mann Whitney test was used to statistically compare the raw Ct values and Δ Ct values of each microRNA between good and poor prognosis groups.

A	Good vs Poor Prognosis	MicroRNA	Good Prognosis		Poor Prognosis		Δ Ct	Fold change	Unpaired Non-parametric T-Test P Values:	
			Mean Raw Ct value	$\pm$ SEM	Mean Raw Ct value	$\pm$ SEM			Raw Ct values	Δ Ct Values
Upregulated Expression		191	33.5	0.9	38.0	1.9	-4.48	16.57	0.061	0.05
		490-5p	33.4	0.8	36.9	1.4	-3.45	8.08	0.066	0.14
		124	34.0	0.2	38.7	2.3	-4.69	19.07	0.074	0.10
		30e	33.0	0.4	35.0	0.6	-2.08	3.14	0.016	0.01

B	Good vs Poor Prognosis	MicroRNA	Good Prognosis		Poor Prognosis		Δ Ct	Fold change	Unpaired Non-parametric T-Test P Values:	
			Mean Raw Ct value	$\pm$ SEM	Mean Raw Ct value	$\pm$ SEM			Raw Ct values	Δ Ct Values
Downregulated Expression		218-2*	39.5	1.0	35.3	0.5	4.14	0.04	0.005	0.008
		3122	34.7	0.3	33.7	0.2	1.08	0.31	0.010	0.05
		1237	37.0	0.7	34.4	0.4	2.52	0.11	0.012	0.001
		4269	34.9	0.7	32.4	0.5	2.52	0.12	0.016	0.002
		138-1*	35.3	0.4	33.4	0.6	1.98	0.18	0.027	0.02
		378*	37.4	0.9	35.0	0.6	2.48	0.13	0.045	0.07
		654-5p	39.4	1.4	35.7	0.9	3.74	0.05	0.056	0.02

Table 3C: Expression of the selected microRNA candidates identified in the SYBR Green microRNA microarray that showed different expression between the good versus poor prognostic patient cohorts.

For each microRNA mean $\pm$ SEM Ct values are shown for both good and poor disease prognosis, by comparing these two groups the Δ Ct and fold difference were calculated. Both raw Ct and Δ Ct values were statistically compared by a Mann Whitney test to sort microRNA candidates. MicroRNA candidates were selected from the SYBR Green microRNA microarray for further validation that showed:

A) Upregulated expression in good versus poor disease progression cohorts

B) Downregulated expression in good versus poor disease progression cohorts

For the majority of the microRNAs tested in the microarray the raw Ct values detected were >30. This was expected as the concentration of circulating microRNA in serum is very low. The raw Ct values and the Δ Ct values of the prognosis pools were statistically compared to take into account this variability and to ensure the candidates with the most robust difference between prognosis groups were selected. The downregulated microRNA candidates exhibited the more robust differences between prognosis groups and this is reflected in the P values. MicroRNA-4269 and microRNA-1237 have the same Δ Ct values but different fold change values, this is because the RT-qPCR experiments for each microRNA were run on different plates. As shown in table 3B the geometric mean for C.elegans is slightly different across the three plates and because this was used to produce the fold change calculation it explains why the fold change for microRNA-4269 and microRNA-1237 are different.

Validation of SYBR green microRNA microarray results

To validate the observations recorded in the SYBR Green microRNA microarray, TaqMan RT-qPCR was conducted to determine the expression of the 11 microRNAs in all 80 patient baseline samples used in pilot cohort. Spike in C.elegans was again used as a normalisation control. Table 3D shows A) the upregulated B) the downregulated microRNA candidates in good versus poor disease prognosis groups. The expression of the microRNA candidates was normalised to the spike in C.elegans Ct value for each sample which generated individual Δ Ct values of the 11 microRNA candidates in each patient. The mean Δ Ct for each microRNA across a prognosis group was calculated and the fold change determined by using the following method ($2^{\text{POWER} - \Delta\text{Ct}}$). An unpaired non-parametric T-test (Mann Whitney) was used for statistical comparison of the Δ Ct values from each prognosis group. The fold change and P values results for each microRNA from the TaqMan validation experiments are shown alongside that of the microRNA microarray experiments for comparison.

A	Good vs Poor Prognosis	microRNA	Mean Δ Ct		Fold change		Unpaired Non-parametric T-Test P Values:	
			Good Prognosis	Poor Prognosis	Validation	Array	Validation	Microarray
Upregulated Expression		191	9.9	9.8	0.92	16.57	0.67	0.05
		490-5p	17.9	18.1	1.15	8.08	0.25	0.14
		124	17.4	17.5	1.10	19.07	0.92	0.10
		30e	10.3	10.1	0.91	3.14	0.59	0.01

B	Good vs Poor Prognosis	microRNA	Mean Δ Ct		Fold change		Unpaired Non-parametric T-Test P Values:	
			Good Prognosis	Poor Prognosis	Validation	Array	Validation	Microarray
Downregulated Expression		218-2*	Undetermined	Undetermined	-	0.04	-	0.008
		3122	18.7	18.4	0.82	0.31	0.36	0.05
		1237	10.4	10.3	0.93	0.11	0.68	0.001
		4269	18.4	18.3	0.92	0.12	0.81	0.002
		138-1*	18.0	17.8	0.88	0.18	0.54	0.02
		378*	11.5	11.7	1.15	0.13	0.04	0.07
		654-5p	16.7	16.8	1.03	0.05	0.28	0.02

Table 3D: Validated expression of the microRNA candidates that were selected from the SYBR Green microRNA microarray in good versus poor disease progression cohorts as tested by TaqMan RT-qPCR.

For each microRNA the mean Δ Ct values from the 40 patients in each prognosis group are shown after normalisation to individual spike in *C.elegans* expression. The fold change was calculated from these values and Mann Whitney test was used to statistically compare the raw Ct values for each microRNA and the Δ Ct values for the 11 microRNA candidates. The data shown is split into two tables based on the expression found in the SYBR Green microRNA microarray:

A) Upregulated expression in good versus poor disease progression cohorts

B) Downregulated expression in good versus poor disease progression cohorts

The SYBR Green microRNA microarray produced a range of candidates with significant differences in expression between the two prognosis groups, but these results did not translate to the validation experiments. Of the 11 microRNA candidates that were tested by TaqMan RT-qPCR in the 80 serum samples selected for the pilot study, none produced a fold change in expression that was close to the corresponding value observed in the microarray. The greatest fold change value of any candidate was microRNA-490-5p with 1.15, which is compared to a fold change of 8.08 for the same microRNA in the microRNA microarray. The statistical comparison of microRNA-378* ΔC_t values generated lower P values in the validation experiment compared to the microRNA microarray. This is likely due to be a statistical anomaly, the fold change after validation was 1.15 in good versus poor prognosis groups (the opposite direction of change observed in the microarray) and the mean raw C_t values for the good and poor prognosis cohorts are 31.9 ± 0.08 and 32.0 ± 0.06 respectively. Therefore the statistical significance is mostly likely attributed to the very low variation in the two datasets and not a physiological difference in the expression of microRNA-378*.

Grouped analysis

Other circulating microRNA biomarker studies in the literature use a process of grouping the RT-qPCR data of multiple microRNA candidates together (Meder et al., 2011) (Calin and Croce, 2006). This can have the effect of improving the statistical power when comparing patient groups or distinguishing healthy versus disease. Circulating microRNA expression is variable, so by grouping multiple microRNAs together it can also reduce how much this variability can impact on the reliability of a comparing two groups. The method used for comparing groups of microRNA candidates in this study generated a microRNA score value in order to compare multiple microRNAs simultaneously (Goren et al., 2012). Firstly the normalised value for a microRNA candidate (ΔC_t , normalised to *C.elegans*) was subtracted from a fixed numerical value (50). The lowest value in each prognosis group was then subtracted from the other values in the group so that the range of all the values start at 0 and this was the microRNA score value. The mean microRNA score value of all patients in a prognosis group was calculated and displayed in table 3E. The difference between the prognosis groups (Δ) was compared by unpaired non-parametric T-test (Mann Whitney).

Group	MicroRNA Candidate Grouping	Good Prognosis		Poor Prognosis		Δ	P Value
		Mean miR Score	$\pm$ SEM	Mean miR Score	$\pm$ SEM		
A	3122, 378*, 490-5p, 138-1*, 124	6.05	0.63	9.16	0.99	-3.11	0.018
B	3122, 378*, 490-5p, 138-1*	5.05	0.63	8.86	0.66	-3.81	0.0005
C	3122, 378*, 490-5p,	4.67	0.42	5.69	0.49	-1.03	0.12
D	3122, 378*	2.49	0.16	1.87	0.16	0.62	0.008
E	3122, 490-5p	4.89	0.43	5.31	0.46	-0.43	0.50

Table 3E: Grouped analysis of the microRNA candidates whose expression that were validated by TaqMan RT-qPCR.

5 permutations of microRNA groups were tested and a microRNA score system was used to group the expression of microRNA candidates together (Goren et al., 2012). The difference between prognosis groups (Δ) was statistically compared by Mann Whitney test.

The microRNA candidates that were validated by TaqMan RT-qPCR were combined into groups for analysis; firstly the data from the 5 candidates that had the highest fold change were combined in an attempt to distinguish the good and poor prognosis patient groups with more statistical reliability than the individual microRNA candidates. The candidate with the lowest fold change of the 5 was removed and the data from the remaining 4 were compared. This pattern was continued until only 2 candidates remained. The results in table 3E show that groups A ($P=0.018$), B ($P=0.0005$) and D ($P=0.008$) were able to distinguish between good and poor prognosis groups with statistical significance. The delta value for group D is smaller compared to that of A and B and statistical significance between good and poor prognosis could be attributed to the lower variation ($\pm$ SEM) of both groups.

TaqMan microRNA microarray

Due to the paucity of individual microRNA candidates identified in the SYBR Green microRNA microarray that were successfully validated (table 3D), a second microarray was conducted which used TaqMan rather than SYBR Green RT-qPCR technology. In theory a TaqMan based platform could potentially address some of the sensitivity issues associated with SYBR Green RT-qPCR technology. The TaqMan microRNA microarray was used to screen 667 microRNAs in the patient baseline serum samples, but using only one pool of RNA from 5 patients per disease prognosis group. Spike in *C.elegans* expression was determined in the same manner as the SYBR Green microRNA microarray to yield a Geometric mean value for each prognosis group. The TaqMan microRNA microarray produced 17 downregulated and 22 upregulated candidates in the good versus poor disease prognosis pools. After assessing the amplification plots for accuracy and the plausibility of the difference between plots, the number was reduced to 7 downregulated and 5 upregulated candidates. The results for the 12 microRNA targets are shown in table 3F; the raw Ct values together with difference between raw Ct values (Δ Ct) are shown for each prognosis group. The fold change for each microRNA target was calculated in the same manner as the SYBR Green microRNA microarray for consistency ($2^{\text{POWER} - (\Delta\text{Ct} \times \text{fold change of Spike In } C.elegans \text{ between good and poor progression groups for the RT-qPCR plate})}$).

A

Good vs Poor Prognosis	MicroRNA	Raw Ct Value		Δ Ct	Fold Change
		Good Prognosis	Poor Prognosis		
Upregulated Expression	1260	29.66	33.58	-3.92	15.07
	345	30.98	33.98	-3.00	7.99
	590-5p	32.81	35.44	-2.63	6.18
	378 (3p)	30.32	32.52	-2.20	4.57
	660	30.27	32.45	-2.17	4.50

B

Good vs Poor Prognosis	MicroRNA	Raw Ct Value		Δ Ct	Fold Change
		Good Prognosis	Poor Prognosis		
Downregulated Expression	495	36.52	31.50	5.02	0.031
	378* (5p)	35.48	32.02	3.45	0.091
	409-3p	35.34	32.33	3.01	0.124
	539	34.90	31.96	2.93	0.131
	193b	34.78	31.93	2.85	0.139
	27b	33.75	31.91	1.84	0.279
	483-5p	31.19	29.49	1.70	0.306

Table 3F: Expression of the selected microRNA candidates identified in the TaqMan microRNA microarray that were found to have different expression between the good versus poor prognostic patient cohorts.

For each microRNA the Δ Ct value between the prognosis groups is shown. The fold change was calculated from this value and the Δ Ct of *C.elegans*.

MicroRNA candidates were selected from the TaqMan microRNA microarray for further validation that showed:

A) Upregulated expression in good versus poor disease progression cohorts

B) Downregulated expression in good versus poor disease progression cohorts

The raw Ct values obtained from the TaqMan microRNA microarray were typically lower than those of the SYBR Green microRNA microarray, this is due to the pre-amplification step conducted as part of the protocol. No statistical analysis was conducted on the results because n=1 for each prognosis group. There was some cross-over in microRNA candidates that met the inclusion criteria of both microarrays; microRNA-378* and microRNA-483-5p were identified in the both microarrays as downregulated in good versus poor prognosis groups and attempts were previously made to validate microRNA-378* (table 3D).

Validation of TaqMan microarray results

Due to the unavailability of statistical analysis for sorting of the 12 candidates an alternative method was required to create a shortlist from the 12 microRNA candidates in table 3F. Three microRNA candidates were selected for further validation based on the existence of studies in the literature that had investigated the role of the microRNAs in the heart together with concurrent research being conducted in Professor Thum's laboratory. The three microRNA candidates selected for further validation in the baseline samples of all 80 patients in the pilot group were as follows:

MicroRNA expression downregulated in good versus poor prognosis cohorts in the TaqMan microRNA microarray:

microRNA-27b: the microRNA-23 / 27 / 24 cluster plays a key role in angiogenesis and endothelial apoptosis in cardiac ischaemia (Bang et al., 2012). Overexpression of microRNA-27b has been shown to induce cardiac hypertrophy (Wang et al., 2012).

microRNA-483-5p: identified as an intronic microRNA encoded within insulin-like growth factor 2 (IGF2). MicroRNA-483-5p has been shown to inhibit angiogenesis, an important process that is downregulated after cardiac ischaemia (Qiao et al., 2011).

MicroRNA expression upregulated in good versus poor prognosis cohorts in the TaqMan microRNA microarray:

microRNA-1260: a microRNA of interest at Professor Thum's laboratory.

MicroRNA-483-5p was identified in both microRNA microarrays with downregulated expression in good versus poor prognosis groups and this made it a stronger candidate for validation. Therefore it was hypothesised that the likelihood of the observed difference in expression being validated was greater because it was detected by both microarrays. TaqMan RT-qPCR was conducted for the three

microRNAs in the baseline samples of the 80 patient pilot cohort to validate the observations of the TaqMan microRNA microarray. Table 3G shows the expression of the three microRNA candidates in good versus poor disease prognosis groups. The expression of each microRNA was normalised to the individual spike in C.elegans Ct value of the patients' samples tested. This generated ΔCt values for each microRNA for each patient and the mean ΔCt for each microRNA across a prognosis group was then calculated. The fold change was calculated in the same manner as for table 3D. An unpaired non-parametric T-test (Manny Whitney) was used for statistical comparison of the ΔCt values from each prognosis group. The fold change for each microRNA from the TaqMan validation experiments are shown alongside that of the TaqMan microRNA microarray experiments for comparison.

Good vs Poor Prognosis	microRNA	Mean Δ Ct		Fold Change		P Value
		Good Prognosis	Poor Prognosis	Validation	Array	Δ Ct
Downregulated	27b	15.85	16.69	1.79	0.28	0.08
	483-5p	13.47	13.65	1.14	0.31	0.60
Upregulated	1260	11.11	11.36	1.18	15.07	0.43

Table 3G: Validated expression of the microRNA candidates that were selected from the TaqMan microRNA microarray in good versus poor disease progression cohorts as tested by TaqMan RT-qPCR.

For each microRNA the mean Δ Ct values from the 40 patients in each prognosis group are shown after normalisation to *C.elegans*. The fold change was calculated from these values and a Mann Whitney test was used to statistically compare the Δ Ct values for the three microRNA candidates.

Good vs Poor Prognosis	microRNA	Mean Δ Ct		Fold Change		P Value
		Good Prognosis	Poor Prognosis	Validation	Array	Δ Ct
Downregulated	27b	16.03	16.69	1.58	0.28	0.40
	483-5p	13.88	13.42	0.72	0.31	0.28
Upregulated	1260	11.54	11.16	0.77	15.07	0.01

Table 3H: Validated expression of the microRNA candidates in the serum from the 10 patients that were included in the TaqMan microRNA microarray only (5 patients for the good and poor prognosis patient cohorts respectively).

For each microRNA the mean Δ Ct values from the 5 patients in each prognosis group are shown after normalisation to *C.elegans*. The fold change was calculated from these values and a Mann Whitney test was used to statistically compare the Δ Ct values for the three microRNA candidates.

As with the 11 microRNA candidates validated after the SYBR Green microRNA microarray the differential in expression between prognosis groups of these three microRNA candidates could not be validated. The fold change for microRNA-27b was calculated to be 1.79, which is the greatest difference detected from all 14 microRNA candidates validated. The TaqMan microRNA microarray and validation results are contradictory; in the microarray the poor prognosis patients had greater microRNA-27b expression than the good prognosis patients (0.279 fold difference). In the validation experiment there was a greater microRNA-27b expression in the good prognosis patient cohort versus the poor prognosis cohort (1.79 fold difference). The raw Ct values from the validation experiment (not shown) were 34.8 ± 0.17 and 35.5 ± 0.18 for good and poor prognosis groups respectively. With such high Ct values the number of the microRNA sequences is extremely low, it is at limit of what is reliably detected by RT-qPCR making it a potentially unstable and a challenging recording to replicate. This means the result is not strong enough as a viable option for further study in a much larger population of patient serum samples.

As with the previous validation experiments both spike in *C.elegans* and global normalisation techniques were applied to the data and as before the differences in the datasets after each normalisation was minimal. The TaqMan microRNA microarray was conducted on one pool of 5 patients serum for each prognosis group, rather than using 5 pools per prognosis group as in the SYBR Green microRNA microarray. With a smaller sample size of patients any variation in the data will have a greater impact on the reliability of the result; this could explain the disparity between microarray and validation data. Table 3H shows the TaqMan validation results of the same three microRNA candidates, but only the expression of the 10 patients that were selected for the TaqMan microRNA microarray. This was an attempt to reduce the impact of the variability of pooling RNA. Interestingly, the difference in expression of microRNA-1260 was statistically significant when only the 5 patients from each prognosis groups are included for analysis, but the fold change in expression is in the opposite direction compared to when all 40 patients from each prognosis group were analysis. Overall, the results in 3H do not support any of findings in the microarray. It could be concluded that the P value for microRNA-27b in table 3G that is the closest to significance could be due to a larger sample size, rather than any real difference in the data between the two prognosis groups. This is the nature of sampling, where the reduced variability associated with greater sample sizes can allow smaller differences between two groups to emerge as significant.

Discussion

Microarray and validation results

The SYBR Green and TaqMan microRNA microarrays enabled the expression of 1, 113 and 667 microRNAs to be analysed respectively in the serum of the heart failure patients enrolled in the Copernicus trial. The principle of a microarray is to determine the expression of a large range of microRNAs that is then narrowed down to a shortlist to study in greater detail. This was successfully achieved by both microarray experiments; a number of microRNAs were found whose expression was upregulated or downregulated in good versus poor disease prognosis groups (tables 3C and 3F). Either statistical analysis or researching the literature was used to select which candidates to take forward for further investigation. After validation of the 11 and 3 microRNA candidates chosen from the SYBR Green and TaqMan microRNA microarrays, none showed any reproducible difference in expression between good and poor disease prognosis groups (tables 3D and 3G).

Other circulating microRNA studies in the literature use analytical techniques to normalise the expression of ≥ 1 microRNAs to a standard scale. The microRNAs are then grouped together in order to simultaneously compare the expression of multiple microRNAs between two patient groups. This technique has enabled the expression of several microRNAs grouped together to distinguish healthy versus disease patient groups where a single microRNA could not. This type of analysis was conducted using the RT-qPCR data of the microRNA candidates identified in the SYBR Green microRNA microarray that were validated (table 3E) (Goren et al., 2012). It showed that different combinations of microRNA were able to distinguish the good versus poor disease prognosis Copernicus patients in the pilot group. Although this is a viable methodology of analysis, the differential of the microRNA ΔCt between the two prognosis groups was small and within the natural range of fluctuation in expression of microRNA which could make the grouped analysis result a false positive. If the results of the validation experiment yielded larger differences between the ΔCt values, then this form of analysis would be applicable to the Copernicus results.

Both types of RT-qPCR microarrays to identify circulating microRNAs have been conducted with other clinical trial serum samples by Thomas Thum's group at Hannover Medical School. Typically ~25% of the microRNA candidates identified in microarrays show the same pattern of expression in a larger cohort of samples after validation by TaqMan RT-qPCR. Therefore, the question remains as to why the microRNA microarray results of this study did not translate in the validation experiments.

One possibility is that the null hypothesis is correct, circulating microRNAs are unable to act as a prognostic marker of heart failure disease prognosis in patients. Although this should not be ignored as an explanation, other studies particularly those comparing a healthy, age matched population compared to disease group have proven successful at distinguishing the two groups. This would suggest that circulating microRNA expression is linked to disease and can be used to identify patients (Ellis et al., 2013) (Widera et al., 2011) (Zampetaki et al., 2012). Besides the null hypothesis, there are other possible explanations for the results presented in this study, these will be discussed in the following sections.

Experimental or technical error

The serum samples used to derive RNA and study microRNA expression were from heart failure patients enrolled in the Copernicus clinical trial (Packer, 2002). The samples had been frozen for at least 10 years and the number of times they were thawed and refrozen during that time is unknown. During this time the microRNA sequences may have degraded. Although serum microRNA expression has been found to be stable after multiple freeze/ thaw cycles (Gilad et al., 2008). Whether this is the case or to what extent RNA degradation has occurred in the Copernicus samples is impossible to determine. The typical yield of microRNA from plasma or serum is 1-10ng (Pritchard et al., 2012), this is too low to measure the concentration and determine if the RNA has degraded. The isolation of RNA from the serum was successful, as illustrated by consistency in spike in *C.elegans* expression (table 3B) across pooled RNA samples. What factor RNA degradation plays in affecting microRNA expression stability is not clear in the literature. It has been shown that microRNA expression can be accurately quantified in human tissue samples where the RNA has degraded by heating, even samples where the level of mRNA can not be determined (Jung et al., 2010). This would suggest that the stability of RNA is not linked to that of microRNAs, and a sample can undergo RNA degradation but microRNA expression is unaffected.

Another study found that mouse liver and duodenum samples that were stored on ice for defined periods before RNA isolation resulted in RNA that was highly degraded with microRNA expression in the same samples found to be two to eight fold less compared to control samples (Ibberson et al., 2009). Conversely, collaborators at the Hannover Medical School have stored serum samples at room temperature for 24 hours and found microRNA expression to be unchanged. RNA degradation could have potentially contributed to variable microRNA expression found in this study and clearly degradation of RNA is a risk when handling biological samples. Without intimate knowledge of how

the serum samples were prepared, it is impossible to know whether the samples were handled in a manner to minimise contamination and prevent RNA degradation. Another factor that should be considered is that the laboratory in which the work was conducted focuses heavily on studying the expression of both cellular and circulating microRNA in a variety of human and animal samples. In such an environment, cross contamination of microRNAs from other samples and RT-qPCR plates is a theoretical possibility. All feasible attempts were made to reduce this risk but when dealing with such minute concentrations of RNA it is very difficult to retain 100% purity of a sample.

Regarding the length of time the Copernicus samples were stored before this study, variable results have been found when isolated RNA is stored at low temperatures before analysing gene expression. There is not a published study that has determined if microRNA expression is stable in frozen samples over a period of years comparable to the samples used in this study. Bravo et al recorded degradation in the expression of four microRNA sequences from different microRNA families after three days of storage at -80°C (Bravo et al., 2007). Other studies have found that microRNA expression is stable after 10 months of storage at the same temperature (Mraz et al., 2009). From these studies in the literature, it can be suggested that a simple explanation for the results obtained is that the microRNA sequences in the serum samples have degraded due to prolonged storage or contamination. Resulting in the loss of any real patterns in expression between the two prognosis groups.

The first microarray conducted used SYBR Green RT-qPCR technology, which is known to have a lower sensitivity for detecting expression compared to TaqMan (Ginzinger, 2002). The SYBR Green microarray has a larger coverage in terms of microRNAs analysed (1, 113 vs 667) and has a lower cost hence why it was conducted first. Various studies have found that the expression of circulating microRNAs is lower compared to cellular, which makes the expression more difficult to quantify by RT-qPCR (Kroh et al., 2010). Therefore, it is possible that the differences recorded in microRNA expression between good and poor disease prognosis groups observed in the SYBR Green microRNA microarray did not translate to validation experiments because the sensitivity of the microarray was not high enough. This shortcoming was addressed by the second microRNA microarray which used TaqMan RT-qPCR technology, however, the second microRNA microarray also produced results suggesting differences in the expression of microRNAs between the two disease prognosis groups that could not be validated. This would suggest that it might not be a lack of RT-qPCR sensitivity that was the reason why none of the expression of the microRNA candidates could be validated.

The method to select the 80 patients included for the pilot group from the 2289 that were available from the Copernicus trial was designed to reflect their prognosis over time. The time to event was a measure of the number of days that occurred before a patient was hospitalised, died or the end of the trial. Therefore the greater the value for a patient's time to event number, the longer they survived in the trial and the better their prognosis. The patients that exited the trial early were deemed to have a poor prognosis. This method of selecting patients based on their prognosis did not include any other parameters, which means that a patient may have exited the trial due to hospitalisation by a cause that was not fatal or related to a worsening of their condition. The patient's heart failure prognosis may have been good, which would mean that the poor prognosis group was made up of patients that did not all have a poor prognosis. This could have increased the variability of the circulating microRNA expression of the poor prognosis group.

The influence of patient heterogeneity and temporal variability on circulating microRNA levels

After the discovery of the existence of microRNAs (Lee et al., 1993), further studies soon identified that microRNA expression could be altered by disease (Takamizawa et al., 2004). This has led to numerous studies looking at how microRNA expression differs in disease and whether this change in expression is intrinsically linked to the cause of the disease. The presence of microRNAs in the circulation and their link to disease is a more recent discovery and adds a new angle to research in the field (Gilad et al., 2008). What has proved difficult is showing consistency in circulating microRNA expression in disease versus healthy, particularly in heart failure. As discussed in the Introduction section: Circulating microRNAs in heart failure, studies have shown that microRNA-423-5p expression to be significantly upregulated in a population of heart failure patients versus controls, whereas another study showed the expression of the same microRNA to be unchanged (Tijssen et al., 2010) (Fukushima et al., 2011). These publications would suggest the link between the levels of circulating microRNA and disease is complicated and not always consistent. In this study, there is variability in circulating microRNA expression even between two groups of patients that have been classified to have the same disease.

The heart failure patients that were enrolled in the Copernicus trial the study had to pass the following inclusion criteria:

'Patients were enrolled if they had dyspnea or fatigue at rest or on minimal exertion for ≥ 2 months and a left ventricular ejection fraction $< 25\%$ as a result of an ischemic or non-ischemic cardiomyopathy.' (Packer, 2002)

As discussed in the Introduction section: Heart failure, there is a great amount of heterogeneity in the population of heart failure patients. There is a range of clinical symptoms associated with the disease that are caused by a number of malfunctioning physiological mechanisms. There are a variety of aetiologies for chronic heart failure and rates of disease severity progression e.g. MI vs DCM vs anthracycline-induced cardiomyopathy (Cowie et al., 1999). What is clear from this complicated picture of heart failure pathophysiology is the sheer number of variables and permutations associated with the disease. The broad nature of the inclusion criteria for the Copernicus trial permitted patients with a range of clinical backgrounds to be included. As shown by the studies discussed earlier (Tijssen et al., 2010) (Fukushima et al., 2011), there is variation in microRNA expression even within heart failure patients and this is likely to be caused by the heterogeneity of the disease phenotype. With circulating microRNA expression variable between heart failure patients and the inclusion criteria of the Copernicus trial being relatively broad, the experiments conducted in this study faced a very difficult challenge of identifying patterns in variables that were innately inconsistent in the two groups. With each prognosis group composed of patients that had a large distribution in the expression of each microRNA, the difference in the mean value between prognosis groups would have to be large enough account to overcome this variability to achieve statistical significant.

Another element of the inclusion criteria of patients to the Copernicus trial was:

'the study imposed no stability criteria with regard to the use of background medications. (Packer, 2002)

The effect of pharmacological intervention on microRNA expression has previously been investigated, for example; *in vitro* treatment of human colon cancer cells resulted in altered expression of 22 microRNAs (Rossi et al., 2007). The expression of several microRNAs were found to be different in tumour biopsies from 35 patients with rectal cancer before therapy compared to

tumour biopsies from 31 patients two weeks after starting preoperative chemoradiotherapy treatment (Svoboda et al., 2008). Although patients selected from the Copernicus trial were only from the placebo group, their existing medication was not accounted for. This adds a further layer of complexity to the issue of patient heterogeneity, because the effect of any clinical treatment on circulating microRNA expression is unknown in the Copernicus trial patients.

The heart failure patients enrolled in the Copernicus trial were in a relatively stable clinical condition, this was further evidenced by the fact that some patients survived throughout the trial 18 months after being enrolled, because of this an assumption was made that the circulating microRNA expression profile was also stable. This enabled a comparison between the two prognosis groups, but if the microRNA expression profile of the patients was temporally variable, then any patterns or differences identified between the two groups would only be applicable to that time point and may not be mirrored at any other time point. The literature has examples of both consistency and inconsistency in circulating microRNA expression over time. In post MI patients the expression of microRNA-21 decreased 2 days after the event, then increased 5 days post MI before finally returning to control values at later time points. In the same patients, microRNA-29a increased after 5 days and then decreased to control levels at later time points (Zile et al., 2011). It would be reasonable to suggest that 5 days after a physiologically traumatic event like a MI the change in microRNA expression could be transient or reactionary. The circulating microRNA could be attributed to necrotic cardiomyocytes shedding cellular microRNAs into the bloodstream inactively, or apoptotic cardiomyocytes actively releasing microRNAs through exocytosis for communication to other cells. In another study, changes in the circulating microRNA profile of ischaemic stroke patients remained stable several months after the initial ischaemic period (Tan et al., 2009).

These studies illustrate that circulating microRNA expression is affected by disease but any changes are not always stable over prolonged periods. If the circulating microRNA profile in heart failure is acutely variable over time, then circulating microRNAs could not be a prognostic disease biomarker when measuring expression at a single time point. Therefore one explanation for the results observed in this study is that circulating microRNA expression fluctuates acutely in heart failure. Only the baseline samples of Copernicus were measured and therefore it is unknown whether the circulating microRNA expression profile is consistent over time in the patients used in this study. The point at which the samples were taken from the patients is only snap shot in time and may not reflect a consistent expression profile of circulating microRNAs over the previous and subsequent

weeks or months. If this were true, comparing a single time point would be irrelevant as the expression profile of the same patient could be different the following day.

Expanding upon this hypothesis, the expression profile could be variable day to day but exhibit an overall shift over a longer time. This more macroscopic temporal change could be correlated with or even predicted disease prognosis. If a patient's expression profile varied day to day, then a network analysis of serial measurements taken over time could be used to quantify the temporal change in expression. A shift of the expression profile over time relative to a reference could indicate whether the prognosis of the patient is on declining or stable path. Given the availability of serum samples from Copernicus enrolled patients over several months this experiment is possible future study.

Evidence suggests microRNAs are not just present in circulation by chance or at random concentrations; it is known that microRNAs are released through active mechanisms (Diehl et al., 2012) (Kosaka et al., 2010). Studies have gone beyond these findings to show that both endogenous and exogenous microRNAs can be transported by high-density lipoprotein (HDL) to recipient cells where their delivery results in a change to cellular function (Vickers et al., 2011). This would imply microRNAs are released into circulation with a designated purpose. Communication between cells has been suggested as the primary purpose of microRNA transport in circulation with the aim of altering gene expression in the target cells. It is unclear how this would operate in concert with other physiological communication methods such as neuronal and hormonal pathways. With the concentration of circulating microRNAs is extremely low; the yield of microRNA from plasma or serum is 1-10ng (Pritchard et al., 2012). It is also unknown whether such a small amount of microRNA could have the potency to change the expression of its target genes to such an extent that it results in a change in function to more than just a collection of cells but on a organ level. In other words, whether circulating microRNA could change the contractile capacity of the heart by affecting a critical mass of cardiomyocytes.

Conclusion

The heart failure patients enrolled in the Copernicus trial were a well characterised cohort. The availability of the serum samples from this large population presented the opportunity to determine the circulating microRNA expression profile of heart failure patients in an attempt to identify a novel biomarker that could be a prognostic marker for disease progression. The baseline serum samples from a pilot group were chosen, split into good and poor disease prognosis groups, and the

expression of a range of microRNAs was quantified by two different RT-qPCR based microRNA microarrays. From each microarray a shortlist of microRNA candidates were selected for further analysis in order to validate the results of the microarray. Unfortunately, none of the shortlisted microRNA displayed the same pattern in expression and were therefore unable to consistently distinguish the good from poor prognosis heart failure patients. The underlying cause of the results is unknown, but could be attributed to degradation of the microRNA sequences in the serum samples after stored for over 10 years at -80°C. Alternatively the heterogeneity of the heart failure patients' pathophysiology, aetiology and medication regimes meant that their circulating microRNA expression profile was too variable for any patterns in expression to be identified.

Chapter 4: The effect of increasing microRNA-1 expression on cardiomyocyte functionality by *in vitro* transfection

Introduction

The combination of microRNAs that are most prominently expressed by a cell represent the signature microRNA expression profile. When the microRNA expression profile of healthy human adult cardiomyocyte was mapped it revealed that microRNA-1 is highly expressed in the myocardium (Thum et al., 2008). In heart failure there is a global shift in the myocardial microRNA expression profile and as part of this change myocardial microRNA-1 expression is disrupted. Interestingly the heart failure expression profile bears many similarities to that of the fetal microRNA profile (Thum et al., 2007). Reduced myocardial microRNA-1 expression has been found in the *in vivo* model of heart failure used by Professor Harding group; surgical proximal coronary ligation of adult rats in order to induce chronic MI followed by 16 weeks for heart failure to develop (Kumarswamy et al., 2012).

After it was discovered that the expression of microRNAs changed in disease, the next stage of microRNA research was to understand what effect this change may have in causing the development of disease. In the majority, but not all studies conducted to date, microRNA-1 expression has been found to be decreased in some human heart failure patients and *in vivo* models. There have also been a number of studies that have found evidence that microRNA-1 has the capacity to affect a number of components of cardiomyocyte calcium homeostasis that are known to malfunction in heart failure. For example, microRNA-1 has been found to influence the expression of the regulatory subunits of protein phosphatase type 2a, connexin43 and the subunit of $K_{ir2.1}$, as described in the section Introduction: MicroRNA-1 and excitation contraction coupling.

In a study by a collaborator of Professor Harding's group, delivery of a microRNA-1 AAV9 vector that upregulated cardiac microRNA-1 expression in a trans aortic constriction model of heart failure also normalised SERCA2a protein expression back to healthy levels (Kumarswamy et al., 2012). As part of work conducted in a previous Masters project, heart failure was replicated by reducing microRNA-1 expression in healthy adult rat cardiomyocytes through antisense RNA oligonucleotide transfection. This resulted in increased expression of NCX and improved functionality; with calcium extrusion from the cytoplasm faster compared to controls. The mechanism by which microRNA-1 affects NCX is clear as there is a direct match between the sequences of microRNA-1 and the 3' UTR of NCX. This is not the case for SERCA2a and the mechanism by which microRNA-1 alters SERCA2a expression remains to be determined.

The cardiomyocyte pathological phenotype of heart failure is complex and not confined to malfunctioning calcium handling and contractility. Remodelling of cardiomyocyte structure and size, responsiveness to circulating catecholamines and intracellular signalling pathways also become dysfunctional in heart failure. MicroRNA-1 is known to have the highest expression of any microRNA in the myocardium and there are ~800 known gene targets of microRNA-1. MicroRNA-1 has already been found to have a regulatory influence over a number of components of cardiomyocytes that are important for cell functionality. This occurs either through post transcriptional inhibition of an mRNA sequence, or indirectly through targeting a component of a regulatory cascade, but the full range of cardiac gene targets affected by the decreased microRNA-1 expression in heart failure is still to be explored. High myocardial microRNA-1 expression in healthy cardiomyocytes, the change in microRNA-1 expression in heart failure *in vivo* models and patients and that microRNA-1 has already been found to regulate a number of important cardiomyocyte gene targets together formed the basis of the hypothesis for this chapter; that microRNA-1 plays a role in regulating a number of important cardiomyocyte components that malfunction in heart failure and that normalising microRNA-1 expression may reverse these changes. A strategy of investigating the effect increasing microRNA-1 expression in heart failure on calcium handling and the other aspects of the heart failure phenotype was developed.

Aims

- Determine how increasing microRNA-1 expression in a range of cardiac cells types may be able to affect the expression of the key calcium handling protein SERCA2a.
- Investigate the effect of increasing microRNA-1 expression on heart failure cardiomyocytes calcium handling capability through measuring contractility.
- Determine if microRNA-1 can also affect other cardiomyocytes functions that are affected by heart failure cardiomyocyte phenotype such as T-tubular regularity and β -adrenoceptor function.

Methods

Adenovirus and lipofection transfection methods

Two methods to modulate transgene expression *in vitro* were used in the results of this chapter: lipofection and adenoviral transfection. Of these two options, adenoviral transfection is the more frequently used in the literature because it is associated with the higher transfection efficiency (Djurovic et al., 2004). In some cases this has been shown to be 100% efficient in cultured adult and neonatal cardiomyocytes (Kass-Eisler et al., 1993). Adenoviruses have a non-enveloped structure; this means the viral capsid is made up of a large set of proteins (rather than a lipid envelop) and gives adenoviruses a greater resistance to heat and acid, and higher virulence than enveloped viruses. Adenoviruses can be modified with inserts of linear double stranded genetic sequences of up to 35kb in size. Although the transgene expression caused by an adenovirus is only transient (typically around 2 weeks), this timescale is more than suitable for *in vitro* studies, particularly with adult cardiomyocytes (Louch et al., 2011) and only limits their transferability into clinical application. The main difficulty associated with the application of adenoviruses is toxicity due to the induction of an inflammatory and immunogenic response caused by the proteinaceous capsid (Kay et al., 2001) (Patil et al., 2005). To ensure the amount of virus delivered is consistent between experiments the Multiplicity of Infection (MOI) is calculated. The MOI is the number of viral particles per cell, an MOI of 100 would mean that for every cell in the dish there are 100 viral particles. Cells that are readily transfectable this can be around 10, the MOI values stated in this chapter are much higher than this. This is most likely to be caused by the adenoviral stocks for adeno.miR-1 and adeno.GFP containing 'empty' viral particles. The viral capsid is formed but the gene sequence is not housed within it, this means that to reach an adequate proportion of cells that are GFP positive requires adding more adenovirus, which increases the MOI value.

The other technique used here was lipofection, a non-viral method which is not associated with a such a significant toxicity risk as adenoviruses. Lipofection is based on the principle of liposomes containing genes of interest fusing to cell membranes and transferring the DNA into the host cell. The liposome is a cationic lipid which forms a complex with negatively charged DNA resulting in a positively charged structure. This fuses with the anionic cell surface and the gene of interest is transferred into the cell through endocytosis (Felgner et al., 1987). This simple, non-toxic technique is widely used in the literature, typically with immortal cell lines such as HEK293. The transfection efficiency in adult cardiomyocytes is much lower compared to adenoviruses (0-20%).

H9c2 cells

H9c2 is a commercially available cell line that was originally derived from embryonic rat heart ventricle. Despite their cardiac origin they do not possess heart-specific morphological structures (Zordoky and El-Kadi, 2007) but plated H9c2 cells will simultaneously contract and produce APs when exposed to electrical stimulation or acetylcholine (Hescheler et al., 1991). Two of the benefits of using H9c2 cells are that they can be maintained in culture for longer periods than adult or neonatal cardiomyocytes, and it is possible to achieve high transfection efficiency by viral or non-viral methods. In this study, H9c2 were plated and after 24 hours treated with adeno.miR-1 or adeno.GFP, then cultured for 24, 48 and 72 hours post transfection. After testing a range of adenovirus MOIs (data not shown) to determine an acceptable level of transfection efficiency, the optimum MOI was determined to be 7000.

Rat and mouse neonatal cardiomyocytes

Two properties of neonatal cardiomyocytes that makes them more advantageous model than adult cardiomyocytes for studying gene transfer are that they survive in culture over prolonged periods and are more readily transfectable (Ehler et al., 2013). The contractile apparatus and sarcomeric structure of adult cardiomyocytes is different to that of neonates, meaning that results from functional experiments with neonatal cardiomyocytes must be approached with a certain degree of caution. In this study, 48 hours after plating rat neonatal cardiomyocytes were exposed to a MOI 1400 of adeno.miR-1 or adeno.GFP (after testing a range of adenovirus MOIs, data not shown) then cultured for 24, 48 or 72 hours. The transgenic neonatal mouse cardiomyocytes that expressed the EPAC1 FRET sensor were also transfected after 48 hours then cultured for another 48 hours before conducting FRET experiments.

Adult rat cardiomyocytes

Adult rat cardiomyocytes were transfected using both lipofection and adenoviral methods, these are detailed in the sections Methods: MicroRNA-1 adenovirus and Methods: Lipofection. For both, cardiomyocytes were cultured for 48 hours only, before the cells were studied in functional experiments or the cells were saved for RT-qPCR experiments.

RNA isolation and RT-qPCR

Both SYBR Green and TaqMan RT-qPCR techniques were used in the experiments in this chapter. Together with the isolation of RNA from cells, the methods used were conducted as stated in the methods chapter.

Functional studies using adult rat cardiomyocytes

PTX culture and contractility

PTX is an inhibitor of intracellular G α i activation, it targets the cysteine residue of G α i protein and the A-protomer of PTX ADP-ribosylates the cysteine residue of G α i which results in the G α i signal being blocked (Mangmool and Kurose, 2011). The net effect of this activity is the receptor becoming uncoupled from the G α i protein, inhibiting any activity through this pathway. In the experiments presented in this chapter, this is the isoprenaline induced β_2 -adrenoceptor activation of G α i signalling. PTX is widely used as a pharmacological tool to identify G α i protein activity at the plasma membrane. In this study it was used in culture over 24 hours to block the influence of G α i activity on cardiomyocyte contractility. To validate whether the inhibition of G α i was successful, cardiomyocytes were first exposed to 3×10^{-8} M isoprenaline to stimulate a positive inotropic response. This was followed by exposure to 5 μ M Carbachol, an agonist at the M2 receptor which activates the G α i pathway (Gong, 2002). If G α i inhibition by PTX was not comprehensive, the G α i pathway will be activated by Carbachol and this results in a reduction in contractile amplitude of the cardiomyocyte. This was tested with the 250 ng/ml PTX exposure of 24 hours and resulted in no decrease in contractile amplitude (data not shown) meaning that no G α i signal was detectable and the PTX inhibition was successful. The Methods: β_2 -adrenoceptor specific studies protocol was followed in cardiomyocytes after 24 hours of PTX culturing.

Results

The effect of increasing microRNA-1 expression by adenovirus on SERCA2a

Adeno.miR-1 transfection of a range of cardiac cells types

Collaborators of Professor Harding's group had previously shown that in a rat aortic banding model of heart failure, microRNA-1 transfer by AAV9 resulted in a normalisation of SERCA2a expression comparable to levels of a healthy control (Karakikes et al., 2013). The authors did not further investigate the mechanism by which microRNA-1 regulated SERCA2a gene expression. Before proceeding with any functional or gene expression experiments to investigate how increasing microRNA-1 expression improves SERCA2a expression, the ability of adeno.miR-1 to increase microRNA-1 expression by *in vitro* transfection needed be verified. To do this a range of cell types were transfected with adeno.miR-1 and adeno.GFP (to act as a control) and the expression of microRNA-1 measured by RT-qPCR.

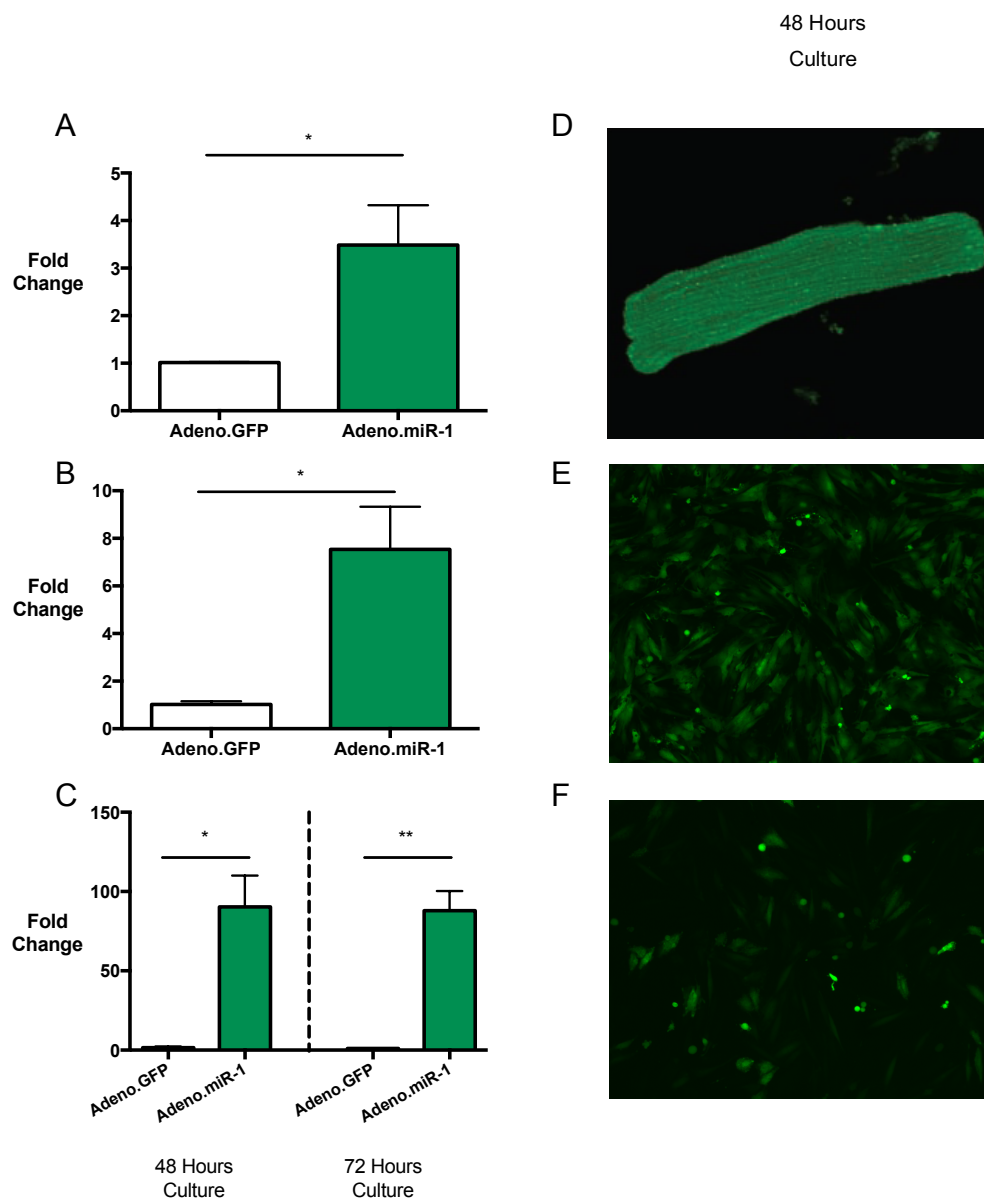


Figure 4A: TaqMan RT-qPCR analysis of microRNA-1 expression after transfection with adeno.GFP or adeno.miR-1.

MicroRNA-1 expression is shown as the fold change of adeno.miR-1 transfected cells compared to adeno.GFP after 48 hours (unless stated). RNU6B was used as a housekeeping gene for normalisation in both groups. Mean fold change $\pm$ SEM, Mann Whitney test for statistical comparison, n=9 for all groups:

A) Adult rat cardiomyocytes: 1.01 ± 0.01 versus 3.49 ± 0.84 , *P=0.04

B) Neonatal rat cardiomyocytes: 1.02 ± 0.13 versus 7.54 ± 1.791 , , *P=0.02

C) H9c2 cells: 48 hours: 1.57 ± 0.72 versus 90.3 ± 19.7 , P=0.011. 72 hours: 1.03 ± 0.18 versus 87.9 ± 12.4 , , *P=0.002

D) Representative image of a fluorescent adult rat cardiomyocyte after adeno.miR-1 transfection

F) Representative image of fluorescent neonatal rat cardiomyocytes after adeno.miR-1 transfection

E) Representative image of fluorescent H9c2 cells after adeno.miR-1 transfection

In all three cell types tested, adeno.miR-1 transfection resulted in a significant increase in the fold expression of microRNA-1 compared to transfection with adeno.GFP. Due to their ability to survive for longer periods in culture, H9c2 cells were maintained with the adenoviruses for 72 hours to allow a longer time window for microRNA-1 expression to increase. As shown by figure 4A-C, microRNA-1 expression had already increased after 48 hours transfection with adeno.miR-1. Visual inspection using fluorescence microscopy after transfection (figure 4A-D, E and F) showed that the GFP was well expressed, another indicator that transfection had been successful. There is a risk that the microRNA-1 expression detected by the RT-qPCR experiment is not entirely caused by the intracellular microRNA-1 sequences present in the cells. Instead the microRNA-1 from the adeno.miR-1 present in the media of the cells could have been included in the material used for amplification by RT-qPCR. This would be a false positive, with the RT-qPCR not a true representative of microRNA-1 expression in the cells tested.

In a Masters project conducted prior to this study, healthy adult rat cardiomyocytes were transfected with Pre miR-1 by lipofection which yielded a significant increase in microRNA-1 expression of ~15 fold ($P=0.038$). This result validates lipofection as an alternate methodology to adenovirus for altering microRNA-1 expression in cardiomyocytes *in vitro*. As the adeno.miR-1 was conjugated to GFP the fluorescent tag enables more accurate selection of cardiomyocytes that have been transfected for single cell studies. The Pre miR-1 construct used for transfection by lipofection does not have a detectable label, this means there is no guarantee that the cell being studied has been transfected and is therefore an experimental weakness of the technique. The opportunity to use the adenoviruses only occurred mid way through this study, hence there is a mix between transfection experiments conducted with adenovirus and lipofection.

SERCA2a mRNA expression after transfection with microRNA-1 adenovirus

The next stage was to determine if the elevated microRNA-1 expression could influence SERCA2a mRNA expression. SYBR Green RT-qPCR was used to measure SERCA2a mRNA expression in neonatal rat cardiomyocytes and H9c2 cells.

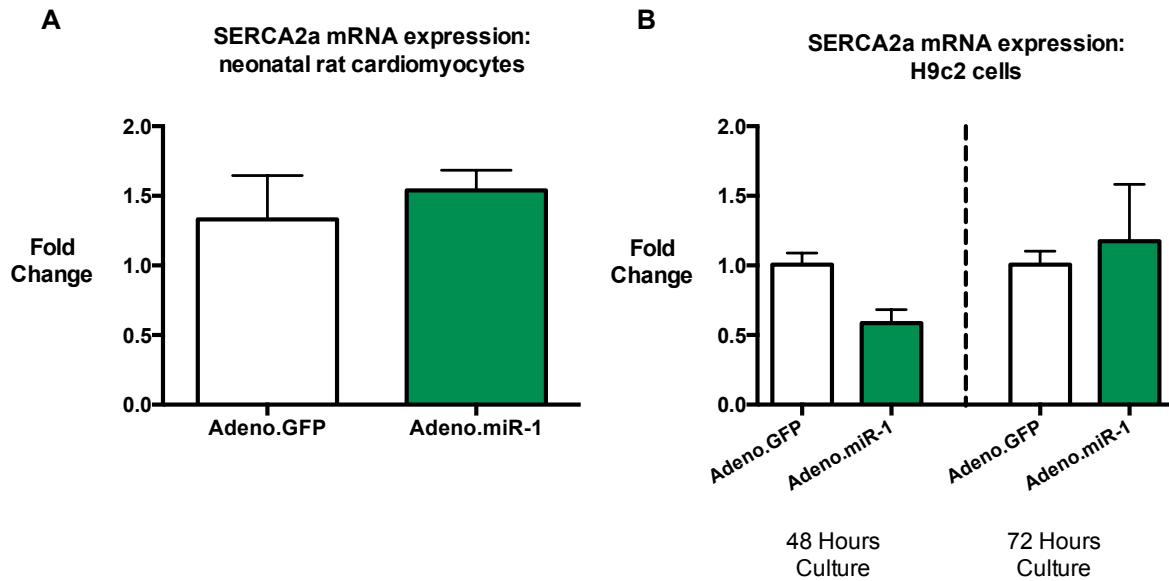


Figure 4B: SYBR Green RT-qPCR analysis of SERCA2a mRNA expression 48 hours (unless stated) after transfection with adeno.GFP or adeno.miR-1.

SERCA2a expression is shown as the fold change of adeno.miR-1 transfected cells compared to adeno.GFP with GAPDH used as a housekeeping gene for normalisation in both groups. Mean fold change $\pm$ SEM, Mann Whitney Test, $n=3$ for all groups, all data was non significant:

A) Neonatal rat cardiomyocytes: 1.33 ± 0.32 versus 1.54 ± 0.14

B) H9c2 cells: 48 hours: 1.01 ± 0.082 versus 0.59 ± 0.095 , $P=0.1$. 72 hours: 1.01 ± 0.096 versus 1.173 ± 0.41

In both neonatal rat cardiomyocytes and H9c2 cells, increased microRNA-1 expression did not cause an increase in SERCA2a mRNA expression. After 48 hours after adeno.miR-1 transfection the expression of SERCA2a mRNA was slightly reduced compared to adeno.GFP transfected cells, while at 72 h the levels in adeno.GFP and adeno-miR-1 were similar. From this result it is possible to conclude in healthy cells, driving up microRNA-1 expression does not have a similar effect on SERCA2a mRNA.

The effect of increasing microRNA-1 expression on cardiomyocyte functionality

β -adrenoceptor contractile response of adult rat heart failure cardiomyocytes

Because the increased microRNA-1 expression did not influence SERCA2a mRNA expression in a range of healthy cardiac cell types, a different experimental approach was necessary to understand

how microRNA-1 might influence SERCA2a. The importance of SERCA2a functionally on calcium handling in cardiomyocyte contractility is well established and is an area where Professor Harding's laboratory has a long standing interest. β -adrenoceptors are also important for cardiomyocyte contractility and respond to catecholamines to influence a number of cardiomyocyte targets such as SERCA2a in order to have a positive inotropic effect. Added to this, both SERCA2a and the β -adrenoceptors expression and function have been found to be disrupted as part of the heart failure disease phenotype. Therefore it was hypothesised that microRNA-1 may regulate the functionality of SERCA2a and the β -adrenoceptors and when the expression of microRNA-1 decreases in heart failure this causes SERCA2a and the β -adrenoceptors to malfunction. In order to measure their functionality, the contractile response of adult rat cardiomyocytes (healthy and heart failure) to a concentration range of isoprenaline after transfection with Pre miR-1 by lipofection was studied. A concentration range of isoprenaline was used that started at 1×10^{-10} M and increased in accumulative fashion by half log concentrations to 1×10^{-6} M. Each data point represents the difference between the baseline as a % of peak height (BI%PeakH) before isoprenaline was added and the BI%PeakH after the contractile amplitude had plateaued after an addition of isoprenaline. For example, if the baseline BI%PeakH was 5% and this increased to 8%, then the difference (3%) would be shown in the graph for that concentration of isoprenaline. The lowest concentration of isoprenaline did not cause an increase in the contractile amplitude of the cardiomyocytes hence why the peak contractile amplitude minus basal is 0% in figure 4C-A. This protocol is used for all cardiomyocyte contractility data shown in this study unless stated.

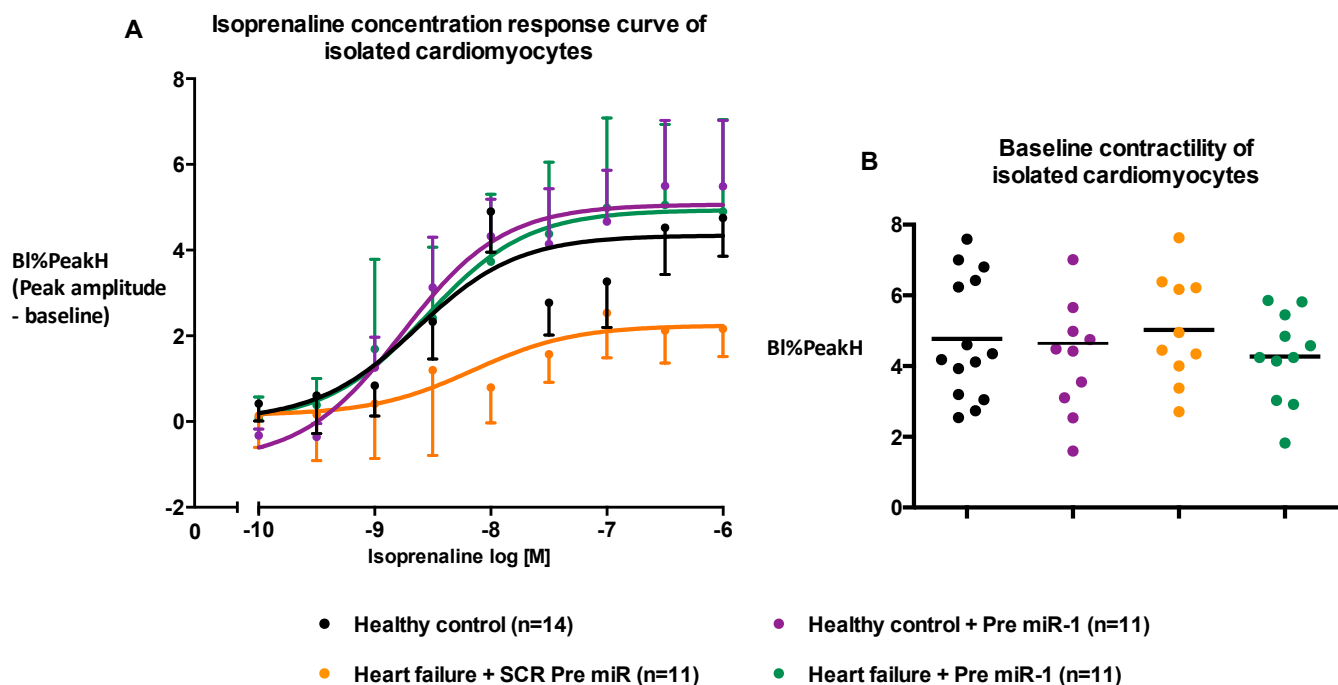


Figure 4C: Contractile parameters of isolated healthy and heart failure (MI + 16-20 weeks) cardiomyocytes before and after exposure to a concentration range of the β -adrenoceptor agonist isoprenaline 48 hours after culture.

Lipofection was used as the transfection technique for all cell types (n = number of cells tested, 3-4 animals per group):

A) BI%PeakH (Peak contractile amplitude – baseline) in response to a concentration range of isoprenaline. *** $P < 0.0001$ (F-test)

B) Baseline BI%PeakH prior to any addition of isoprenaline. All data was non significant (One way ANOVA)

Transfection of heart failure cardiomyocytes with the Pre miR-1 construct by lipofection resulted in a significantly enhanced contractile response to the concentration range of isoprenaline compared to Scrambled Pre miR treated heart failure cardiomyocytes ($P < 0.0001$). The contractile response of heart failure + Pre miR-1 to isoprenaline was restored to a level comparable with that of healthy cardiomyocytes. The heart failure + Pre miR-1 concentration response curve mirrors that of healthy cardiomyocytes; the EC_{50} values of the two curves are very similar; healthy (2.31×10^{-9} M) versus heart failure + Pre miR-1 (2.79×10^{-9} M) and the first concentration of isoprenaline that triggers an increased contractile response is similar ($\sim 1 \times 10^{-9}$ M). As part of a previous Masters project healthy adult cardiomyocytes were transfected with Scrambled Pre miR as a control group (data not shown) but this did not cause a change to the isoprenaline concentration response curve of healthy adult cardiomyocytes. Reduced β -adrenoceptor sensitivity to catecholamines has been well established as an aspect of heart failure cardiomyocyte phenotype (Bristow et al., 1986). This was observed in

figure 4C-A where the heart failure + Scrambled Pre miR concentration response curve has an EC₅₀ value of 7.05×10^{-9} M and the top BI%PeakH value was only 2.24% (versus 4.34% for healthy).

Transfection with Pre miR-1 transfection did not further increase contractility in healthy cardiomyocytes. This would suggest that the effect of Pre miR-1 is to improve a function of cardiomyocytes that is defective in heart failure but functioning at normal capability in healthy cardiomyocytes. The baseline contractility of the both healthy and heart failure cardiomyocytes was unaffected by the increase in microRNA-1 expression (figure 4C-B). This would suggest that restoration of the isoprenaline response is an effect that is caused by activation of the β -adrenoceptors and their downstream pathways, rather than a general improvement in cardiomyocyte calcium homeostasis. Isoprenaline is a non-selective agonist for the β -adrenoceptors, it binds to β_1 , β_2 and β_3 subtypes and triggers activation of the distinct receptor specific downstream pathways of all three. The result in figure 4C-A shows that increased microRNA-1 expression in heart failure must improve β -adrenoceptor expression or counteract β -adrenoceptor desensitisation in some way. The next stage was to narrow down on the specific effect of increased microRNA-1 expression on β -adrenoceptors in heart failure.

As described in the Methods section: Isolation of adult rat ventricular cardiomyocytes, after a heart was removed from the thoracic cavity of the animal the left ventricle was removed used for isolation of cardiomyocytes. This included cardiomyocytes from both the base and apex of the left ventricle, differences in the cardiomyocytes from the two regions have been identified, particularly important for this experiment is that apical cardiomyocytes have a higher β_1 -adrenoceptor density and β_2 -adrenoceptor mediated contractility (Paur et al., 2012). These variations in cardiomyocyte behaviour and structure were not accounted for in this study as both apex and base cardiomyocytes were used for all adult cardiomyocyte experiments. This could have affected the results shown in figure 4C; if the cardiomyocytes selected from the heart failure + Pre miR-1 group were apical rather than base then the contractile response would be greater simply due to the area of the heart from which they originated rather than the effect of Pre miR-1.

RT-qPCR analysis of adult rat heart failure cardiomyocytes after microRNA-1 transfection

MicroRNAs are known to operate through a post transcriptional mechanism of regulating mRNA sequences, but as shown by figure 4B, the mRNA expression of SERCA2a in two healthy cardiac cells types was unaffected by increased microRNA expression. In another study, the *in vivo* delivery of

microRNA-1 by AAV9 has been shown to improve SERCA2a mRNA and protein expression in a TAC model of heart failure (Karakikes et al., 2013). Therefore a subset of the adult rat heart failure cardiomyocytes transfected with Scrambled Pre miR or Pre miR-1 that were studied in figure 4C for their contractile response were saved for RT-qPCR analysis of gene expression. The mRNA expression of the following genes were tested; SERCA2a, β_1 and β_2 -adrenoceptors. β_3 -adrenoceptor expression and protein level is very low in healthy rat cardiomyocytes so its expression was not measured. The aim was to determine if increased microRNA-1 could affect SERCA2a and β_1 and β_2 -adrenoceptor mRNA expression in heart failure and if this was the case it could be the explanation for the increased contractile response to isoprenaline observed in figure 4C-A. Increased SERCA2a expression could improve diastolic loading of the SR with calcium for release during systole, which would mean improved cardiomyocyte contractility. In heart failure both β_1 and β_2 -adrenoceptors have been shown to activate the G α s-protein signalling pathway, resulting in phosphorylation of PLB by PKA and the release of PLB mediated inhibition on the calcium pumping functionality of SERCA2a (Kaumann et al., 1999). Therefore an increased β -adrenoceptor mediated release of SERCA2a inhibition could result in greater calcium restoration to the SR and therefore a greater contractile force. This means that either an increase in SERCA2a or β_1 and β_2 -adrenoceptor mRNA expression, or an increase in all three could be the reason why increased microRNA-1 expression caused the improved contractility in figure 4C-A.

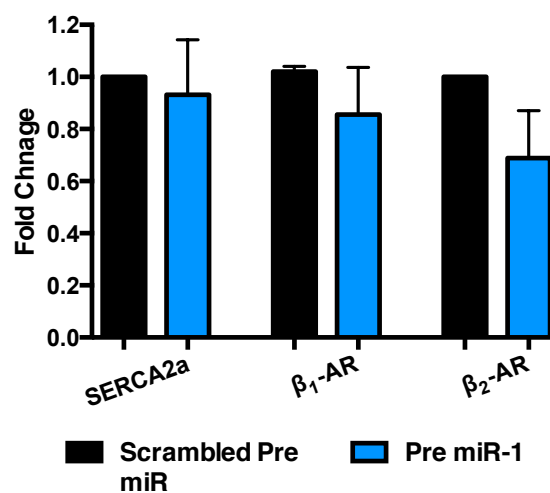


Figure 4D: TaqMan RT-qPCR analysis of SERCA2a, β_1 and β_2 -adrenoceptor mRNA expression in Scrambled Pre miR and Pre miR-1 transfected heart failure adult cardiomyocytes by lipofection. Expression is shown as mean fold change $\pm$ SEM, this was calculated using GAPDH as a housekeeping gene for normalisation. Mann Whitney test for all statistical comparisons, all groups n=4, all data was non significant.

As observed in figure 4D, SERCA2a, β_1 - and β_2 -adrenoceptor mRNA expression is unchanged 48 hours after microRNA-1 transfection of the heart failure cardiomyocytes. RT-qPCR in cultured adult cardiomyocytes is technically challenging in the sense that when extracting cultured cardiomyocytes from a culture dish there is a large percentage of dead cells. In a RT-qPCR experiment they effectively act as noise and cloud the expression signal of the remaining viable cardiomyocytes. This can be minimised by plating the cardiomyocytes with laminin and washing dead cardiomyocytes away (not done in this experiment) but this is not a comprehensive method of removing all dead cardiomyocytes.

Taking into account the degree of uncertainty caused by presence of dead cells, this result would suggest that a simple increase in the mRNA expression of SERCA, β_1 and β_2 -adrenoceptor expression was not responsible for the improved contractile response observed in figure 4D. As part of the cardiomyocyte heart failure phenotype it has been established that both a loss of β -adrenoceptor expression and functional sensitivity occurs. Therefore the improved response to isoprenaline could be attributed to a change in how the β -adrenoceptors are functioning after microRNA-1 transfection rather than a change in expression of receptors at the sarcolemma of cardiomyocytes.

β_2 -adrenoceptor specific contractile response after a single isoprenaline concentration in adult rat heart failure cardiomyocytes

The β_1 - and β_2 -adrenoceptor behave in different ways in heart failure, with the β_1 -adrenoceptor exhibiting a lower expression and desensitised function whereas the β_2 -adrenoceptor shows alterations to its sensitivity and spatial distribution. Before investigating how the receptors are functioning differently after microRNA-1 transfection, the first step was determine which receptor was responsible for the increased contractile response to isoprenaline. From the isoprenaline concentration response curve (figure 4C-A) a single, sub maximal concentration was selected for further experiments (30 nM). This was a more efficient method than testing a full concentration range and by selecting a sub maximal concentration it allowed an increase or decrease in contractility to be detected. CGP20712A was used for pharmacological blockade of the β_1 -adrenoceptor, which when used in conjunction with isoprenaline meant the agonist activity of isoprenaline was focussed on the β_2 -adrenoceptor alone, yielding a β_2 -adrenoceptor specific contractile response.

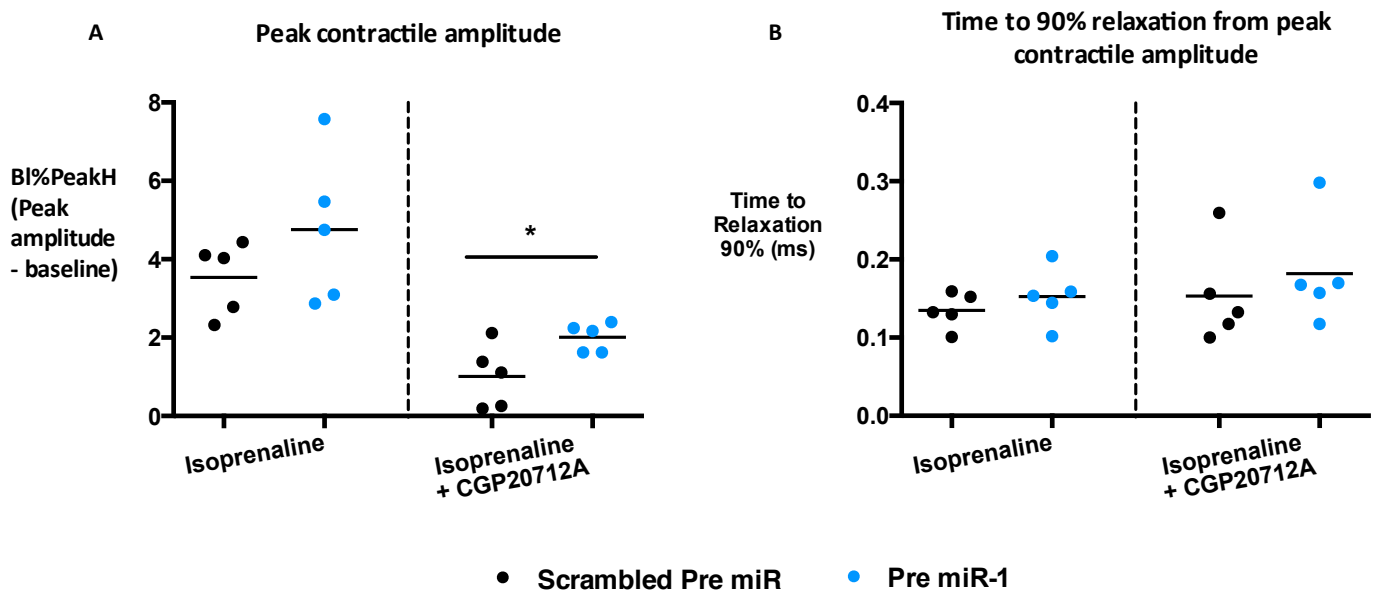


Figure 4E: Contractile parameters of adult heart failure rat cardiomyocytes transfected with Scrambled Pre miR or Pre miR-1 by lipofection and cultured for 48 hours. Cardiomyocyte contractility was measured after the addition of 30 nM isoprenaline following by washout then 30nM isoprenaline + 300nM CGP20712A, a β_1 -adrenoceptor selective antagonist. Mann Whitney test for all statistical comparisons. n=5 animals for all groups (2-4 cells per animal):

*A) Peak contractile amplitude (Bl%PeakH: Peak contractile amplitude – baseline), *P=0.032*

B) Time to 90% relaxation from contractile peak amplitude (ms)

The time to 90% relaxation after isoprenaline and isoprenaline + CGP20712A was unaffected by transfection with Pre miR-1 compared to Scrambled Pre miR (figure 4E-B). This would suggest that release of calcium binding from cardiomyocyte myofilaments and its subsequent removal from the cytoplasm is not affected by increased microRNA-1 expression in heart failure on a cardiomyocyte scale. SERCA2a and NCX are the two principle components that drive calcium removal from the cytoplasm and the resulting relaxation after contraction. Together they clear calcium from the cytoplasm to the extracellular space and SR respectively in a 92% to 7% split in rats (Bers et al; 2002). The remaining 1% is cleared by the 'slow systems'; the sarcolemmal Calcium-ATPase and the mitochondrial Ca uniport (Bers et al., 1996). Given the dominance of SERCA2a over calcium clearance during relaxation, the result observed in figure 4E-B would suggest that SERCA2a is not linked to the effect of increased microRNA-1 expression on improved β -adrenoceptors functionality. This concurs with the SERCA2a mRNA expression results observed in figures 4B and 4D.

Isoprenaline alone resulted in a non-significant increase in the contractile response of Pre miR-1 versus Scrambled miR transfected heart failure cardiomyocytes (mean $\pm$ SEM, 4.75 $\pm$ 0.86 vs

3.54%±0.41, P=0.22) (figure 4E-A). In the presence of CGP20712A the Pre miR-1 transfected cardiomyocytes exhibited a significantly greater β_2 -adrenoceptor specific contractile amplitude compared to Scrambled miR transfected cardiomyocytes (2.01%±0.16 vs 1.01%±0.36, P=0.032). The β_1 -adrenoceptor contractile response was calculated by subtracting the β_2 -adrenoceptor response from the combined β -adrenoceptor response. This was 2.52%±0.57 versus 2.74%±0.99 for Scrambled Pre miR versus Pre miR-1 respectively. This result would suggest that the increased contractile response after Pre miR-1 transfection has resulted in an improved β_2 -adrenoceptor specific function alone and that the β_1 -adrenoceptor is unaffected. The β_2 -adrenoceptor behaves differently in a number of ways in heart failure, with both reduced sensitivity to catecholamines and a more diffuse spatial distribution from the T-tubules to the cell crest detected. MicroRNA-1 could be influencing one, several or all of these mechanisms, such is the nature of how microRNAs operate with a potentially large range of target sequences. Therefore the next stage was to tease out exactly how microRNA-1 is able to exert increased contractility through the β_2 -adrenoceptor in heart failure.

Investigation of the β_2 -adrenoceptor function after microRNA-1 transfection

β_2 -adrenoceptor specific FRET response of neonatal EPAC1 mouse cardiomyocytes

The first approach to further understanding the mechanism by which microRNA-1 was able to influence β_2 -adrenoceptor functionality was to measure the cAMP produced after agonist activation of the β_2 -adrenoceptor. This is an indication of how effectively the β_2 -adrenoceptor is able to couple to PKA, the enzyme that phosphorylates a range of downstream targets that can increase cardiac inotropy. For this experiment, transgenic neonatal mouse cardiomyocytes that expressed the EPAC1 FRET sensor were used instead of adult rat cardiomyocytes. This is because simultaneous transfection with microRNA-1 adenovirus and EPAC1 adenovirus is very technically challenging. After 48 hours of culture with microRNA-1 or GFP adenoviruses the neonatal cardiomyocytes were exposure to isoprenaline + CGP20712A and the FRET response recorded.

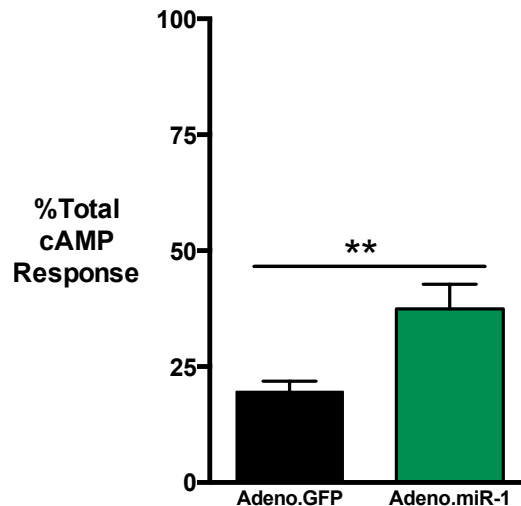


Figure 4F: The FRET response of neonatal EPAC1 mouse cardiomyocytes transfected with adeno.GFP or adeno.miR-1.

*The mean±SEM of the β_2 -adrenoceptor specific FRET response is shown (isoprenaline + CGP20712A) as a % of the 100% cAMP FRET Response after exposure to NKH477. Mann Whitney test, **P=0.0042, n=27 cells for both groups derived from 4 preparations of neonatal EPAC1 mouse cardiomyocytes.*

The data presented in figure 4F was conducted in conjunction with Dr Peter Wright.

Neonatal EPAC1 mouse cardiomyocytes that were transfected with adeno.miR-1 exhibited a β_2 -adrenoceptor FRET response (relative to the 100% FRET response) that was significantly greater compared to cardiomyocytes transfected with adeno.GFP (P=0.0042, 37.5%±5.3 versus 19.7%±2.2). The FRET response to isoprenaline + CGP20712A is expressed here as a percentage of the maximal FRET response (after NKH477) rather than the raw FRET response. The latter is variable from cell to cell and preparation to preparation, whereas the raw response relative to the maximal is better for comparing between cells and preparations. If the raw total FRET response for adeno.miR-1 transfected cells was lower than that of adeno.GFP transfected cells, then the data could be biased so that the β_2 -adrenoceptor FRET response would appear to be a greater percentage of the maximum, but the raw maximum FRET responses were 15.39±2.3 and 14.22±2.4 for adeno.GFP and adeno.miR-1 respectively. Therefore using the percentage of the maximum FRET response is a valid method of analysis and the result is not a statistical abnormality.

The result in figure 4F would suggest that increased microRNA-1 expression in cardiomyocytes has enabled β_2 -adrenoceptors to elicit a greater production of cAMP when activated by an agonist. These are healthy neonatal cardiomyocytes, not adult heart failure cardiomyocytes and so the result must be treated with a degree of caution. To generate cAMP the β_2 -adrenoceptors activate the

enzyme AC with the AC5 and AC6 isoforms being the most relevant cardiac isoforms. This occurs when a β_2 -adrenoceptor couples to the $G_{\alpha s}$ G protein after activation by an agonist such as isoprenaline. It has been found that the β_2 -adrenoceptor is able to activate either $G_{\alpha s}$ or $G_{\alpha i}$ G proteins, and in heart failure of $G_{\alpha i}$ activation becomes greater (Brodde et al., 2006). Therefore it is possible that microRNA-1 is able to improve contractility in heart failure cardiomyocytes by shifting β_2 -adrenoceptor coupling from $G_{\alpha i}$ to $G_{\alpha s}$, causing more cAMP to be generated and enhancing phosphorylation of the downstream targets of PKA.

β_2 -adrenoceptor specific contractile response in the presence of PTX in adult rat heart failure cardiomyocytes

The following experiment was used to determine if the hypothesis that increased microRNA-1 expression elicited increased heart failure cardiomyocyte contractility through shifting the balance of β_2 -adrenoceptor agonist activated intracellular coupling from $G_{\alpha i}$ to $G_{\alpha s}$. To investigate the viability of this hypothesis, adult rat heart failure cardiomyocytes were incubated with PTX for 24 hours in combination with transfection by adeno.miR-1. PTX causes an inhibition of $G_{\alpha i}$ protein activity at the plasma membrane and nullifies its inhibitory influence on cardiomyocyte contractility. If the hypothesis is correct then in microRNA-1 transfected heart failure cardiomyocytes the effect of $G_{\alpha i}$ disinhibition on contractility by PTX should be minimal, as the increased microRNA-1 expression has already shifted β_2 -adrenoceptor coupling away from $G_{\alpha i}$ so there is nothing left for PTX to block. In untransfected heart failure cardiomyocytes PTX will result in an increased contractility that is comparable caused by increased microRNA-1 expression as it is effectively replicating the effect caused by increased microRNA-1. Therefore, heart failure cardiomyocytes were incubated with either PTX alone, PTX in conjunction with adeno.miR-1 transfection or adeno.miR-1 transfection alone. These three groups were compared to untreated heart failure cardiomyocytes using the same protocol as in figure 4E.

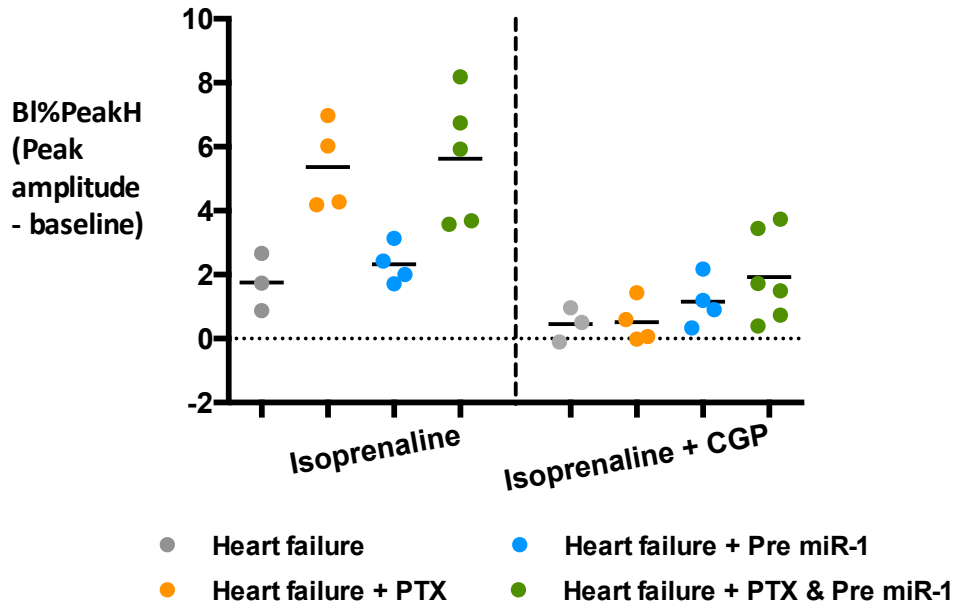


Figure 4G: Peak contractile amplitude (BI%PeakH: peak contractile amplitude – baseline) of heart failure cardiomyocytes, heart failure cardiomyocytes cultured with Pertussis Toxin (PTX), heart failure cardiomyocytes transfected with Pre miR-1 or Pre miR-1 and PTX and cultured for 48 hours. β -adrenoceptor mediated heart failure cardiomyocyte contractility was measured after the addition of 30nM isoprenaline following by washout then 30 nM isoprenaline + 300 nM CGP20712A, a β_1 -adrenoceptor selective antagonist. One way ANOVA with Kruskal Wallace post-hoc test: all data were non-significant. $n=3-6$ animals (2-4 cells per animal).

When compared using a one way ANOVA with Kruskal Wallace post-hoc test none of the groups exhibit significant results, but there are trends in the data from which suggestions can be put forward as possible explanations for the results. The mean ($\pm$ SEM) β_2 -adrenoceptor specific contractile response after Pre miR-1 transfection ($1.15\% \pm 0.38$) is greater than both heart failure ($0.45\% \pm 0.31$) and PTX incubation ($0.52\% \pm 0.33$). The combination of Pre miR-1 transfection and PTX incubation produced the largest β_2 -adrenoceptor response ($1.923\% \pm 0.56$).

Although the results in figure 4G are not significant, the trend in the data would suggest that the positive effect of $G\alpha_i$ inhibition by PTX on the contractility of heart failure cardiomyocytes is in addition to that caused by increased microRNA-1 expression. If this is the case, then the mechanism by which microRNA-1 improves contractility is not by shifting β_2 -adrenoceptor coupling from $G\alpha_i$ to $G\alpha_s$, as PTX incubation caused an improvement in contractility on top of increased microRNA-1 expression. As the data is not significant this statement can not be made with the required level of

certainty, to form a stronger argument for this to be the case, repetitions of the experiment are required.

T-tubule regularity of healthy adult rat cardiomyocytes after adeno.miR_1 transfection.

The previous result in figure 4G could suggest that switching of β_2 -adrenoceptor G protein coupling was not affected by increased microRNA-1 expression in heart failure, but this is not clear from the data. Therefore investigation into alternate mechanisms for the observed result in figures 4E and 4F was required. In healthy cardiomyocytes, the cAMP response of β_2 -adrenoceptors is tightly clustered around the T-tubules (Nikolaev et al., 2010) and in heart failure this becomes more diffuse. This is linked to the breakdown in the T-tubule network structure that occurs as cardiomyocytes progress from hypertrophy into heart failure. With this degradation in T-tubular structure, the close spatial proximity of the β_2 -adrenoceptor to AC and PKA is disrupted.

To investigate whether increased microRNA-1 expression could reduce the degradation of the T-tubule network structure, healthy adult cardiomyocytes were transfected with either adeno.GFP or adeno.miR-1 then stained with CMO to visualise the T-tubules under a fluorescent microscope. Although heart failure cardiomyocytes were not used as a model, culturing healthy cardiomyocytes over time results in a degradation of their T-tubular structure and therefore replicates heart failure in this respect. In a study by Banyasz et al after 48 hours in culture the T-tubule area relative to cell area of healthy cardiomyocytes was found to be significantly decreased compared controls (Banyasz et al., 2008). This technique was used as a surrogate model of heart failure and also coincided with a gap in the availability of heart failure cardiomyocytes derived from MI rats.

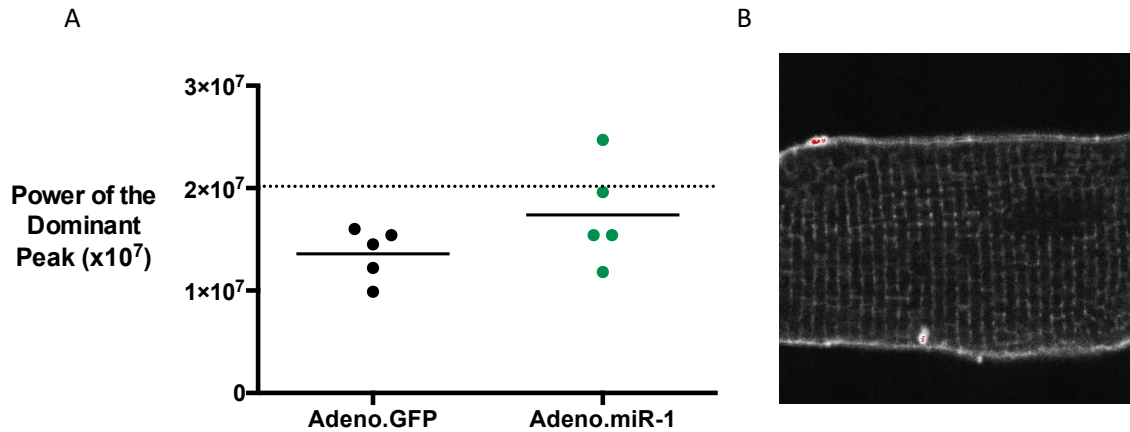


Figure 4H: The regularity of CMO stained healthy adult rat cardiomyocyte T-tubular membranes.

Healthy adult rat cardiomyocytes were transfected with either adeno.GFP or adeno.miR-1 and cultured for 48 hours before staining with CMO. The dotted horizontal line represents the typical Power of the Dominant Peak (x10⁷) of freshly isolated healthy adult rat cardiomyocytes. Mann Whitney test $P=0.3016$, $n=5$ animals, 8-12 cells per animal; 42 cells in total for the adeno.GFP group and 52 cells for the adeno.miR-1 group. The data was non-significant:

A) T-tubular regularity as represented by the metric Power of the Dominant Peak (x10⁷) as calculated by Fourier transform analysis.

B) Representative fluorescent microscopy image of a CMO stained adult rat cardiomyocyte.

Figure 4H-A shows the Power of the Dominant Peak (x10⁷) as calculated by Fourier transform analysis. This is a measure of the density of CMO staining and represents the regularity of the T-tubules network of the cardiomyocytes. The more dense the CMO staining, the greater the amplitude of the power peak and this corresponds to a higher regularity of T-tubules. The T-tubule regularity as measured by this method of freshly isolated adult rat cardiomyocytes is typically $\sim 2.0 \times 10^7$ (horizontal dotted line in figure 4H-A) and this decreases the longer cardiomyocytes are kept in culture. In this experiment adeno.miR-1 transfected cardiomyocytes had a smaller reduction in T-tubule regularity compared to adeno.GFP ($1.74^7 \pm 2.2^6$ versus $1.36^7 \pm 1.1^6$), but this is a non-significant difference by Mann Whitney test ($P=0.3016$). The small n number prevents a normalisation test of the data, however the $\pm$ SEM for both groups was small (12.7% and 8.31% of the respective totals). By conducting an unpaired T test on the individual cells results (data that passed the D'Agostino & Pearson omnibus normality test) the $P=0.073$. The window for increased microRNA-1 expression to limit the reduction in T-tubule regularity after a period of culture is limited, which together with the expression of microRNA-1 already being high in healthy

cardiomyocytes limits the potential change to T-tubule regularity that can occur with adeno.miR-1 transfection.

As this experiment was conducted in healthy rather than heart failure cardiomyocytes the ability to explain the other results that were observed in heart failure cardiomyocytes is limited. Putting this caveat aside, what conclusions can be drawn from this result? It is not possible to know if this is the cause of the improved contractile and cAMP responses observed in figures 4E and 4F as this experiment only assessed structure not function of the cardiomyocytes. There is a range of components localised at the T-tubule that have functional roles relating to the β_2 -adrenoceptor cAMP response and calcium handling, and others that are involved in T-tubule formation and maintaining its structure. It has been shown that in heart failure components from both these groups malfunction in different ways. If microRNA-1 were able to affect T-tubule regularity through targeting the components linked to T-tubule structural competency. It could also restore ECC efficiency and as a result cardiomyocyte contractility would also be improved, this will be covered further in the discussion section.

Discussion

MicroRNA-1 affects β_2 -adrenoceptor mediated contractility and cAMP response

The focus of the results in this chapter was on the effect of increased expression of microRNA-1 in adult rat cardiomyocytes derived from a MI model of heart failure. The first result of note was the improved contractile amplitude of Pre miR-1 transfected heart failure cardiomyocytes in response to a concentration range of isoprenaline (figure 4C-A). Isoprenaline is a non specific agonist of the β -adrenoceptors and it has been well established that the β -adrenoceptors ability to respond to circulating catecholamines is dramatically reduced in heart failure patients, as well as *in vivo* and *in vitro* models. For transfection of microRNA-1 to reverse this characteristic is an interesting finding and to understand the mechanism behind this result further investigation was undertaken.

An increase in the mRNA expression of either β_1 or β_2 -adrenoceptor was ruled out as the cause after RT-qPCR results revealed that expression was the same as cardiomyocytes transfected with Scrambled Pre miR (figure 4D). A key calcium handling protein that has an influence over contractility and is linked to the downstream pathway of the β -adrenoceptors is SERCA2a, but the mRNA expression of SERCA2a was also unchanged in heart failure cardiomyocytes after Pre miR-1 transfection. This result was corroborated by the mRNA expression of SERCA2a in neonatal rat cardiomyocytes and H9c2 cells also being unchanged after transfection by adeno.miR-1 (figure 4B). This would suggest that the mechanism that microRNA-1 operates by does not include the capacity to influence the expression of SERCA2a, β_1 or β_2 -adrenoceptor in these models.

These results are contradictory to a study of a collaborator (Karakikes et al., 2013), in their study 2 weeks after conducting TAC surgery in adult rats to generate a model of heart failure, a single dose of an AAV9 that carried the precursor sequence of microRNA-1 was administered. Then after a further 7 weeks it was found that left ventricular expression of SERCA2a mRNA and protein had improved significantly compared to sham animals. The most notable difference between the Karakikes et al study and the work presented here is that increasing microRNA-1 expression was done in an *in vivo* not *in vitro* setting. A different model of heart failure was also used and the animals were administered with the AAV9 at the point of hypertrophy (2 weeks after TAC) and not after having developed heart failure. The cardiomyocytes used in the experiments of this chapter were transfected after the animal's hearts had progressed from hypertrophy into heart failure. Heart

failure compared to hypertrophy is a different phenotype entirely both in terms of whole organ and cellular function (Hasenfuss, 1998), and microRNA expression profiles (Condorelli et al., 2010). The authors did not look at the cardiomyocyte contractile response to isoprenaline in addition to SERCA2a expression and so the improvement in SERCA2a expression was not connected to a functional change in the cardiomyocytes. For these reasons the results here and from the Karakikes et al study are not entirely comparable.

From the combined results of figures 4C and 4D it can be concluded that the improved contractility after Pre miR-1 transfection is linked to a change in β -adrenoceptor function/ downstream pathways and not mRNA expression. By blocking the effect of the β_1 -adrenoceptor with CGP20712A and applying isoprenaline, the effect of Pre miR-1 transfection on cardiomyocytes contractility was found to be β_2 -adrenoceptor specific (figure 4E). This was only particular to cardiomyocyte contractile amplitude and not relaxation time. A similar protocol was applied to neonatal EPAC1 mouse cardiomyocytes in order to assess β_2 -adrenoceptor specific cAMP production by FRET after adeno.miR-1 transfection (figure 4F). This experiment found that the β_2 -adrenoceptor cAMP response was greater after adeno.miR-1 transfection. The combined conclusion of these last two experiments is that microRNA-1 is able to influence β_2 -adrenoceptor functionality and by affecting the receptors' capacity to elicit cAMP production microRNA-1 is able to affect contractility, so that when microRNA-1 expression is increased it results in improved contractility after β_2 -adrenoceptor agonist activation.

Investigating Gai and Gas switching of the β_2 -adrenoceptor

To develop a better understanding of the connection between microRNA-1 and cAMP production at the β_2 -adrenoceptor, an investigation was conducted into β_2 -adrenoceptor functional selectivity. The β_2 -adrenoceptor has been found to couple to both Gai and Gas proteins after agonist activation, with opposite effects on cAMP production and contractility. In heart failure the weight of the dynamic shifts from Gas to Gai and is associated with a dampening of cAMP production (Brodde et al., 2006). This alteration in the signalling behaviour is a molecular hallmark of β -adrenoceptor desensitisation in heart failure and is present in all forms of the disease independent of aetiology (Bristow, 1993). The reduced cardiomyocyte β -adrenoceptor sensitivity to catecholamines has the effect of dampening contractility, resulting in a weakened ability of the heart to pump blood around the body in response to increased demand.

Therefore the hypothesis was formulated that increased microRNA-1 expression reversed the trend in heart failure of elevated G α i signalling associated with β -adrenoceptor desensitisation. In theory this would improve coupling of the β ₂-adrenoceptor to the G α s pathway resulting in greater cAMP production, which in turn would activate PKA and whose downstream targets would improve cardiomyocyte contractility. This hypothesis is based on the assumption that switching from G α s to G α i coupling is a causative aspect of the heart failure phenotype. There is debate as to whether G α s to G α i switching is a pathological or protective effect of heart failure. For example, increased G α i activity after long term β ₂-adrenoceptor activation in MI rats resulted in reduced left ventricle dilation and ejection fraction decline. The anti-apoptotic effect of increased G α i activity was deemed to be the main reason for attenuation of disease progression (Ahmet et al., 2004). Furthermore, long term isoprenaline treatment in rats caused both increased G α i expression and a reduction in isoprenaline or forskolin induced arrhythmias (Eschenhagen et al., 1996). In contrast, when the expression of G α i was increased by a conditional tetracycline transactivator system in mice, this resulted in a lethal cardiomyopathy that was shown to have a wide QRS complex arrhythmia (Redfern et al., 2000). Despite the lack of clarity over the pathological impact of G α s to G α i switching in heart failure, the ability of microRNA-1 to influence β ₂-adrenoceptor coupling after agonist activation in a heart failure model was still investigated.

In the experiment conducted in this study the β ₂-adrenoceptor mediated contractility was tested in adult rat heart failure cardiomyocytes transfected with adeno.miR-1 after 24 hours of G α i inhibition by PTX (figure 4G). The experiment did not produce any significant results and so the effect of increased microRNA-1 expression in heart failure remains unknown. If the effect of PTX incubation had improved β ₂-adrenoceptor mediated contractility to a similar amplitude as that caused by adeno.miR-1, one could conclude that PTX has replicated the effect of adeno.miR-1 and therefore the two could be operating via the same mechanism. Alternatively, if the effect of PTX incubation and adeno.miR-1 had been additive on contractility this would suggest they are operating by a different mechanism. The effect of PTX is to cause maximum inhibition of G α i, therefore increased microRNA-1 would not be able increase contractility via G α i to G α s switching as G α i is blocked. MicroRNA-1 would have to be operating through a separate mechanism to G α i to G α s switching. As the results were not significant, neither of these two conclusions can be drawn from the data. To cross examine the hypothesis the expression of the G α i and G α s proteins could have been measured as G α i expression is increased in heart failure (Eschenhagen et al., 1992). There may not be a change in expression, just in how the distinct populations of G proteins couple to the β ₂-adrenoceptor. Alternatively the FRET response of neonatal EPAC1 mouse cardiomyocytes that had undergone the

same adeno.miR-1 / PTX treatment protocol could be tested as this would give a function measure of β_2 -adrenoceptor coupling to $G_{\alpha s}$.

The switch in β_2 -adrenoceptor signalling to $G_{\alpha i}$ from $G_{\alpha s}$ has been linked to GRK2 phosphorylation (Zhu et al., 2012). In experiments where GRK2 is blocked by an inhibitory peptide called β ARKct, both β -adrenoceptor responsiveness and cardiomyocyte contractility have been found to increase. This paradigm occurs in cardiomyocytes derived from β ARKct transgenic mice (Koch et al., 1995), adenoviral β ARKct delivery to *in vivo* models of heart failure (Shah et al., 2001) and transfection of human heart failure cardiomyocytes (Williams et al., 2004). β ARKct blocks endogenous GRK2 binding to the β_2 -adrenoceptor at the $\beta\gamma$ -subunits and therefore prevents phosphorylation of the β_2 -adrenoceptor (Rengo et al., 2009). When phosphorylated by GRK2 this prompts the β_2 -adrenoceptor to signal via the $G_{\alpha i}$ rather than the $G_{\alpha s}$ G protein and induced overexpression of GRK2 results in a PTX sensitive depression of cardiomyocyte contractility in transgenic mice. This would suggest the influence of GRK2 on β_2 -adrenoceptor desensitisation in heart failure is critical to reduced cardiomyocyte contractility in response to catecholamines. Given the uncertainty of the conclusion of the data in figure 4G, the effect of microRNA-1 on GRK2 represents a viable future line of investigation.

T-tubule regularity and the link to cardiomyocyte contractility

With the $G_{\alpha i}$ to $G_{\alpha s}$ switching hypothesis proving inconclusive, alternative explanations were sought for the mechanism for how microRNA-1 was able to influence β_2 -adrenoceptor mediated contractility. T-tubules play an important role in cardiomyocyte functionality by providing the structure for close spatial association of RyR and LTCCs which enables efficient CICR. The cAMP response of β_2 -adrenoceptors is also tightly clustered at the T-tubules (Nikolaev et al., 2010), together with a number of targets of the downstream β_2 -adrenoceptor – cAMP pathway that are involved in CICR: RyR, LTCCs and PLB. Agonist activation of β_2 -adrenoceptor results in phosphorylation of these targets by PKA and this causes greater calcium mobilisation and ultimately increased contractility, because of this link between the T-tubules and β_2 -adrenoceptor signalling, the effect of increased microRNA-1 expression on cardiomyocyte T-tubule regularity was studied in figure 4H.

The hypothesis for this experiment was that the increased microRNA-1 expression was able to reverse the breakdown of the T-tubule structure and in the process restore some localisation of the

β_2 -adrenoceptor cAMP response to the T-tubule. This would focus the effect of PKA activation on the targets that are involved in calcium mobilisation located at the dyad. The theoretical end result would be that β_2 -adrenoceptor activation causes more calcium to be mobilised and contractility is improved. The results in figure 4H show that although there was a trend in the data towards increased microRNA-1 expression resulting in an improvement in T-tubule regularity this was not a significant result.

The experiment was conducted using healthy rather than heart failure cardiomyocytes, with the effect of culturing used to breakdown T-tubular structure and replicate the progression from hypertrophy to heart failure. MicroRNA-1 expression is greater in healthy cardiomyocytes compared to heart failure and attempting to cause a functional change by increasing microRNA-1 expression in this context is challenging because of this. As shown by the contractile response to the isoprenaline concentration range in figure 4C-A, when microRNA-1 expression is increased in healthy cardiomyocytes it may not result in a functional change. A possible explanation is that in healthy cardiomyocytes microRNA-1 inhibition of target sequences is already happening. Increasing microRNA-1 expression further in this environment does not have a large effect as the endogenous microRNA-1 is already fulfilling its function.

There are a number of components that have been identified in the literature that contribute to T-tubule structuring and could therefore be affected by microRNA-1 and linked to the result in figure 4H. Amphiphysin 2 (BIN1) has been found to be involved in T-tubule biogenesis through inducing membrane invagination in skeletal muscle cells (Frost et al., 2008) and its expression is significantly reduced in heart failure (Lyon et al; 2012). In its capacity as a membrane scaffolding protein in the Bin, Amphiphysin, Rvs (BAR) domain superfamily, BIN1 is able to deliver the LTCC 1.2 to the membrane via microtubules and tether LTCC 1.2 to the T-tubule. This enables LTCC 1.2 to be in close proximity to RyRs that gate the SR and initiate the intracellular calcium transient when activated (Hong et al., 2010). Junctophilin 2 (JP2) is another important protein found at the T-tubule whose expression is reduced as cardiac hypertrophy develops into heart failure (Lyon et al., 2012). JP2 acts as a bridge between the plasma membrane and the SR, by doing so it contributes to the formation of the cardiac dyad/T-tubule/SR coupling (Guo et al., 2013). A stable complex between these constituents is essential for normal ECC. JP2 deficient embryonic cardiomyocytes exhibit dyads that are abnormal; with the SR uncoupled from the T-tubule this resulted in unstable calcium transients with reduced amplitudes (Takeshima et al., 2000). Quantifying the expression of these two genes

would be the first step to determine if they are affected by increasing microRNA-1 expression in heart failure cardiomyocytes.

In order to better understand if microRNA-1 is able to influence the regularity of T-tubule structure and have the knock on effect of re-localising the β_2 -adrenoceptor cAMP response to the T-tubules a different experiment is required. A localised EPAC1 sensor in conjunction with a scanning ion conductance microscopy (SICM) / FRET setup would provide a higher resolution of cAMP production compared to the EPAC1 FRET method used here because SICM / FRET system is able to visualise the localised cAMP response in small regions of a cardiomyocyte rather the whole cell cAMP response (Nikolaev et al., 2010). A possible protocol would be to take isolated cardiomyocytes, transfect these cells with adeno.miR-1 and split them into two populations, one for CMO staining and the other for SICM/ FRET. If the first group exhibited improved T-tubular structure and the other a greater, more localised cAMP response compared to control groups, then the conclusion could be made that microRNA-1 can affect the cAMP through retaining T-tubular structure.

Possible mechanisms for improved β_2 -adrenoceptor mediated contractility

The results in this chapter provide an interesting new discovery: a connection between microRNA-1 and the cardiac β_2 -adrenoceptor function. What has not been answered is the mechanism by which microRNA-1 is able to improve β_2 -adrenoceptor mediated contractility in heart failure. Figure 4F revealed that increased microRNA-1 expression was associated with an elevated cAMP response of the β_2 -adrenoceptor. cAMP is generated by AC in the cytoplasm and the levels of cAMP are primarily kept in check by PDEs. cAMP is a substrate of PKA in cardiomyocytes, an enzyme with a number of different targets that influence calcium handling and contractility (see figure 1C).

Any number of the targets that are connected to this cascade could be affected by microRNA-1 and their investigation would represent valid lines of inquiry. With the range of downstream intracellular targets of PKA being so broad, what approach can be adopted to determine which targets are most pertinent? As discussed in the Introduction section: MicroRNA mechanism of action, there are a number of tools that match microRNA sequences to complimentary sections in the 3' UTR of genes. These tools also give an indication of the accuracy of the match and therefore the likelihood of an effect on expression of the gene. In circumstances like this study, these techniques can be used to focus investigation on particular targets where expression is predicted to be inhibited by the microRNA in question. For microRNA-1 there are ~800 sequence matches (www.targetscan.org)

which makes a large population of targets to consider. The relevant cardiac targets that could offer an explanation for the observed results in this chapter are discussed in the subsequent sections.

Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-Kinase)

There are three classes of PI3-Kinase based on their sequence homology and substrate preference (Engelman et al., 2006). In both rats and humans microRNA-1 matches two sequences from the PI3-Kinase family and an additional third sequence in humans only. PIK3C2A (PI3K-C2 α) and PIK3R3 (p55- γ) matched for both species and PIK3CG (p110 γ) in humans only. PI3K-C2 α is a class II catalytic PI3-kinase whilst p55- γ is a regulatory subunit for the class Ia p110 δ catalytic PI3-kinase. The p110 δ subtype is primarily expressed in leukocytes (Vanhaesebroeck et al., 1997) but PI3K-C2 α has been found to be highly expressed in the heart (Domin et al., 1997) (Falasca and Maffucci, 2007). The functional activity of class II PI3-kinases in the heart is understudied and the influence of PI3K-C2 α in the heart relating to contractility is unknown. Interestingly a study by (Wang et al., 2006) found that in vascular smooth muscle cells PI3K-C2 α regulated contractility through a calcium dependent mechanism, with small interfering RNA inhibition of PI3K-C2 α resulted in reduced contractility. In that study, the reduced contractility was suggested to be caused by the impaired activation of the small GTP-ase Rho which caused the reduced phosphorylation of myosin light chain.

Although p110 γ has been found to only be expressed in relatively low levels in the heart (Alloatti et al., 2004), changes in its expression have been found to result in altered cardiac phenotypes. Activation of p110 γ is associated with increased PDE activity and a reduction in cytoplasmic cAMP concentration. In the literature there are examples of transgenic p110 γ knockout mice and it has been found that there was a significantly greater cAMP present in the cytoplasm (Marcantoni et al., 2006) (Patrucco et al., 2004), increased LTCC current and an overall improvement in cardiac contractility in cardiomyocytes derived from the transgenic mice compared to controls (Crackower et al., 2002). Depending on the study, p110 γ is proposed to influence PDE 3 or 4 (Kerfant et al., 2007).

As well as directly affecting the levels of cAMP through targeting PDEs, p110 γ has also been shown to be involved with eliciting some of the effects of β -adrenoceptor signalling *in vivo*. In transgenic p110 γ knock out mice, the extent to which chronic β -adrenoceptor activation by isoprenaline triggered cardiac dysfunction was reduced compared to controls (Oudit et al., 2003). Specifically, in p110 γ knock out mice the effect of chronic isoprenaline on fractional shortening, peak aortic velocity

and left ventricular end-diastolic pressure was far less compared to controls. Together with affecting cAMP levels, a possible explanation for these findings could be provided by a separate study where activation of the β_2 -adrenoceptor by salbutamol in p110 γ null mice resulted in a greater LTCC current compared to controls (Marcantoni et al., 2006). These findings suggest that the p110 γ activity is linked to β -adrenoceptor signalling in cardiomyocytes and p110 γ is able to influence the intracellular pathways that affect the contractile machinery of cardiomyocytes.

A possible hypothesis to explore based on these findings in the literature is that increased microRNA-1 expression is able to restore inhibition of p110 γ expression in heart failure back to the level observed in healthy cardiomyocytes. This would mean a reduction in the p110 γ driven activation of PDE and an overall positive effect on cardiac function. More cAMP would be present in the cytoplasm to trigger PKA activation which would mean increased activation of any PKA targets. Greater calcium entry through LTCCs would provide a greater stimulus for CICR and more calcium would be mobilised from the SR. The net effect would be improved contractility compared to untransfected heart failure cardiomyocytes.

Mitogen-activated protein kinase (MAPK)

The MAPK network is a large family of proteins but in cardiomyocytes the primary focus on research has been on extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinases (JNKs), and p38-MAPKs. Between them these MAPKs have been linked to a wide array of cellular behaviours in response to extracellular stimuli and various regulatory pathways in cardiomyocyte pathophysiology (Clerk and Sugden, 2006). MAPKs are phosphorylated and activated by upstream MAPK kinases (MKKs), these in turn are phosphorylated and activated by MKK kinases (MKKKs). Below is a table of microRNA-1 target matches in human and rat that are MAPK relevant:

Species	Human ortholog of target gene	Gene name	Alternative name
Human	MAP4K2	mitogen-activated protein kinase kinase kinase kinase 2	MEKKK2
	MAPK1	mitogen-activated protein kinase 1	ERK2
	TAOK1	TAO kinase 1	
	TAOK3	TAO kinase 3	
Rat	MAP3K1	mitogen-activated protein kinase kinase kinase 1	MEKK1
	MAP4K2	mitogen-activated protein kinase kinase kinase kinase 2	MEKKK2
	MAP4K3	mitogen-activated protein kinase kinase kinase kinase 3	MEKKK3
	MAPK3	mitogen-activated protein kinase 3	ERK1
	TAOK1	TAO kinase 1	

Table 4A: Selected matches between the mature human and rat microRNA-1 sequence and the 3' UTR of MAPK relevant gene sequences as identified by www.targetscan.org.

TAO kinase 1 and 3 were attributed to being part of the MKKK group because their kinase domain is similar to the yeast Ste20p MKKK. Both TAO kinase 1 and 3 are regulators of MKK3/6 (Raman et al; 2007), which in turn are known to directly activate p38-MAPK (Kerkela and Force, 2006). p38-MAPK expression in the heart is known to be limited to the p38 α and p38 γ isoforms and it has been linked to a range of cardiac pathophysiology; cardiomyocyte death during ischemia, cardiac hypertrophy, cardiac fibrosis and malfunction post ischemia (Marber et al., 2011). What makes p38-MAPK particularly relevant for this study is that activation of p38-MAPK by adenoviral gene transfer has been found to have an inhibitory affect on cardiomyocyte contractility. This finding was further confirmed by pharmacological inhibition of p38-MAPK restoring contractile function. The change in contractility by p38-MAPK was found to be exclusive of altered calcium homeostasis (Liao et al; 2002) and the authors suggest that p38-MAPK is able to have a negative inotropic affect by reducing cardiomyocyte myofilament response to calcium. Interestingly, in a study by Paur et al the inhibition of p38-MAPK by an antagonist resulted in an increase in cardiomyocyte contractility. The authors found that the increase was comparable to that of PTX inhibition and that the two effects were not additive suggesting that the p38-MAPK was activated through a G α i dependent mechanism (Paur et al., 2012).

Therefore from the research conducted in the literature on p38-MAPK a hypothesis can be made that could explain the results in this chapter. Increased microRNA-1 expression restores the

inhibition of TAO 1 and 3 expression back to normal levels, this limits the activation of the MKK3/6 – p38MAPK pathway and removes the dampening effect of p38-MAPK on contractility in a $G\alpha_i$ dependent manner. The ability of TAO kinases to influence p38-MAPK function through MEKK3/6 has not been studied in cardiomyocytes. Results have been focussed on how TAO kinases interact with p38-MAPK in response to cellular stress and DNA damage and the subsequent effect on the cell cycle and DNA repair (Craig et al., 2008). It has been found that in embryonic chick ventricular cardiomyocytes β_2 -adrenoceptor activation caused a dose dependent phosphorylation and activation of p38-MAPK (Magne et al., 2001). This complicates the hypothesis as in theory the increased microRNA-1 expression would therefore be suppressing p38-MAPK activity through TAO kinase whilst simultaneously increasing its activity through improved β_2 -adrenoceptor function. There is enough evidence to make this a possible explanation and there has been speculation in the literature about the influence of TAO kinase on p38-MAPK in the context of cardiac function.

ERK1/2 are also cardiac relevant targets identified in the list of microRNA-1 sequence matches. ERK1/2 is activated in response to a large range of stress and agonist-induced hypertrophic stimuli (Lorenz et al., 2009), this results in the induction of cardiac hypertrophic responses including increased fetal-gene expression, cardiomyocyte growth, and/or reorganisation in neonatal cardiomyocyte cytoskeleton (Heineke and Molkentin, 2006). Upstream of ERK1/2 is Ras, a member of the small G protein Ras-mediated signalling pathway it has the ability to activate ERK1/2 as part of its downstream signalling spectrum. In a study where Ras was overexpressed in transgenic mice there was a significant reduction in cardiomyocyte calcium transient amplitude through reduced SERCA2a mRNA expression and hypophosphorylation of PLB (Zheng et al., 2004). There was also a negative effect in the cardiac performance of these animals, the contractile force exerted (dP/dt_{max}), relaxation (dP/dt_{min}) and end-diastolic pressure of the transgenic hearts after treatment with increasing concentrations dobutamine were significantly worse compared to controls. Although total ERK protein was increased in the hearts of the transgenic animals, Ras also activates other targets such as Akt, so it is not known whether the results observed can be attributed entirely to changes in ERK.

From the research conducted on ERK1/2 and the direct match to microRNA-1 sequence it is possible to derive the following hypothesis. Increased microRNA-1 depresses ERK1/2 expression which restores contractile performance by improving the calcium transient amplitude and SERCA2a and PLB phosphorylation. SERCA2a mRNA expression was tested (figure 4B) and found to be unaffected by microRNA-1 transfection and there are no examples of ERK1/2 inhibiting β_2 -adrenoceptor

induced cAMP production in the literature, these two caveats weakens the strength of this possibility that ERK1/2 is the target of microRNA-1. As well as this it has been found that transgenic mice overexpressing ERK1/2 develop stable concentric hypertrophy with few indications of histopathology or interstitial cell fibrosis at 6 months (Bueno et al., 2000). Isolated working heart preparations of the transgenic hearts had improved ventricular function and were considered as a physiological rather than pathological hypertrophic phenotype. This would suggest that increased ERK1/2 activity is not necessarily associated with a negative influence on contractility. Understanding whether microRNA-1 operates through this mechanism to cause the results in this chapter would need to be investigated.

Conclusion

The results presented in this chapter are from the *in vitro* transfection of a range of cardiac cell types to increase microRNA-1 expression and the effect that had on the expression and function of a number of cardiomyocyte components. The mRNA expression of SERCA2a, β_1 - and β_2 -adrenoceptor were unchanged in adult rat heart failure cardiomyocyte after the expression of microRNA-1 was increased, but the contractile amplitude of the same cardiomyocytes to the adrenoceptor agonist isoprenaline was improved. Upon investigation this was found to be a β_2 -adrenoceptor specific effect and this discovery led to further experiments in the attempt to understand the mechanism of the microRNA-1 influence on β_2 -adrenoceptor activity. The first experiment found that the ability of the β_2 -adrenoceptor to trigger cAMP production via AC was upregulated after adeno.miR-1 transfection. It was hypothesised that microRNA-1 may regulate the functional selectivity of β_2 -adrenoceptor coupling and cause a switch from the $G_{\alpha i}$ to the $G_{\alpha s}$ pathway. When tested the results were inconclusive and this remains an uncertainty. β_2 -adrenoceptors are tightly distributed at the T-tubules, but in heart failure they become more diffuse due to a breakdown in T-tubular structure. The regularity of healthy adult rat cardiomyocytes was tested after adeno.miR-1 but this was not improved by the increased microRNA-1 expression. Although the results in this chapter present a novel finding; that of increased β_2 -adrenoceptor mediated contractile response in heart failure after microRNA-1 expression is normalised, the mechanism of this relationship has yet to be determined.

Chapter 5: Cardiac targeted delivery of microRNA-1 to an *in vivo* model of heart failure by an adeno-associated virus (AAV9)

Introduction

The role microRNA-1 plays in regulating key components of cardiomyocyte function and how this may be linked to heart failure is an emerging field. In the previous chapter the effect of increased microRNA-1 expression by *in vitro* transfection was studied in cardiomyocytes derived from a model of heart failure. When examining the functionality of the cardiomyocytes the increased microRNA-1 expression was able to restore β_2 -adrenoceptor mediated contractility, a finding that has expanded upon the current level of understanding of the role of microRNA-1 in heart failure. As discussed in the previous chapter, the underlying mechanism of the relationship between microRNA-1 and β_2 -adrenoceptor function must be determined. As well as understanding the molecular mechanism it is important to replicate this result *in vivo*.

There are multiple differences between a cardiomyocyte that is one of millions of others that make up the myocardium and one that is in a culture dish after enzymatic isolation. Cardiomyocytes in the heart are exposed to electrical, hormonal and physical influences as well as contracting and relaxing up to several hundred times a minute in rodents. Many of these influences are lost when cardiomyocytes are cultured *in vitro* and consequently how much credence can be attached to a result is always limited because these are important influences on cardiomyocyte functionality. By studying freshly isolated cardiomyocytes the cellular function should adhere more closely to when part of the myocardium compared to after 48 hours of deterioration in culture. Therefore after conducting the experiments presented in the previous chapter the next step was to repeat the increased microRNA-1 expression in an *in vivo* model of heart failure. This would provide the opportunity to study the effect of increased microRNA-1 in heart failure over a longer period of time compared to *in vitro* where adult cardiomyocytes typically survive up to 48 hours.

To increase microRNA-1 expression in a cardiac targeted manner in an animal model required a delivery mechanism. Professor Giacca's team at the AAV Vector Unit of the International Centre for Genetic Engineering and Biotechnology Trieste kindly produced two AAVs, one containing LacZ (AAV9.LacZ) as a control and a second with microRNA-1 (AAV9.miR-1). These were delivered via jugular vein injection to animals that had undergone MI surgery around 16 weeks earlier and were considered to have progressed to heart failure. The exact method for MI surgery and jugular vein injection are detailed in the Methods sections: Myocardial infarction surgery and AAV9 jugular vein injections. The time course of surgery, injections and subsequent experiments is shown in the Methods section of this chapter. The animals were subject to weekly echocardiography

measurement in order to determine whether the increased microRNA-1 expression could initiate an improvement or at least offset the decline in cardiac performance associated with heart failure. Then after sacrifice a range of experiments were conducted on isolated cardiomyocytes in order to repeat and expand upon the results observed after *in vitro* transfection to increase microRNA-1 expression. The primary target of these additional experiments was SERCA2a because of its key role in maintaining calcium homeostasis and due to previous studies having shown that upregulation of microRNA-1 expression in a model of heart failure resulted in an improvement in SERCA2a expression (Karakikes et al., 2013). The authors did not examine whether the ability of SERCA2a to pump calcium back into the SR during diastole had also improved and so this represented an opportunity to expand upon their findings.

Aims

- Explore the effect of β_2 -adrenoceptor mediated contractility after increased microRNA-1 expression in an *in vivo* model of heart failure.
- Determine whether increased microRNA-1 expression is able to affect the cardiac performance of adult heart failure rats over time by echocardiography.
- Take isolated cardiomyocytes from adult heart failure rats that have been treated with AAV9.miR-1 and measure SERCA2a function, T-tubular regularity and the expression of a range of cardiac relevant genes.

Methods

Study design

The results in this chapter are derived from two groups of heart failure adult rats that were administered with a single intravenous dose of either AAV9.LacZ or AAV9.miR-1. The methods for the individual experiments are detailed in the methods chapter, whereas the chronological order of the experiments that were conducted is illustrated in table 5A:

Week	0	→	16-18	→	21-27	26-28
List of procedures/ experiments	- Myocardial infarction surgery: 10x adult rats	- Hypertrophy progresses into heart failure	- Baseline echocardiography - Staggered AAV9 LacZ/ miR-1 jugular vein injections	- Wound healing	- Weekly echocardiography	- Sacrifice - β_2 -Adrenergic Receptor Contractility - Intracellular calcium transients - T-tubular regularity - Gene Expression

Table 5A: The study protocol for the in vivo AAV9.LacZ/ miR-1 treated heart failure adult rats.

At week 0, 10 adult rats underwent MI surgery and then were allowed to progress over 16 weeks from hypertrophy into heart failure. A baseline echocardiography measurement was taken at this point to confirm reduced cardiac performance before the procedure for jugular vein injection of AAV9.LacZ or AAV9.miR-1 was conducted. These procedures were ably assisted by Dr Laura Roca Alonso. After 5 further weeks to allow wound healing from the surgery, a weekly echocardiography programme was conducted for a period of 5 weeks. At the end of this programme the animals were sacrificed, left ventricular tissue samples were taken and cardiomyocytes enzymatically isolated. The cardiomyocytes were then tested for β_2 -adrenoceptor mediated contractility, intracellular calcium transients and T-tubular regularity. The jugular vein injections were staggered over a two week period to allow the subsequent sacrifices and cardiomyocyte isolations to be conducted over several days. As a comparison, terminal echocardiography measurements from 8 age-matched controls were included in the results, these measurements were collected by Dr Markus Sikkell.

Results

LacZ staining of tissue from AAV9.LacZ treated healthy adult rats

In order to determine the optimum AAV9 dose for the two groups of heart failure adult rats, a range of AAV9.LacZ doses were tested in healthy adult rats over a timecourse of 3 weeks. Four treatment arms were included in this initial experiment, assaying three doses of LacZ in a logarithmic scale and using saline injections as a control. After the three weeks the animals were sacrificed, tissue from the heart removed and stained for LacZ.

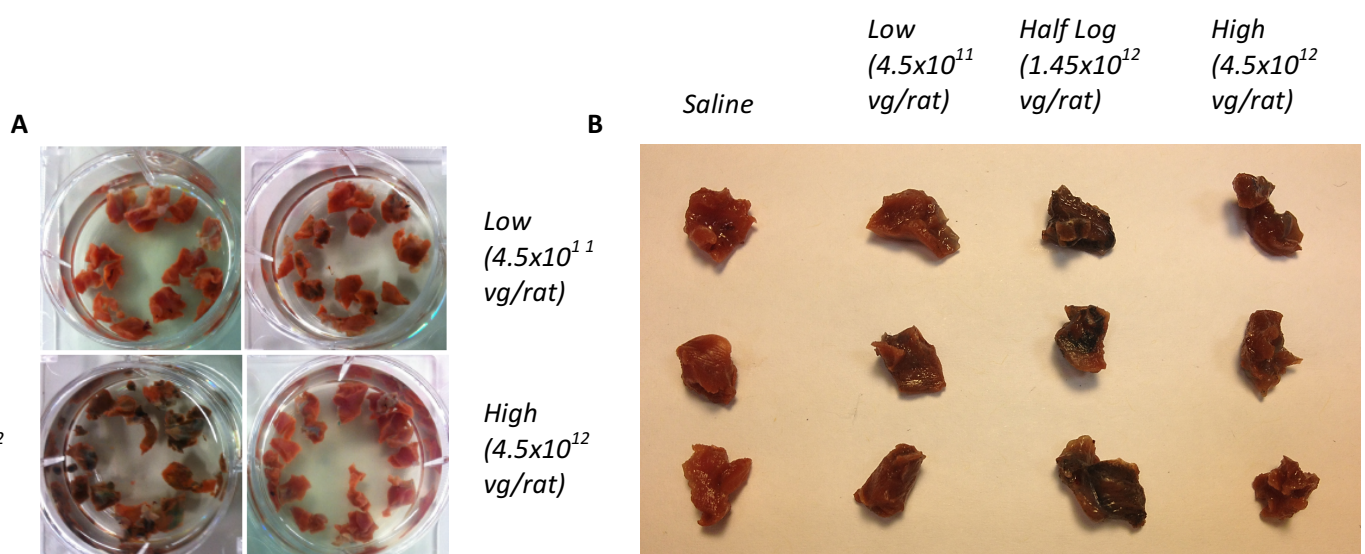


Figure 5A: LacZ staining detection in pieces of hearts from AAV9.LacZ treated healthy rats.

A) Tissue LacZ staining performed on minced treated heart tissue. One image per dose (Saline control, Low (4.5×10^{11} vg/rat), Half Log (1.45×10^{12} vg/rat) and High (4.5×10^{12} vg/rat) as representative of n=3 animals.

B) One stained tissue piece per heart disposed according to the viral dose received.

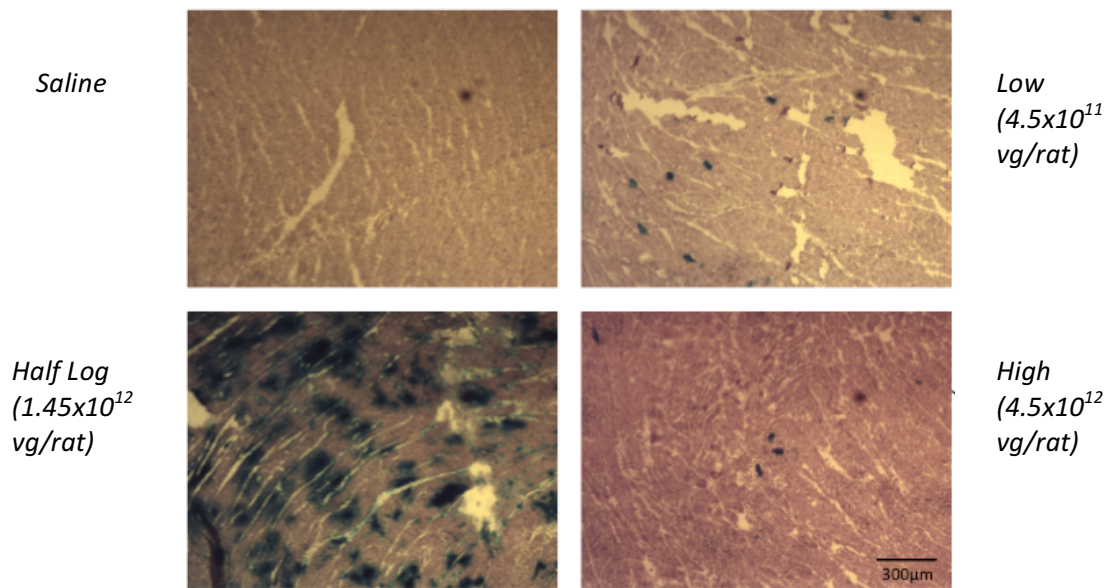


Figure 5B: Representative images of stained heart slices harvested from AAV9.LacZ treated healthy rats.

Tissues were embedded in OCT compound and 10 μ m thick sliced were cut using a cryostat, prior to LacZ staining.

Quantification and comparison across dosing groups of the LacZ stained pieces of heart tissue such as those show in figure 5A is difficult. To resolve this issue, histochemistry for LacZ reporter detection was conducted on sliced heart tissue; the relative percentage of LacZ stained cells detected by histochemistry (figure 5B) was the highest in the half log dose (1.45×10^{12} vg/rat) group, something that was corroborated by visual inspection of the pieces of heart tissue (figure 5B). This was the dose selected for AAV9.miR-1 and AAV9.LacZ treatment of the adult heart failure rats. Although not visible in the images shown in figure 5B, in the high dose group there was a substantial infiltration of nuclei appearing to be inflammatory cells (figure 5B) which further enhanced the case to use the half log dose.

MicroRNA-1 expression after AAV9 treatment of adult heart failure rats

After determining the optimum dose for AAV9.LacZ and AAV9.miR-1 treatment (figure 5A and 5B) the next step was to examine if the intravenous delivery of microRNA-1 by AAV9 to the heart was successful and if the expression of microRNA-1 was affected by the AAV9.miR-1. When the two cohorts of adult heart failure rats had completed their 10 weeks post jugular vein injections of

AAV9.LacZ or AAV9.miR-1, the cardiomyocytes from the hearts were isolated and the levels of microRNA-1 expression tested.

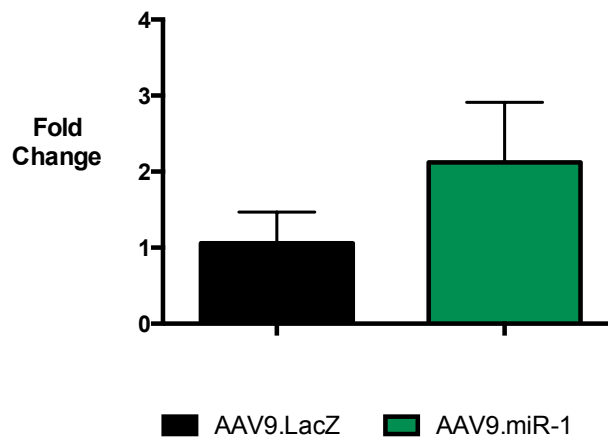


Figure 5C: RT-qPCR analysis of microRNA-1 expression in isolated cardiomyocytes from AAV9.LacZ and AAV.miR-1 treated adult heart failure rats.

MicroRNA-1 expression is shown as fold change, this was calculated using RNU6B as a housekeeping gene for normalisation. Mann Whitney test used for statistical comparison, n=4 animals per group, data was non significant.

Expression of microRNA-1 was higher in the AAV9.miR-1 group (2.12 ± 0.4) versus AAV9.LacZ group (1.06 ± 0.2). Although the 1 fold change value of the AAV9.miR-1 group was double that of AAV9.LacZ it was not a significant result ($P=0.054$). It is possible that this is because the experiment was underpowered by a low n number; when the individual triplicates from each animal were included for statistical comparison the $P=0.0003$. This aside, compared to AAV9.LacZ, AAV9.miR-1 has resulted in a non-significant increase in microRNA-1 expression in adult heart failure rats. Prior to the jugular vein injections for AAV9.LacZ and AAV9.miR-1 being administered two rats died, although no autopsy was conducted this was most likely due to the effects of the MI.

Echocardiography measurements of cardiac performance after AAV9 treatment

At 16 weeks post MI surgery the heart failure rat model displays a relatively stable cardiac performance that can be maintained for several further weeks. This period of stability was used to act as a baseline in order to see if increased microRNA-1 expression after AAV9.miR-1 treatment could cause a detectable change to cardiac performance that developed over time. Producing echocardiography results is heavily dependent on the operator; the positioning of the animal, the heart and the angle of the ultrasound probe are dependent on the operator achieving consistency

from measurement to measurement. This is difficult to achieve and makes collecting reliable data over time that is statistically comparable technically challenging. Each animal was used as its own baseline which enabled the use of paired measurements and does increase the likelihood of identifying any statistical differences in the data. Any emerging trends between the two treatment groups could also be tracked week by week. This would be considered more reliable at the end of the time course of several weeks compared to a difference detected in a one off measurement prior to sacrifice. The cardiac parameters that were measured are listed below:

LVIDd	Left ventricle internal diameter end diastole
LVIDs	Left ventricle internal diameter end systole
EF	Ejection fraction
FS	Fractional shortening
SV	Stroke volume
BW	Body weight
TL	Tibia length
ESV	End systolic volume
EDV	End diastolic volume

Table 5B: The range of cardiac parameters measured by echocardiography in the adult heart failure rats.

Serial left ventricle internal diameter and volume measurements after AAV9 treatment

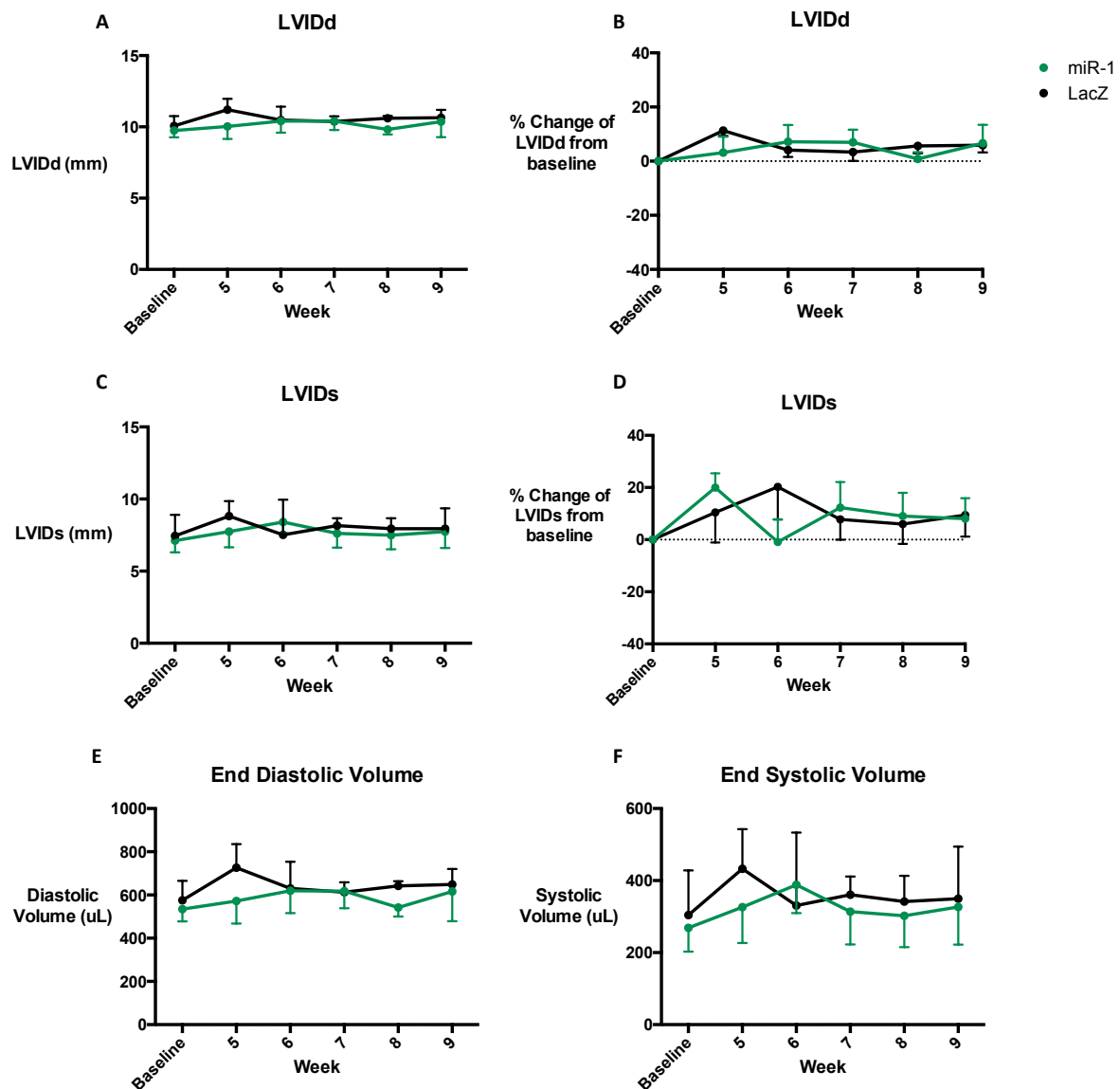


Figure 5D: Serial echocardiography measurements of a range of cardiac parameters over a 5 week period from AAV9.miR-1 and AAV9.LacZ treated heart failure rats.

$n=4$ animals per data point for both groups. F-test used for comparison of curves, all data was non significant:

A) Raw left ventricle internal diameter (LVID) end diastole measurements (mm)

B) LVIDd normalised to baseline and displayed as the percentage change (%)

C) Raw LVIDs measurements (mm)

D) LVIDs normalised to baseline and displayed as the percentage change (%)

E) End systolic volume (μL)

F) End diastolic volume (μL)

Serial ejection fraction, fraction shortening, stroke volume and body weight measurements after AAV9 treatment

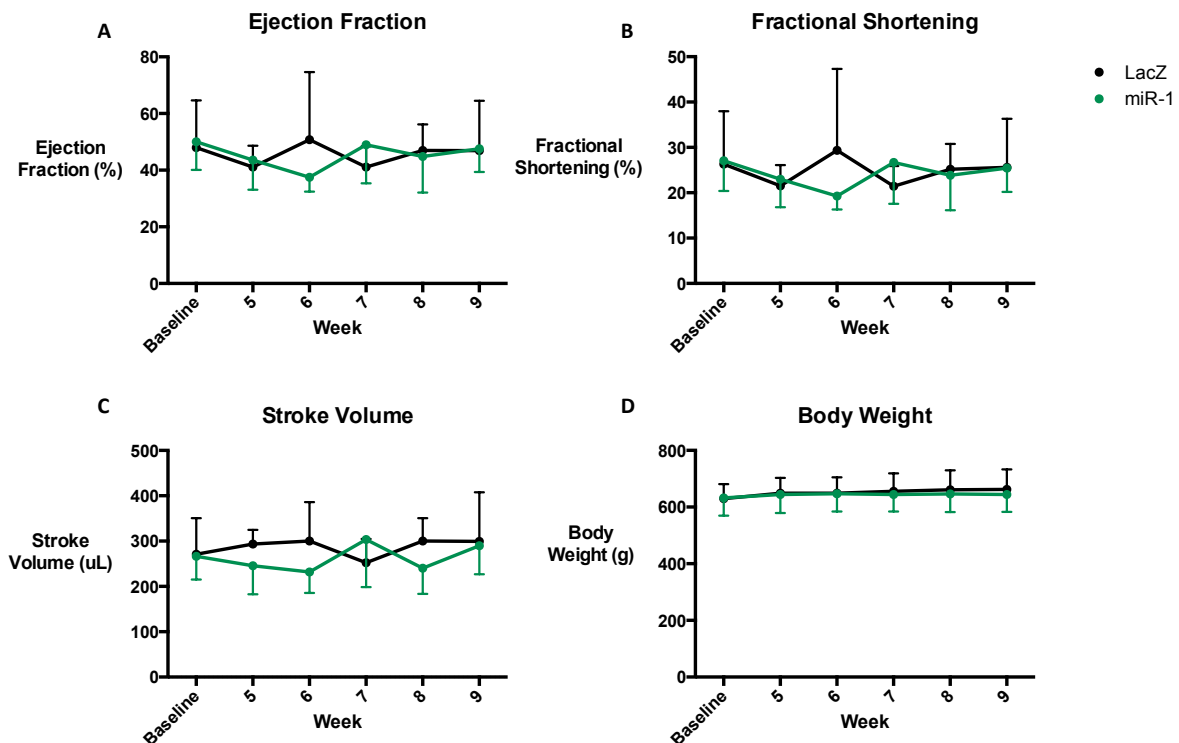


Figure 5E: Serial physiological, and echocardiography measurements of a range of cardiac parameters over a 5 week period from AAV9.miR-1 and AAV9.LacZ treated heart failure rats. $n=4$ animals per data point for both groups. F-test used for comparison of curves, all data was non significant:

A) Ejection fraction (%)

B) Fractional shortening (%)

C) Stroke volume (μ l)

D) Body weight (g)

For all the metrics displayed in figures 5D and 5E none show a significant difference between the AAV9.LacZ treated group compared to the AAV9.miR-1 group. In terms of trends, LVIDd showed a slight increase over time as would be expected with heart failure (figures 5D-A and -B) whilst LVIDs appeared to be unchanged between the two treatment groups. Ejection fraction, fractional shortening and stroke volume are all relatively consistent over the 5 week period whilst the range of body weights within each group is large making it difficult to draw any trends from the data (figure 5E-D).

Terminal echocardiography measurements compared to age matched controls

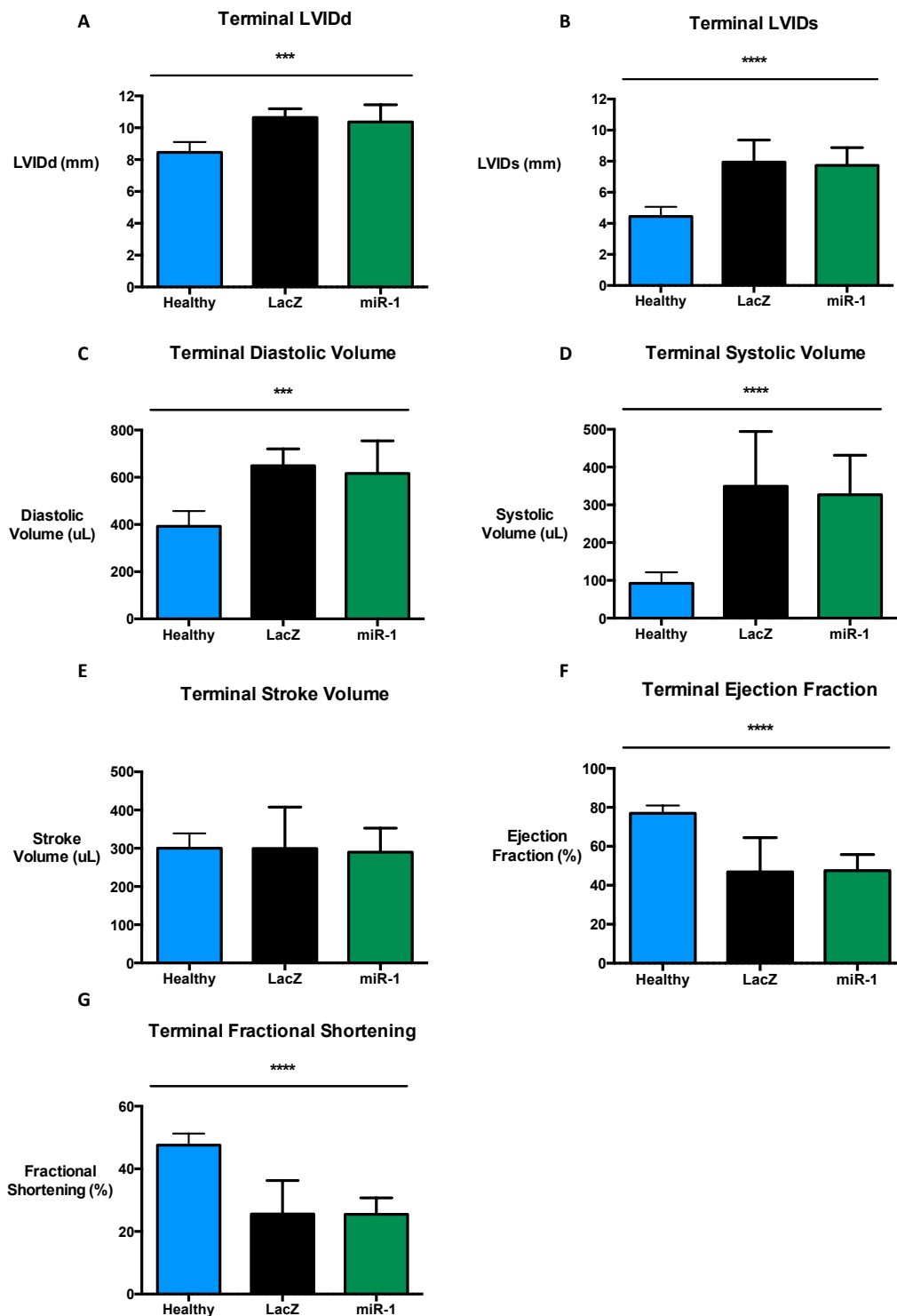


Figure 5F: Terminal echocardiography measurements of a range of cardiac parameters compared to age matched controls.

$n=4$ animals for both AAV9.LacZ and AAV9.miR-1 groups, aged matched controls (healthy) are included for comparison ($n=8$ animals). One-way ANOVA with Tukeys post-hoc test for statistical comparison of the terminal measurements in A-G:

- A) LVIDd ($***P=0.0001$)
- B) LVIDs ($****P<0.0001$)
- C) End diastolic volume (EDV) ($***P=0.0001$)
- D) End systolic volume (ESV) ($****P<0.0001$)
- E) Stroke volume ($P=0.03966$)
- F) Ejection fraction (EF) ($****P<0.0001$)
- G) Fractional shortening (FS) ($****P<0.0001$)

In order to give context to the echocardiography data, the terminal measurements of the AAV9.LacZ and AAV9.miR-1 groups are shown in figure 5F together with age-matched controls (healthy) for comparison, the terminal measurements produced the same result as in figures 5D and 5E, with no significant difference between the AAV9.LacZ and AAV9.miR-1 treated groups. Compared to aged-matched controls (healthy) it is clear that at sacrifice both the AAV9.LacZ and AAV9.miR-1 animals had the pathological characteristics of heart failure. LVIDd and EDV (5F-A and -C) were higher in heart failure animals as the hearts showed diastolic dysfunction and dilation. Ejection fraction was lower in heart failure animals (figure 5F-F) and ESV was increased due to both dilation and poor ejection fraction (figure 5F-D). Unfortunately the non-significant increase in microRNA-1 expression detected in the populations of isolated cardiomyocytes from AAV9.miR-1 treated animals was unable to improve the cardiac performance of the heart failure. This would suggest that on a global scale this dose of microRNA-1 is not effective in reversing the pathological aspects of heart failure on a whole organ scale.

Single cardiomyocyte experiments from AAV9 treated adult heart failure rats

The serial and terminal echocardiography experiments did not show any differences in the cardiac performance between AAV9.LacZ and AAV9.miR-1 treated animals but it is possible that a more subtle effect could be observed at a single cardiomyocyte level. This is a rational hypothesis given the results observed in chapter 4 in single heart failure cardiomyocytes after *in vitro* transfection to increase microRNA-1 expression.

Baseline and β_2 -adrenoceptor receptor specific contractile response

After the increased β_2 -adrenoceptor receptor specific contractile response observed in chapter 4, this was the first protocol conducted with the cardiomyocytes isolated from the AAV9 treated adult heart failure rats and was conducted as described in the Methods section: β_2 -adrenoceptor specific studies. The aim was to see if the increased microRNA-1 expression caused by AAV9.miR-1 treatment could cause an improvement in the β_2 -adrenoceptor receptor specific contractile response.

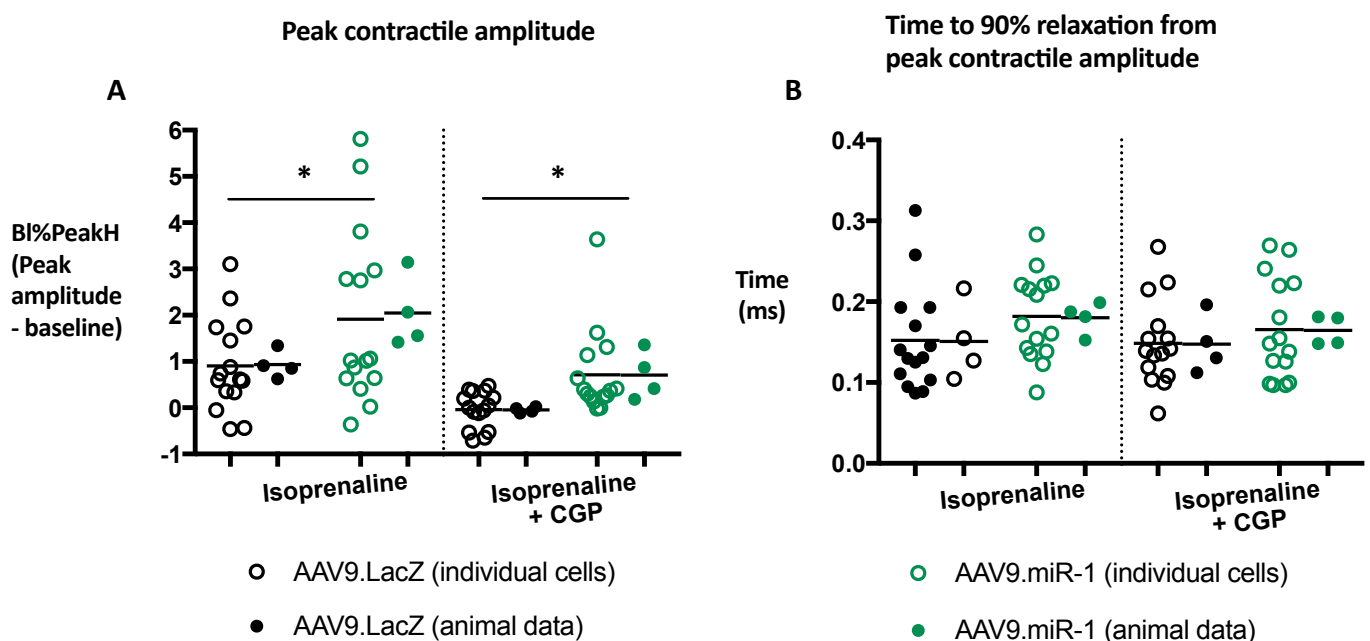


Figure 5G: β -adrenoceptor mediated contractility of freshly isolated cardiomyocytes isolated from AAV9 treated adult heart failure rats.

Cardiomyocyte contractility was measured after the addition of 30 nM isoprenaline following by washout then 30 nM isoprenaline + 300 nM CGP20712A, a β_1 -adrenoceptor selective antagonist. Mann Whitney test for all statistical comparisons. Closed circles represent the mean value for cells from one animal ($n=4$ animals), open circles represent individual cell data from all cells tested ($n=15$), $*P<0.05$.

A) BI%PeakH (Peak contractile amplitude – baseline)

B) Time to 90% relaxation from contractile peak amplitude (ms)

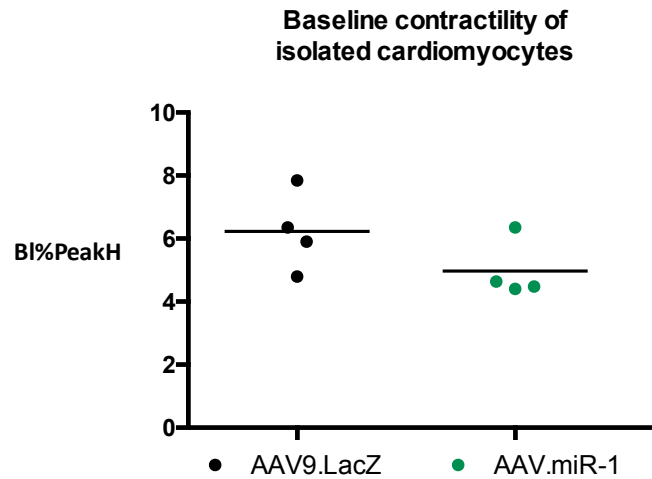


Figure 5H: Baseline BI%PeakH of freshly cardiomyocytes from AAV9.LacZ and AAV9.miR-1 treated adult heart failure rats.

Cardiomyocyte contractility was recorded before the addition of isoprenaline was added. Mann Whitney test for all statistical comparisons. $n=4$ animals for all groups (3-4 cells per animal), data was non significant.

In figure 5G-B the time to 90% relaxation of the cardiomyocytes after both isoprenaline alone and in conjunction with CGP20712A was unaffected by the increased microRNA-1 expression after AAV9.miR-1 treatment. This replicated the result that was recorded with heart failure cardiomyocytes transfected *in vitro* (chapter 4). AAV9.miR-1 treatment has resulted in an increase in the contractile amplitude compared to AAV9.LacZ after both isoprenaline alone (mean±SEM; $2.07\% \pm 0.39$ vs $0.93\% \pm 0.15$, $P=0.029$) and when isoprenaline was used with CGP20712A (mean±SEM; $0.71\% \pm 0.26$ vs $-0.047\% \pm 0.03$, $P=0.029$) (figure 5G-A). By replicating the *in vitro* result, this improves the strength of the hypothesis that increased microRNA-1 expression is able to affect how β_2 -adrenoceptor receptor functionality impacts cardiomyocyte contractility. Before the cardiomyocytes were exposed to isoprenaline the baseline contractility was recorded. The cardiomyocytes from both AAV9.LacZ and AAV9.miR-1 do not appear to have a reduced function, with the BI%PeakH $6.22\% \pm 0.63$ and $5.00\% \pm 0.46$ respectively, $P=0.11$ (figure 5H).

Intracellular calcium transient parameters

In order to measure changes in intracellular calcium the cardiomyocytes were loaded with Fura-2, a dual excitation dye (ratiometric) that changes its emission fluorescence from 380 nm to 340 nm when it becomes bound to calcium. Changes in 340 nm emission can therefore be used to measure

changes in the level of calcium within a cell, see Methods section: Intracellular calcium measurements transients for the detailed protocol. Exposure of cultured adult heart failure rat cardiomyocytes to caffeine can be technically challenging, the emptying of the SR can overwhelm the cells resulting in arrhythmia and cell death. In a previous Masters project, the calcium transients were successfully recorded from heart failure cardiomyocytes that were cultured for 24 hours then exposed to caffeine (Kumarswamy et al., 2012), but this is only a short window for the increased microRNA-1 expression to elicit an effect on its intracellular targets cardiomyocyte function.

The freshly isolated cardiomyocytes from the AAV9 treated animals provided an opportunity to study the calcium handling of the cardiomyocytes after a prolonged period with increased microRNA-1 expression on a heart failure background. One of the primary components of calcium handling that was not studied after *in vitro* transfection of microRNA-1 was SERCA2a function. By restoring calcium to the SR during diastole it maintains the intracellular store of calcium that is essential for CICR (Bers, 2001). Although SERCA2a expression was unchanged after *in vitro* microRNA-1 transfection (chapter 4), this was also the case for the β_2 -adrenoceptor receptor expression. β_2 -adrenoceptor receptor functionality was improved after increased microRNA-1, showing that (as is well established) expression levels of mRNA or protein do not always indicate function. Besides the key role SERCA2a plays in calcium handling in cardiomyocytes and its established malfunctioning during heart failure, the paradigm observed with the β_2 -adrenoceptor receptor provides the justification to study SERCA2a function.

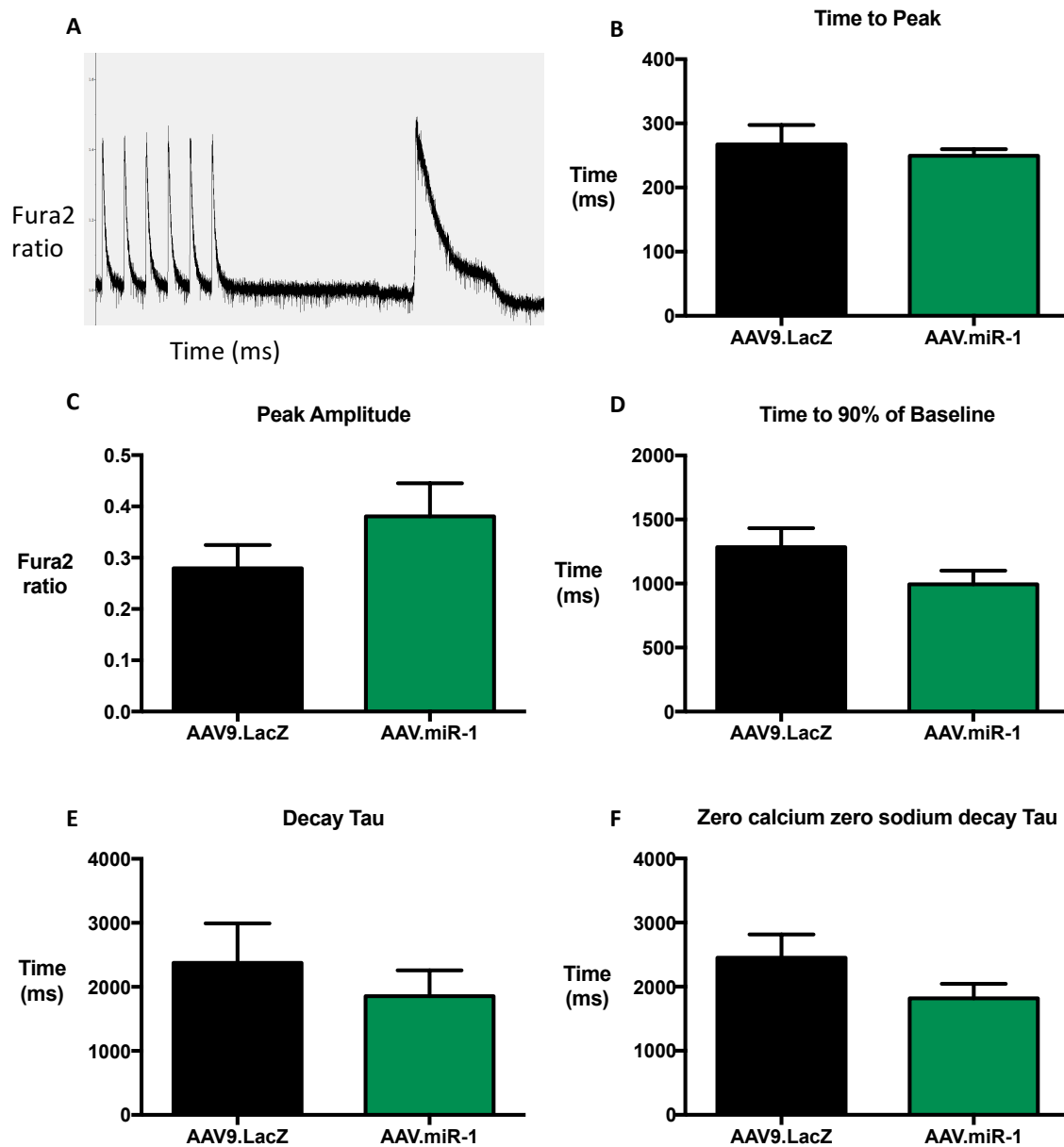


Figure 5I: Intracellular calcium transient parameters of cardiomyocytes isolated from AAV9 treated adult heart failure rats after a caffeine spritz.

Mann Whitney test for all statistical comparisons. $n=4$ animals for all groups (6-7 cells per animal), all data was non significant:

A) Representative calcium transients at 1Hz stimulation followed by a caffeine spritz

B) Time to peak

C) Peak amplitude

D) Time to 90% of baseline

E) Tau decay value derived from the decay in the presence of Caffeine solution

F) Tau decay value in the section of the value in zero sodium zero calcium solution only.

Figure 5I shows a range of parameters calculated from the calcium transients of cardiomyocytes isolated from AAV9.LacZ and AAV9.miR-1 treated adult heart failure rats. They were all derived from the calcium transient triggered by a caffeine spritz and not from the basal 1Hz stimulation (data not shown). This data would suggest that the amount of calcium mobilised and the speed at which it was mobilised from the SR after a caffeine spritz is unchanged after AAV9.miR-1 as both the peak amplitude (figure 5I-C) and time to peak (figure 5I-B) were not statistically different compared to AAV9.lacZ. In terms of clearance of calcium from the cytoplasm after mobilisation from the SR, AAV9.miR-1 does not appear to have an affect either as the time to 90% of baseline (figure 5I-D) and decay Tau (figure 5I-E) data was non significant. Finally, when SERCA2a function was restored in the zero calcium zero sodium (figure 5I-F) the clearance of calcium was still no faster after AAV9.miR-1. The means and the associated P values from conducting Mann Whitney tests after comparing AAV9.miR-1 versus AAV9.lacZ are shown in table 5C. In addition, the same test was used to compare the data from the non-hierarchical groupings.

Parameter	Hierarchical Grouping			Non-Hierarchical Grouping		
	LacZ (n=4)	miR-1 (n=4)	P value	LacZ (n=25)	miR-1 (n=27)	P value
Time to peak (ms)	267±30	249±10	> 0.99	271±33	255±20	0.77
Peak amplitude	0.279±0.05	0.381±0.06	0.34	0.294±0.03	0.406±0.05	0.015*
Time to 90% baseline (ms)	1285±149	995±107	0.20	1321±158	1002±79	0.15
Decay Tau	2376±616	1854±405	0.83	2432±348	1924±240	0.48
0Ca ²⁺ 0Na ⁺ Decay Tau	2451±364	1820±226	0.49	2417±197	1867±140	0.038*

Table 5C: The mean±SEM and P values for the parameters calculated from the calcium transients of cardiomyocytes isolated from AAV9.LacZ and AAV9.miR-1 treated adult heart failure rats when the data is grouped by animal (hierarchical) and by cell (non-hierarchical).

For hierarchical grouping, the mean of all cells tested from an animal represented 1 replicate, there were 4 animals per group and so the n number is 4 for the AA9.LacZ and AAV9.miR-1 groups. In non-hierarchical grouping each cell is treated as an individual replicate and the n number represents all cells tested across the 4 animals. A Mann Whitney test was used for all statistical comparisons,

**P<0.05.*

Table 5C is shown to highlight the difference between the types of data derived from this experiment that can be used for statistical testing. When hierarchical groupings were used none of the parameters were significant, but for non-hierarchical groupings both peak amplitude and zero

sodium zero calcium decay were significant by a t-test (Mann Whitney). Both data sets had small SEM values so it is possible that the test is underpowered due to the low n number of the hierarchical groupings. A larger n number could resolve this issue but without repeating the same experiment with more animals it is impossible to know whether this is the case or not.

If these two results are an accurate representation of the effect increased microRNA-1 expression in heart failure cardiomyocytes they are interesting results. The primary mechanism by which calcium is loaded into the SR during diastole is by SERCA2a; in rats 92% of the calcium removed by from the cytoplasm is by SERCA2a (Bers, 2001). A greater peak amplitude after a caffeine spritz would suggest that more calcium has been loaded into the SR during diastole. When the caffeine renders RyR in the active state, all calcium is emptied from the SR into cytoplasm and this yields the peak amplitude value recorded in figure 5I-C. Clearance of calcium in zero sodium zero calcium solution is attributed to SERCA2a as NCX function is nullified in the solution. The non-hierarchical grouping of the data in table 5C would suggest that SERCA2a is clearing the calcium more quickly after AAV9.miR-1, but these conclusions are not possible when the hierarchical groupings are considered.

T-tubule regularity

Another parameter of the freshly isolated cardiomyocytes from the AAV9 treated heart failure animals that was measured was T-tubule regularity. As discussed in the Introduction section: Impaired calcium mobilisation in systole, the T-tubule represents a key component of CICR, the spatial conduit that groups LTCCs and the SR together that is disrupted in heart failure. In this experiment, freshly isolated cardiomyocytes were used rather than cultured cardiomyocytes as in chapter 4. This meant that rather than measuring the effect of increased microRNA-1 expression against the degradation of cell culture, the effect of microRNA-1 increased expression was measured against degradation of the T-tubular structure due to MI induced heart failure.

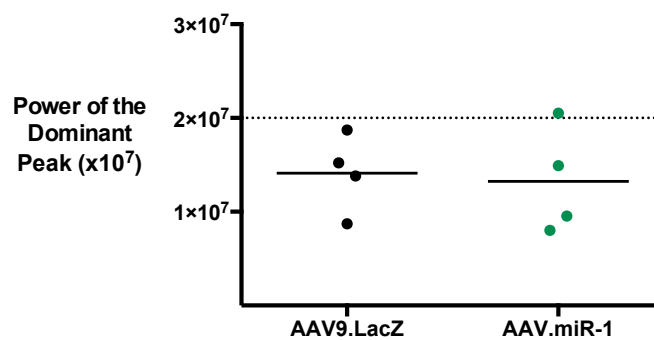


Figure 5J – The regularity of CMO stained adult heart failure rat cardiomyocyte t-tubular membranes isolated from animals treated with AAV9.LacZ or AAV9.miR-1.

The dotted line represented the typical T-tubular regularity of healthy, freshly isolated cardiomyocytes. Mann Whitney test, $n=4$ animals (5 cells per animal), data was non significant.

As observed in figure 5J, AAV9.miR-1 treatment did not appear to have had any effect on improving T-tubular regularity compared to AAV9.LacZ in cardiomyocytes isolated from adult heart failure rats. Healthy, freshly isolated cardiomyocyte typically have a T-tubular regularity of $\sim 2 \times 10^7$ (represented by the dotted line), which deteriorates when cardiomyocytes are kept for prolonged periods in culture as well as part of the pathological phenotype of heart failure. This reduction in regularity is evident in both groups of heart failure animals. The mean values of the two groups were almost the same; $1.41 \times 10^7 \pm 2.1 \times 10^6$ vs $1.32 \times 10^7 \pm 2.8 \times 10^6$ for AAV9.LacZ and AAV9.miR-1 respectively. There was a relatively large $\pm$ SEM for each but this is not to suggest that it is hiding any trend or difference between the two groups. One suggestion that can be drawn from the data is that one animal from each group had a mild heart failure phenotype in the context of T-tubule regularity. The largest value for T-tubule regularity in each group is comparable to healthy cardiomyocytes, with both $\sim 2 \times 10^7$, whereas the other three animals in each group present with valued more expected of heart failure $\sim 1.5 \times 10^7$.

RT-qPCR analysis of a range of cardiac relevant gene targets in isolated cardiomyocytes

As well as conducting functional experiments on the isolated cardiomyocytes from the AAV9.LacZ and AAV9.miR-1 animals, a portion of cardiomyocytes were saved for analysis of the mRNA expression a range of cardiac pertinent genes. β_1 - and β_2 -adrenoceptor receptors, SERCA2a and NCX expression was studied because of results collected as part of this study and previous work conducted (Kumarswamy et al., 2012). Atrial natriuretic peptide (ANP) expression is increased in the ventricles of pressure overloaded adult human myocardium in response cellular hypertrophy (Izumo et al., 1988) and the ventricles of heart failure patients with a range of cardiomyopathies (Saito et

al., 1989). Skeletal muscle α -actin (Acta1) and cardiac α -actinin, (Actc1) are both hypertrophic fetal genes and are markers of hypertrophy. α - and β -myosin heavy chain (Myh6 and Myh7 respectively) expression correlates with contractility in opposite fashions; in the heart failure myocardium increased expression in Myh7 results in decreased power output (Schiaffino and Reggiani, 1996) whilst decreased Myh6 expression has the same effect (Miyata et al., 2000). The aim of this experiment was to understand whether increased microRNA-1 expression was able to reverse the expression pattern observed in heart failure of any of these cardiac gene targets.

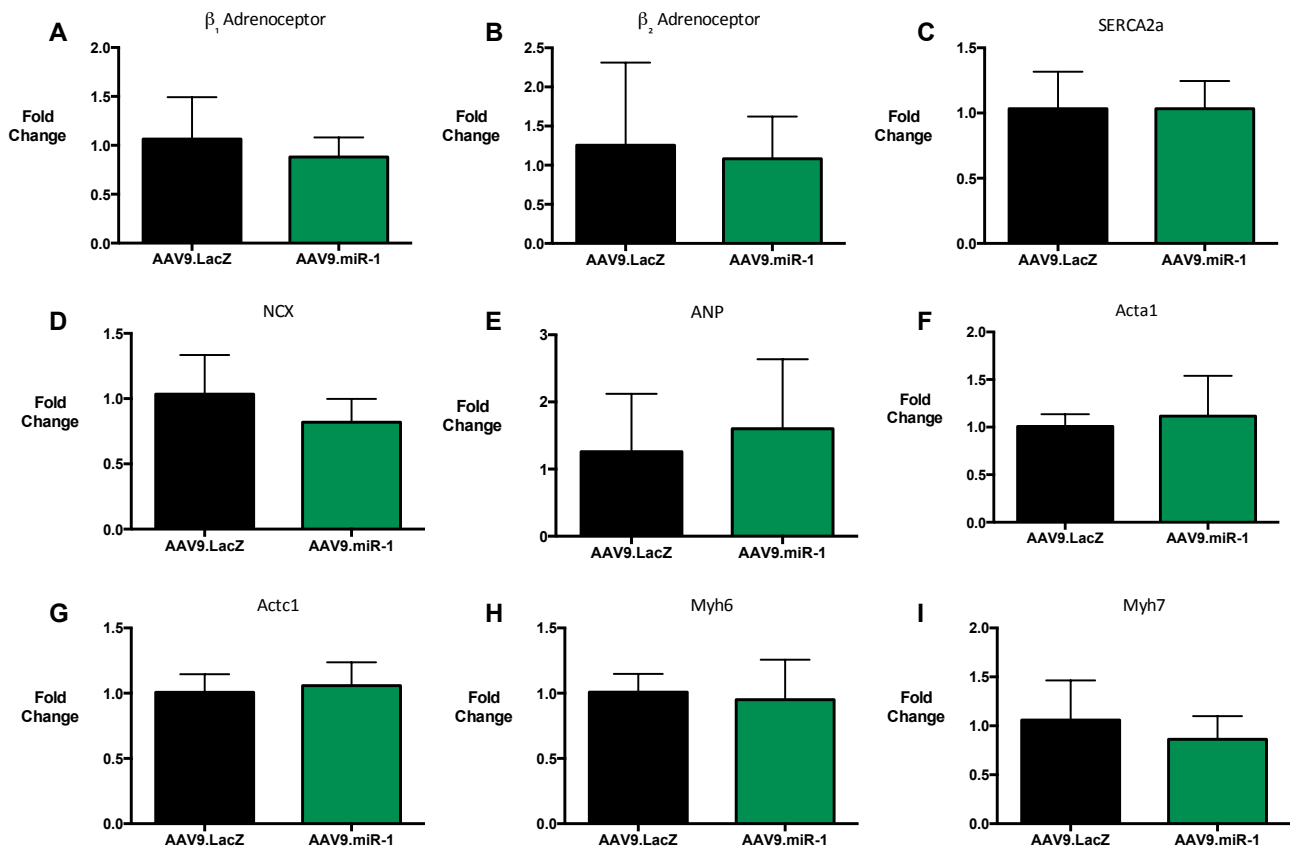


Figure 5K: TaqMan RT-qPCR analysis of the expression of a range of cardiac gene targets from freshly isolated cardiomyocytes from AAV9.LacZ or AAV9.miR-1 adult heart failure rat cardiomyocytes.

Gene expression is shown as the mean fold change $\pm$ SEM of AAV9.miR-1 cardiomyocytes compared to AAV9.GFP with GAPDH used as a housekeeping gene for normalisation in both groups. Mann Whitney Test and $n=4$ for all groups, all data was non significant:

- A) β_1 adrenoceptor receptor
- B) β_2 -adrenoceptor receptor
- C) SERCA2a
- D) NCX
- E) Atrial natriuretic peptide (ANP)
- F) Skeletal muscle α -actin (Acta1)
- G) Cardiac α -actinin, (Actc1)
- H) α -Myosin heavy chain (Myh6)
- I) β -Myosin heavy chain (Myh7)

Of genes tested in figure 5K, none had a significant difference in the fold change of mRNA expression between the AAV9.LacZ and AAV9.miR-1 groups. This would suggest that increased microRNA-1 expression in heart failure does not affect any of these target genes. Without having the expression

of healthy cardiomyocytes to compare against, it is uncertain whether the expression of the target genes have followed the expected changes associated with heart failure that were described earlier.

Discussion

AAV9.miR-1 does not affect cardiac performance or the expression of cardiac gene targets

The echocardiography data (figure 5F) would suggest that both groups are in a state of heart failure compared to the healthy aged matched controls, for example the terminal measurement of ejection fraction in both AAV9.miR-1 (46.9%) and AAV9.LacZ (47.6%) were comparable to previous studies conducted (Lyon et al; 2009), but there does not appear to any change in cardiac performance after AAV9.miR-1 treatment as measured by echocardiography (figure 5D and 5E). This could be expected as AAV9.miR-1 treatment did not result in a non-significant increase in the expression of microRNA-1 in isolated cardiomyocytes from AAV9.miR-1 treated animals versus AAV9.LacZ (figure 5C) and the expression of a known microRNA-1 cardiac gene target (NCX) was also unaffected (figure 5K). As the increase in microRNA-1 mRNA was not statistically significant it is only the LacZ staining and significant functional effects seen on other parameters that indicate that the AAV9 treatment was in any way effective.

There are examples of a single AAV9 dose triggering a functional change in the heart in the literature; AAV9.SERC2a gene transfer in adult heart failure rats (the same model as used in this study) resulted in an improvement in the following Pressure-Volume (PV) loop measured metrics; Emax, energetic efficiency, end-diastolic pressure volume relationship and end-diastolic pressure (Lyon et al., 2011). In another model of heart failure induced by TAC surgery in adult rats where 2 weeks post-surgery the animals are administered a single dose of AAV9.miR-1 (Karakikes et al., 2013). Seven weeks later both the echocardiography and PV measurements of AAV9.miR-1 animals showed a reversal of hypertrophy and deterred cardiac functional deterioration. Finally in a large animal model of heart failure by where the left circumflex coronary artery of domestic pigs by was blocked by balloon occlusion, a single AAV9 dose of the calcium sensor protein S100A1 resulted in improved cardiac performance as measured by echocardiography and PV loops (Pleger et al., 2011). The purpose of stating these other studies is to show that a single AAV9 dose is able to affect cardiac performance in the same model of heart failure as the one used for this study as well as other models of heart failure.

Drawing trends from the echocardiography measurements would require much larger n numbers and so to confirm that AAV9.miR-1 is unable to reverse the detrimental effect of heart failure on cardiac performance would require a repeat study with a larger group of animals. One alternative

non-invasive technique to echocardiography is MRI which has a temporal resolution that is 1.8-fold higher than echocardiography. A recent study found that it would require five times more animals to find a statistically significant difference in repeated measurements of small animal left ventricles by echocardiography than MRI (Stuckey et al., 2008).

Certain parts of the RT-qPCR results observed in figure 5K are more difficult to explain, previous experiments have shown that NCX (a known target of microRNA-1) function in cardiomyocytes is readily affected by changes in microRNA-1 (Kumarswamy et al., 2012). Therefore it was somewhat surprising to see that NCX expression in isolated cardiomyocytes after AAV9.miR-1 treatment is unchanged (figure 5K-D). Mean microRNA-1 expression was 2 fold higher following AAV9.miR-1 treatment compared to control but this difference did not reach statistical significance, whereas in a previous Master project adult cardiomyocytes were transfected with a Pre miR-1 construct by lipofection *in vitro* which caused a ~15 fold increase in microRNA-1 expression. Therefore it is possible that the marginal and non-significant change in microRNA-1 *in vivo* has been too small to affect this or any of the other genes. It would be pertinent to analyse the expression of NCX and the other cardiac gene targets in healthy animals as a comparison against the heart failure animals studied here. This would give an indication as to whether the pathological phenotype of heart failure was also present on a gene expression level as well as some of the other cellular and cardiac performance parameters measured in the chapter. Protein level measurements rather than expression levels might also be more informative, particularly if phosphorylation of key components such as PLB were included.

AAV9.miR-1 improves β -adrenoceptor mediated contractility in heart failure

In a repeat of the result observed with heart failure cardiomyocyte *in vitro* transfection to increase microRNA-1 expression (chapter 4), the AAV9.miR-1 treatment was able to significantly increase the contractile response mediated by the β_2 -adrenoceptor in response to isoprenaline and CGP20712A (figure 5G). There was also a significantly increased total isoprenaline response in the same experiment, which was different to the equivalent experiment after *in vitro* transfection of heart failure cardiomyocytes. There, the combined contractile response of both the β_1 - and β_2 -adrenoceptors was not significantly improved when the cardiomyocytes were exposed to isoprenaline alone. To understand whether the β_1 -adrenoceptor alone was affected, an additional experiment would be required. Instead of inhibiting the β_1 -adrenoceptor with CGP20712A, the β_2 -adrenoceptor would be inhibited with ICI 118, 551 in the presence of isoprenaline. The restored β -

adrenoceptor mediated contractile response does not appear to be caused by an increase in the regularity of the cardiomyocyte T-tubule structure. The T-tubule regularity of cardiomyocytes from AAV9.LacZ hearts was almost identical as that of AAV9.miR-1 (figure 5J). The increased microRNA-1 expression has not appeared to have caused a constitutive increase in cardiomyocyte contractility since the baseline contractility of cardiomyocytes isolated from AAV9.LacZ and AAV9.miR-1 treated hearts was similar (figure 5H).

The split of β -adrenoceptors in healthy human cardiomyocytes is around 70-80% in favour of the β_1 -adrenoceptor (Port and Bristow, 2001), but this has been found to decrease to around 50:50 in cases of dilated and ischaemic cardiomyopathies due to selective down-regulation (Bristow et al., 1986). As described in the Introduction section: Disrupted β -adrenoceptors signalling and distribution in heart failure, the desensitisation of the β_1 -adrenoceptor is dependent on the GRK2 phosphorylation after catecholamine binding to and induced activation of the receptor. This is also coupled to a reduction in the expression of the β_1 -adrenoceptor. The effect of reduced β_1 -adrenoceptor signalling in heart failure is still unclear, with some studies finding that it drives heart failure pathophysiology (Redfern et al., 2000) and others find that it has a protective effect (Ahmet et al., 2004) (Eschenhagen, 1996). What is undisputed is that β_1 -adrenoceptor function and expression both decrease in heart failure. Therefore if the result in figure 5G-A could be validated to show that increased microRNA-1 expression can improve the β_1 -adrenoceptor mediated contractility in a heart failure setting this would represent an interesting result. Particularly as the higher proportion of β_1 to β_2 -adrenoceptors in the heart in healthy cardiomyocytes means there is more scope for restoration of signalling with the β_1 -adrenoceptor.

To determine whether increased microRNA-1 expression is able to reverse the GRK2 induced downregulation of the β_1 -adrenoceptor thereby restoring its capacity to elicit cAMP production, the cAMP response of the activated β_1 -adrenoceptor could be measured by FRET. Direct measurement of GRK2 activity is an alternative experiment; after isolating the cytosolic fraction from cardiac tissue the GRK2 activity could be assessed by light-dependent phosphorylation of rhodopsin-enriched rod outer segment membranes using [γ - ^{32} P]-ATP (Iaccarino et al., 2005). The activity of tissue from AAV9.miR-1 treated hearts versus AAV9.LacZ would be compared to see if reduced GRK2 activity was occurring. Alternatively microRNA-1 may influence expression of the β_1 -adrenoceptor at a translational level, which would result in changes to the protein levels of the receptor.

Increased microRNA-1 expression may cause greater loading of the SR with calcium

Of the intracellular calcium transient parameters presented in figure 5I there are two which suggest that increased microRNA-1 expression was able to influence how the cardiomyocytes handle the movement of intracellular calcium. This is only if the non-hierarchical grouping (cellular) data is considered and not the hierarchical grouping (animal) data. The peak transient amplitude after a caffeine spritz (figure 5I-C) of AAV9.miR-1 cardiomyocytes was significantly greater than the AAV9.LacZ group. Greater calcium release upon caffeine spritz is indicative of greater calcium loading during diastole, a process which is driven by SERCA2a. A more direct measure of SERCA2a function is displayed in figure 5I-F where removal of calcium, the decay section of the transient, occurred in the presence of zero calcium zero solution. With NCX activity nullified this reduction in the calcium signal can almost solely be attributed to SERCA2a as the other 'slow systems' make up only 1% of calcium clearance from the cytoplasm. This assumes that the calcium buffering capacity of the cytosol is unchanged, if buffering capacity was reduced in one set of cardiomyocytes then the fluorescent signal detected after caffeine exposure would inaccurately suggest a greater release of calcium from the SR. As with peak transient amplitude, when using the non-hierarchical data this was significantly faster in AAV9.miR-1 cardiomyocytes. Together these two results are complementary, with the conclusion drawn from both is that the ability of SERCA2a to pump calcium from the cytoplasm into the SR was greater when microRNA-1 expression was elevated. Once again, greater animal numbers would be required to confirm this.

Both SERCA2a expression and function are known to be altered in heart failure. In human heart failure myocardium samples with dilated and ischaemic cardiomyopathies the expression of SERCA2a was reduced compared to healthy myocardium samples (Hasenfuss et al., 1994). The functional capacity of SERCA2a to load the SR with calcium and the associated levels of SR calcium are reduced in models of chronic heart failure (Lyon et al., 2011). To further verify these findings, when SERCA2a mRNA and protein expression were improved by AAV9.SERCA2a treatment in a model of heart failure, the Fura-2 calcium peak amplitude was also improved to levels comparable with controls due to improved SR calcium loading (Del Monte et al., 1999). The effect of increased microRNA-1 expression on the heart failure cardiomyocytes appears to have replicated this result. This would suggest that SERCA2a is part of the spectrum of intracellular cardiomyocyte targets that microRNA-1 is able to influence and that when microRNA-1 expression is increased it corresponds to an improvement in SERCA2a functionality.

Of the two results in figure 5I that were significant using the non-hierarchical groupings, the zero sodium zero calcium decay Tau could be less reliable. This is portion of the decay that occurred when the cardiomyocytes were exposed to zero sodium zero calcium without caffeine. Fitting a curve to that section of the decay (figure 5I-A) is more difficult for the computer software and because it makes up the tail end of the decay it is variable. Together this reduces the consistency and accuracy of the data compared to the time to 90% baseline and decay Tau which are also derived from the decay. The $\pm$ SEM from this data as a percentage of the total response was the lowest out of all the parameters measured, which would suggest there was consistency between the cells measured.

With the caffeine removed from the solution the RyR is no longer held in the active state, and as a result any calcium pumped by SERCA2a into the SR from the cytoplasm is retained in the SR. Therefore this section of the decay curve represents SERCA2a function. The cardiomyocytes isolated from the AAV9.miR-1 treated adult heart failure rats exhibited a faster decay Tau in zero sodium zero calcium solution (figure 5I-F) which complements the peak amplitude result. A greater peak amplitude would be caused by more loading of calcium in the SR, which would be due to improved SERCA2a pumping of calcium during diastole. Therefore together these two results support the conclusion that cardiomyocyte SERCA2a function is improved after AAV9.miR-1 treatment.

The RT-qPCR results displayed in figure 5K-C show that SERCA2a mRNA expression was unaffected by the increased microRNA-1 expression. As well as influencing mRNA expression, microRNAs also have the capacity to alter protein levels through repression of translation (Pillai et al., 2005) (Huntzinger and Izaurralde, 2011) and it is possible that microRNA-1 affects SERCA2a at the protein level exclusively. The SERCA2a protein levels were not examined in this study, to determine if this was affected a Western Blot of SERCA2a could be conducted using tissue from the AAV9.miR-1 and AAV9.lacZ treated animals.

If the level of SERCA2a protein was found to be unaffected by increased microRNA-1 expression in heart failure, then microRNA-1 could be targeting one of the regulatory factors that influence SERCA2a; PLB, HS-1 associated protein X-1 (HAX-1), S100A1, small ubiquitin-related modifier type 1 (SUMO1) and histidine rich calcium binding protein (HRC). Of these, none are direct sequence matches for post-transcriptional regulation by microRNA-1, therefore it would require one or more intermediate targets as part of a sequence of regulation factors linking microRNA-1 to SERCA2a. All could be potentially responsible for the increased SERCA2a capacity to load the SR with calcium

during diastole after the AAV9.miR-1 treatment in the adult heart failure rats. Investigation of each would be required to understand if this is the case but would likely involve additional components as part of a cascade of as none of the factors discussed are direct targets of microRNA-1.

Conclusion

The results presented in this chapter are from an *in vivo* study in which adult heart failure rats were administered with a single dose of AAV9.miR-1 or AAV9.LacZ. The two groups were studied through repeated echocardiography recordings and a range of experiments on isolated cardiomyocytes upon sacrifice. Although the microRNA-1 expression was not significant, and did not result in an improvement in cardiac performance over the course of 9 weeks post AAV9 dosing, some functional effects were seen. β_2 - and possibly β_1 -adrenoceptor mediated contractility was improved along with the capacity of SERCA2a to load the SR with diastole. The former complements the same result observed *in vitro* and together they represent novel results in the study of the role of decreased microRNA-1 expression in heart failure.

Chapter 6: Discussion

Key findings of the study

The first results chapter presented in this study focussed on the determining the capacity of microRNAs circulating in the blood of heart failure patients to act as prognostic markers of the progression of disease severity. In chapter 3, two separate RT-qPCR microRNA microarray formats were used to identify a shortlist of 15 microRNA candidates that exhibited differences in expression between good and poor disease prognosis groups. The same pattern could not be repeated for any of these candidates when the expression was examined more rigorously by TaqMan RT-qPCR validation experiments. The results do not rule out whether microRNAs could fulfil the role of a prognostic marker in heart failure, but the results in chapter 3 would suggest that at least in the Copernicus patient cohort microRNAs this is not the case. The mitigating factors that may have affected the reliability of the Copernicus patient samples for identifying microRNA prognostic markers were discussed in detail in the discussion section of chapter 3. These include the significant amount of disease phenotype heterogeneity in the patient population used in the Copernicus and the prolonged period in frozen storage.

The other results chapters presented in this study are closely aligned in terms of their conceptual aims. The effect of increased microRNA-1 expression in cardiac settings was studied by *in vitro* (chapter 4) and *in vivo* (chapter 5) transfection. Increased microRNA-1 expression by *in vitro* transfection of cultured adult heart failure cardiomyocytes was able to improve the contractile response mediated by the β_2 -adrenoceptor. This result was repeated in freshly isolated adult cardiomyocytes from adult heart failure rats treated *in vivo* by AAV9.miR-1, but the combined contractile response mediated by both the β_1 and β_2 -adrenoceptors was also significantly increased. Malfunction of the β -adrenoceptors is part of the pathophysiological phenotype of heart failure, displaying reduced gene expression (β_1) and impaired sensitivity (β_1 and β_2) to agonist activation. Increased microRNA-1 expression was able to elicit a reverse the depressed capacity of the β -adrenoceptors to trigger a positive inotropic response after agonist stimulation.

Interestingly, when comparing the two contractility data sets from *in vitro* transfected and *in vivo* treated cardiomyocytes it would appear that there are differences in sensitivity to agonist activation by isoprenaline. Upon initial inspection it would appear that *in vitro* transfection resulted in a greater increase in β_2 -adrenoceptor contractile amplitude than that of the cardiomyocytes derived from the *in vivo* AAV9.miR-1 treated animals. The BL%PeakH (peak contractile amplitude – baseline measurements recorded were $2.01\% \pm 0.16$ (*in vitro*) and $0.71\% \pm 0.26$ (*in vivo*). The differential

between the β_2 -adrenoceptor mediated contractile response of the control (AAV9.LacZ or Scrambled Pre miR) and microRNA-1 *in vivo* treated/ *in vitro* transfected groups was actually relatively similar; 0.75 and 1.00 respectively. The same metric for β_1 and β_2 -adrenoceptor combined contractility is also comparable, 1.14 and 1.21 respectively. The $\pm$ SEM of the *in vitro* transfection results were greater than the *in vivo* treated counterparts, this may explain why statistical significance was not achieved in the combined the β_1 and β_2 -adrenoceptor contractile response after *in vitro* transfection. Despite a greater increase in microRNA-1 expression after *in vitro* transfection (~15 fold increase) compared to *in vivo* treatment (2 fold increase) the effect on contractility would appear negligible. From this it can be concluded that a 2 fold increase in microRNA-1 expression in heart failure is sufficient to elicit the maximum improvement in β -adrenoceptor contractility. As even when the expression is increased to ~15 fold the contractile response is not increased further.

The contractility of freshly isolated cardiomyocytes is typically greater than cultured cardiomyocytes, this because cardiomyocyte functionality deteriorates over time whilst in culture. This paradigm does not appear to be true in this study; the isoprenaline induced contractile response of cultured heart failure cardiomyocytes transfected *in vitro* (figure 4E) was greater than the cardiomyocytes freshly isolated from *in vivo* treated heart failure animals (figure 5G). Why this is the case is uncertain, it is possible that without the chronic influence of circulating catecholamines present in heart failure the β -adrenoceptors of cultured cardiomyocytes are able to resensitise at the sarcolemmal membrane, enabling a greater contractile response when activated by isoprenaline. An alternative explanation to the difference in the sensitivity of the cardiomyocytes to isoprenaline is that the animals used in the *in vivo* AAV9 treatment experiments had a more severe heart failure phenotype than those used for the *in vitro* transfection experiments. The baseline contractility of the cardiomyocytes from both experiments was ~5% which would suggest the severity of heart failure was similar.

Another result obtained in this study was the possible improvement in SERCA2a function to pump calcium from the cytoplasm into the SR during diastole after *in vivo* AAV9.miR-1 treatment of heart failure animals. Through measuring the calcium transients of isolated cardiomyocytes exposed to caffeine, increased microRNA-1 expression resulted in faster clearance of calcium from the SR and more calcium loaded into the SR to be released upon stimulation. These effects are most likely attributable to improved SERCA2a functionality as in rats the calcium cleared by SERCA2a in diastole is 92% of the total (Bers, 2001). This conclusion is further enhanced by the understanding that NCX, the second most prolific component of cytoplasmic calcium clearance is a direct target for post

transcriptional inhibition by microRNA-1. With NCX function likely (but not measured) to be reduced and the other slower systems only making up 1% of calcium clearance, any improvement in this aspect of calcium handling during diastole is highly likely to be due to changes to SERCA2a function. Recording SERCA2a function after a caffeine spritz was not performed after *in vitro* transfection of cultured cardiomyocytes. This was attempted in previous work as part of a Masters project, but this was too technically challenging as very few of the cardiomyocytes survived the caffeine spritz. In addition to improving SERCA2a function, increased microRNA-1 also resulted in increased cAMP production after selective activation of the β_2 -adrenoceptor. cAMP production is driven by AC (AC5 and AC6 isoforms are the cardiac isoforms) whose activation is triggered by the $G_{\alpha s}$ G protein of the β -adrenoceptors after agonist induced activation. Increased cAMP results in increased PKA activity and the activity of the targets that are phosphorylated by PKA.

How can the results be explained?

After observing these changes in cardiomyocyte function, the next step was to attempt to understand the mechanism by which microRNA-1 operates. A series of experiments were undertaken to answer this question across the *in vitro* transfection and *in vivo* treatment models. Because individual microRNAs can regulate the expression of multiple mRNA sequences, a valid experimental approach was to measure the expression of a range of cardiac gene targets that are disrupted in heart failure and contribute to the pathological phenotype to see if increased microRNA-1 expression would affect these targets. Both *in vitro* transfection and *in vivo* treatment to increase microRNA-1 expression in heart failure did not cause the mRNA expression of any of the cardiac gene targets tested to be statistically different compared to the control.

It is possible that microRNA-1 did not affect the mRNA expression of the cardiac target genes tested, but instead blocked production of the protein that was translated from the mRNA. Studies have found mRNA sequences in complex with polysomes during microRNA inhibition and that the polysomes dissociate upon exposure to puromycin, a protein synthesis inhibitor (Kong et al., 2008), which means that ribosomes were actively translating the mRNA sequence during microRNA inhibition. It has been suggested that the mechanism for the repression of protein expression is that the microRNA causes the deceleration of translational elongation (Maroney et al., 2006). Testing the protein expression of the cardiac genes targets by Western Blotting would resolve whether this has occurred in this study.

Given the lack of change to the mRNA expression and the absence of protein expression data, the next step was to examine how the functionality of the β -adrenoceptors or SERCA2a was changed after increased microRNA-1 expression. The first potential explanation to be investigated was that microRNA-1 might cause a switch in the G protein coupling of the β_2 -adrenoceptor from $G_{\alpha i}$ to $G_{\alpha s}$. It was hypothesised this was the case as the reverse paradigm occurs in heart failure, with β_2 -adrenoceptor signalling switching from $G_{\alpha s}$ to $G_{\alpha i}$. This results in a greater inhibitory influence of $G_{\alpha i}$ signalling on the production of cAMP and a dampening on cardiomyocyte contractility. In this study PTX was used to block β_2 -adrenoceptor signalling via $G_{\alpha i}$, the β_2 -adrenoceptor mediated contractile response in the absence of $G_{\alpha i}$ was then tested in heart failure cardiomyocytes transfected with Pre miR-1. The effect on contractility of $G_{\alpha i}$ inhibition by PTX and increased microRNA-1 expression was possibly additive at best, suggesting that they operate via different pathways. If increased microRNA-1 expression had no additional effect on contractility it would suggest that PTX has saturated the ability of microRNA-1 to affect contractility, but as this is not the case, it is unlikely to be the explanation.

After transfecting healthy cardiomyocytes *in vitro* with Pre miR-1, the group with increased microRNA-1 expression had a non-significant increase in T-tubule regularity compared to the control group. T-tubules are known to breakdown in heart failure which has significant ramifications on CICR and it is associated with the cAMP signal losing its focus and becoming more diffuse across the T-tubule crest. Culturing cardiomyocytes also causes the same effect and was used to model the effect of heart failure in healthy cardiomyocytes. When the same experiment was conducted in freshly isolated cardiomyocytes from AAV9.miR-1 treated heart failure animals the same result was not repeated. The depression in T-tubule regularity was present in both AAV9.LacZ and AAV9.miR-1 groups, suggesting that the heart failure phenotype was present but there was no difference between the two groups. Therefore it would seem that microRNA-1 does not possess the ability to influence T-tubule regularity and therefore is not the cause of the improvements in heart failure cardiomyocyte function.

The question remains around how microRNA-1 was able to alter the functionality of both SERCA2a and the β -adrenoceptor in heart failure. One intracellular component of cardiomyocyte function that links both these two is PLB. Its activity is decreased by cAMP driven PKA phosphorylation of the Serine 16 residue (Mattiuzzi et al., 2005) and this is associated with an increase in the rate of calcium transport into the SR by SERCA2a. This increases the capability of the β -adrenoceptor to drive cAMP production and PKA activity would cause a decrease in PLB inhibition of SERCA2a and

permit the increased calcium loading of SR during diastole. PLB expression appears to be unchanged in heart failure (Schwinger et al., 1999) but the decrease in SERCA2a results increased functional stoichiometry of PLB to SERCA2a and greater inhibition of SERCA2a. The phosphorylation status of PLB in heart failure is decreased, which would also result in an increase in the PLB inhibitory activity exerted on SERCA2a. Studies have shown that agonist induced β -adrenoceptor activation results in a PLB driven disinhibition of SERCA2a activity (Calaghan et al., 1998). For the effect on SERCA2a function to be uncovered an increase in cAMP to drive PKA induced phosphorylation of PLB is required. Alternatively, microRNA-1 may affect PLB through the HS-1 associated protein X-1 (HAX-1) which is localised at the SR and regulates the activity of PLB. Although its primary function is to regulate PLB, HAX-1 also regulates SERCA2a through affecting the affinity of the SERCA2a for calcium (Kranias and Hajjar, 2012).

A possible conclusion from this study is that microRNA-1 improved the β -adrenoceptor linked cAMP production in heart failure which increased the PKA phosphorylation of PLB and consequently increased SERCA2a function, but the results from the calcium transient experiments conducted in this study do not support this conclusion as in cardiomyocytes from AAV9.miR-1 treated animals a caffeine spritz (rather than isoprenaline) caused a significant increase in SERCA2a clearance of calcium from the cytoplasm (non-hierarchical data). Therefore if the microRNA-1-PKA-PLB hypothesis were to be true, the increased microRNA-1 would have had to have caused a tonic increase in cAMP production resulting in reduced PLB activity but that was not great enough for increased SERCA2a function to be detectable at 1Hz (basal) stimulation but was detectable after a caffeine spritz where the SR was emptied of calcium. An interesting experiment to determine whether this is the case would be to expose the cardiomyocytes to the same concentration of isoprenaline and measure the behaviour of SERCA2a.

The alternative hypothesis is that microRNA-1 is able to influence SERCA2a and β -adrenoceptor function independently rather than through a mutual factor such as PLB. In one study, chromatography and immunoprecipitation experiments were conducted to reveal that the calcium sensor protein S100A1 interacts with SERCA2a and PLB in a calcium dependent manner in the human myocardium (Kiewitz et al., 2003). The expression of S100A1 is reduced in human end stage heart failure (Remppis et al., 1996) and cardiomyocytes with increased S100A1 expression showed improved peak calcium transient amplitudes attributed to greater SR calcium loading and the subsequent release of calcium in systole (Most et al., 2001). In a porcine model of heart failure (balloon occlusion of the left circumflex coronary artery) AAV9 cardiac targeted delivery of S100A1

resulted in normalised calcium cycling and SR calcium loading (Pleger et al., 2011). As S100A1 function is therefore able to influence calcium loading of the SR and is spatially associated with SERCA2a at the SR, it represents a viable target for increased microRNA-1.

The activity of SERCA2a is also modulated by the SUMO1 in a post translation modification known as sumoylation. This is a process of reversible attachment of SUMO family members to target lysine residues and results in changes to protein nucleocytoplasmic translocalisation, interactions with other proteins, protein stability and protein to DNA binding (Schwartz and Yeh, 2012). The lysine residues at positions 480 and 585 of SERCA2a are sumoylated in cardiomyocytes and this has the effect of preserving the ATPase calcium pump capacity of SERCA2a (Kho et al., 2011). SUMO1 expression and the sumoylation of SERCA2a is reduced in heart failure and when SUMO1 expression is restored in a mouse model of heart failure it triggers restored SERCA2a expression and improved cardiac performance. This represents another potential target of microRNA-1 for consideration that influences how SERCA2a function, however given that it has previously been found to improve SERCA2a expression in heart failure as well as function it perhaps less likely to be responsible for the results observed in figure 5I as SERCA2a expression was unaffected by the AAV9.miR-1 treatment. Interestingly SUMO1 is a direct target of microRNA-133a/b, which belongs to the same transcriptional unit as microRNA-1. These two microRNAs together with microRNA-16, -27b, -30d, -126, -143 and the let-7 family are highly expressed, although not exclusively, in the adult myocardium (Thum et al., 2008).

Another regulatory influence on SERCA2a function is through the interaction with HRC on the SR luminal side of the protein. As its name suggests Histidine rich calcium binding protein (HRC) is a calcium binding protein, it has a low affinity and high capacity in similar fashion to calsequestrin and is involved in calcium homeostasis in the SR by regulating calcium loading, storage and release. Where the two proteins differ is the extent to which they are able to influence calcium handling in the cardiomyocytes. A small increase in HRC expression has a far greater potency in terms of reducing calcium clearance rates and SR calcium loading during diastole compared to calsequestrin (Sato et al., 1998). Perhaps in conflict with the calcium transient data presented in figure 5I, when HRC was overexpressed in a transgenic model only the calcium uptake into the SR and calcium decay were disrupted and not peak transient amplitude after a caffeine spritz (Gregory et al., 2006). Continued overexpression of HRC resulted in the progression of hypertrophy to heart failure in ageing and it has been hypothesised that the increased stoichiometry of HRC with SERCA2a is linked

to the development of heart failure pathophysiology. Unfortunately there is no direct sequence match with microRNA-1 and the 3'UTR sequence of HRC.

Of the possible microRNA-1 targets discussed in chapter 4 that may regulate β -adrenoceptor function and increase cAMP production, p38-MAPK now seems less likely to be responsible for the improved β -adrenoceptor linked contractility given the results in chapter 5. In a study in which the whole cell calcium current was measured in cardiomyocytes by perforated patch-clamp technique, the p38-MAPK regulation of cardiomyocyte contractility was not associated with a change to the intracellular calcium transients (Liao, 2001). This does not align with the observed improvement in SERCA2a function and calcium loading of the SR in this study.

The other possibility discussed in chapter 4 was the PI3K p110 γ , a direct sequence target of microRNA-1, the activated form of p110 γ has been found to reduce cellular levels of cAMP through increased PDE 3 or 4 activity (Kerfant et al., 2007). This aligns to the paradigm observed in this study as increased microRNA-1 would decrease p110 γ induced activation of PDE. The use of subtype specific PDE inhibitors to block cAMP degradation could be used in conjunction with a SICM FRET protocol to determine if increased microRNA-1 expression can still cause elevated cAMP production in the presence of PDE inhibition. If this is the case it would suggest that increased cAMP production by greater microRNA-1 expression is due to an alternative mechanism to PDE inhibition. The caveats that weakens this hypothesis are that p110 γ was only found to be a match of microRNA-1 in humans but not in rats, and has relatively low expression in the heart of mice (Alloatti et al., 2004).

In some cases a mRNA sequence that is matched to a microRNA by an algorithm does not mean that a functional relationship exists where the microRNA actually affects expression of the mRNA sequence. The expression of the microRNA could be too low in a particular cell type or the expression of the microRNA is inhibited; both would prevent the microRNA from regulating its gene targets. Therefore it is important to combine these tools with a hypothesis based approach when attempting to understand the mechanism by which microRNA-1 was able to cause the changes in cardiomyocyte function observed in this study. For example, testing the function of all of the known targets of PKA that are linked to contractility would be a valid set of experiments to conduct given the increased cAMP production observed after increasing the expression of microRNA-1.

Limitations of the study

Limitations are part of any scientific experiment and the results presented in this study are no different. In chapter 3 the circulating microRNA profile of serum samples from selected patients enrolled in the Copernicus trial was studied with the aim of identifying reliable prognostic biomarkers of heart failure. Two technical limitations encountered as part of this work were the challenge of using biological material that had been frozen for over 10 years and the accuracy of the RT-qPCR protocols available. A limitation of the scientific approach used for the experiments in chapter 3 was that microRNA expression has been shown to be variable across heart failure patients, as highlighted by inconsistencies in the literature (Tijssen et al., 2010) (Fukushima et al., 2011) and that circulating microRNA expression can vary over time in disease (Zile et al., 2011) (Tan et al., 2009), the impact of this patient heterogeneity and temporal variability on the circulating microRNA expression could mean that identifying reliable differences between good and poor prognosis patients was too difficult in the serum samples available.

In chapter 4 when investigating the effect of microRNA-1 on healthy cardiomyocyte function after microRNA-1 transfection two experiments were conducted; the β_2 -adrenoceptor cAMP response of neonatal EPAC1 mouse cardiomyocytes and the T-tubule regularity of healthy adult rat cardiomyocytes. MicroRNA-1 expression is greater in healthy cardiomyocytes compared to heart failure and attempting to cause a functional change by increasing microRNA-1 expression in this context is not a certain success. In work not presented in this study, a cohort of healthy adult rats were treated with AAV9.miR-1 by jugular vein injection, but this did not result in an increase in the expression of microRNA-1 in isolated cardiomyocytes. In figure 4C, *in vitro* transfection of isolated healthy cardiomyocytes to increase microRNA-1 expression did not result in an improvement in isoprenaline stimulated contractility. Furthermore, the manner in which the T-tubule regularity was examined will not give any indication on changes to β_2 -adrenoceptor function. Although T-tubule regularity is important for ensuring spatial distribution of the β_2 -adrenoceptor cAMP signal, stained surface images of cardiomyocytes will not give any indication of the signal.

The limited effect of increased microRNA-1 expression in healthy cardiomyocytes could be due to the fact that the post transcriptional inhibition of target sequences is already occurring; more simply put, microRNA-1 is already inhibiting the expression of sequences where it matches to the 3' UTR. Increasing the level of microRNA-1 in this environment may not have a great effect as the endogenous microRNA-1 is already fulfilling its function. There may be situations where only partial

inhibition of expression occurs in the healthy cardiomyocyte, e.g. due to imperfect annealment between microRNA and sequence. In these circumstances improving microRNA-1 expression could have an additional effect to endogenous microRNA-1. Reversing the inhibition on target sequence of endogenous microRNA-1 or elevating expression where it is low are both situations where there is greater potential for effect. This is comparable to using a submaximal concentration in a concentration response curve, there potential for an increase or decrease in the response.

All of the experiments conducted in chapter 4 were *in vitro*, this is associated with a limited window for the increased microRNA-1 expression after a transfection is able have an effect on its target sequences. This is the nature of all *in vitro* experiments in cell lines that are not immortal and can be considered as a technical limitation of the results. To cause a substantial change to the phenotype of cultured cardiomyocyte in 48-72 hours is a considerable challenge, but the results in chapter 4 show that it is possible for microRNA-1 to cause a functional effect in adult or neonatal cardiomyocytes in a limited time window. The full extent of its influence to become pronounced may take longer, this provided the rationale for undertaking the *in vivo* study in chapter 5.

The primary limitation of the *in vivo* study conducted in chapter 5 is the number of animals used in each AAV9 treatment cohort. A power calculation was conducted prior to starting the study which suggested that an n number of 4 per cohort would be sufficient to detect a significant difference, but the variable nature of the echocardiography results made it very difficult for any trends within a cohort or differences between to be identified. It is plausible that the increased microRNA-1 expression did not cause an effect in the cardiac performance of the heart failure animals. With a larger n number for each cohort the variability of the echocardiography data would be less of an issue. A common limitation to the results of chapter 4 and 5 is the low transfection efficiency of the viral techniques used and the impact this has on the data from single cell experiments. Only the adeno.miR-1 had a reporter (GFP) which meant positively transfected cardiomyocytes could be accurately selected for experiments. For the cardiomyocytes transfected by lipofection or those isolated from AAV9.miR-1 treated animals there was no way of knowing if they had increased microRNA-1 expression or not. Given that the transfection efficiency of the two techniques is not 100% this made the possibility of generating statistically significant data more challenging as data could be included in the Pre miR-1/ AAV9.miR-1 cohort that was derived from cardiomyocytes that did not have increased microRNA-1 expression.

Future direction

The future direction after the observed effects of microRNA-1 on cardiomyocyte function (β -adrenoceptor mediated contractility and cAMP production, and SERCA2a driven calcium loading of the SR) is to understand the mechanism by which microRNA-1 was able to influence these processes. This was not successfully achieved in this study, but the experiments that were conducted can be used to drive the hypotheses of the future direction. The results obtained can be used in conjunction with studying the targets identified by the predictive microRNA-mRNA sequence matching tool target (www.targetscan.org). As highlighted in the limitations section, a repeated study of the effect of AAV9.miR-1 *in vivo* should be conducted to show that the results are repeatable in a distinct instance. This could also be used as an opportunity to conduct a dobutamine stress test; increasing concentrations of infused dobutamine to animals whilst the cardiac performance is measured by echocardiography (Plante et al., 2005). This would be interesting to see if increased microRNA-1 expression would be able to replicate the improved contractility observed in isolated cardiomyocytes in an *in vivo* setting.

Previous studies in Professor Harding's laboratory have also examined the arrhythmogenic profile of heart failure cardiomyocytes. In this study increased microRNA-1 improved SERCA2a function recorded, SERCA2a has been found to regulate arrhythmogenesis in heart failure through controlling both SR calcium content and RyR phosphorylation (Lyon et al., 2011). Therefore it would be logical to examine the whether the propensity of heart failure cardiomyocytes to develop arrhythmias would be limited by increased microRNA-1 expression. The ongoing clinical trials focussed on delivered SERCA2a to heart failure patients further highlights the importance of SERCA2a and the link between increasing microRNA-1 and arrhythmogenesis (Jaski et al., 2009).

A future direction after the results described in chapter 3 that considered the prognostic value of circulating microRNAs in heart failure for predicting disease severity is not so straightforward. As the results proved negative in detecting a suitable microRNA candidate a comprehensive review of the hypothesis for the experiment is required. It is possible that circulating microRNAs are unable to act as reliable prognostic biomarkers for heart failure and the study should therefore be halted at this point. Given the inconsistency of other smaller studies of a similar nature in identifying one or more circulating microRNAs that can distinguish in heart failure versus healthy patients, it would be reasonable to repurpose the serum samples of the large, well physiologically characterised patient population available from Copernicus study. The samples could be used in a future experiment to try

to identify a microRNA that consistently acts as a biomarker to distinguish heart failure from healthy patients in a comparison against BNP.

Final conclusion

The results presented in this study focus have two focus areas; the effect of increased microRNA-1 expression in heart failure and the capability of microRNAs to act as prognostic biomarkers for heart failure severity. The latter was the more challenging hypothesis to prove, with no reliable biomarker identified in the serum samples from the heart failure patients enrolled in the Copernicus trial. This does not rule out either circulating microRNAs as prognostic biomarkers of heart failure or the validity of the Copernicus serum samples. A different hypothesis would be required in future experiments, for example, looking to identify a circulating microRNA that distinguishes heart failure from healthy patients. The same approach could be taken, but with an alternative method of patient selection in order to reduce the impact of patient heterogeneity on circulating microRNA expression.

The aim of understanding the role that microRNA-1 plays in the pathophysiology of heart failure was more successful. Both *in vitro* and *in vivo* experiments to increase microRNA-1 expression were conducted and produced a number of interesting results relating to cardiomyocyte function. β -adrenoceptor mediated contractility was increased after microRNA-1 expression in heart failure was improved, most likely through the observed increase in cAMP production. Calcium homeostasis was possibly improved through greater SERCA2a driven loading of the SR with calcium. Whether microRNA-1 regulates the expression of a single target that elicits these different effects, or operates through multiple targets is unknown. From the results obtained, the next step of investigation would be to understand the intracellular cardiomyocyte mechanism that is regulated by microRNA-1.

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Appendix

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Figure 1A: A graph showing the time course of a cardiac action potential (AP), a calcium transient ([Ca]_i) and a contraction (Bers, 2014).

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Figure 1B: The range of signalling pathways associated with β -adrenoceptor (β -AR) agonist activation and the resulting Protein Kinase A (PKA) activation (El-Armouche and Eschenhagen, 2009).

&

Figure 1C: Components of the PKA signalling complex and its targets that located at the sarcoplasmic reticulum (El-Armouche and Eschenhagen, 2009).

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Figure 1E: The accuracy of microRNA binding to a complimentary section of the 3' UTR region of a mRNA sequence determines the form of post transcriptional inhibition that occurs (Bartel, 2004).

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Figure 1F: The mechanism by which reduced cytoplasmic (cytosol) calcium drives a Akt-FOXO3A dependent reduction in microRNA-1 expression in heart failure. When SERCA2a expression is increased this cascade is altered and microRNA-1 expression is increased (Kumarswamy et al., 2012).