

The Mechanistic Role of MicroRNAs in Takotsubo Syndrome

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Abstract

Takotsubo syndrome (TTS) is a severe but reversible acute heart failure affecting predominantly post-menopausal women. Extreme adrenaline levels arising with stress cause ventricular apical akinesis with hypercontractility at the base. We have previously shown this is likely to occur due to β_2 AR-G α i coupling, which is dysregulated in TTS. A microRNA (miR) profile, with increased miR-16 and miR-26a in TTS, has been identified that enables differentiation from both healthy control and ST-segment elevation myocardial infarction, which it closely resembles at patient presentation. We hypothesise that miR-16 and miR-26a functionally interact to predispose to or exacerbate the cardiodepression seen in TTS.

miRs were modulated *in vitro* in adult rat cardiomyocytes using Lipofectamine 3000. Cardiomyocyte percentage shortening was measured using an Ionoptix system. TTS was induced *in vivo* by adrenaline bolus injection through the external jugular vein of an adult male Sprague-Dawley rat, and blood sampled at defined timepoints. miRs were modulated *in vivo* by AAV9 injection via tail vein (called AAV-control and AAV-miR) with subsequent TTS induction after 6 weeks.

miR-16 and miR-26a specifically reduce baseline contractility of apical but not basal cardiomyocytes *in vitro*. This includes alterations in calcium handling, with reduced calcium transient amplitude and SR calcium content. Together, miR-16 and miR-26a reduce sensitivity to adrenaline in the apex, and amplify maximal response to adrenaline in the base, but do not change β_2 AR response. *In vivo*, in our established model of TTS, serum levels of miR-16 and miR-26a are not changed acutely by adrenaline. AAV-miR treatment produces greater hypokinesis in the apex and hypercontractility in the base. When investigating the mechanism of action, this includes altered G-protein signalling and calcium current activity.

TTS-associated miRs represent novel molecules that clarify the pathogenesis of this curious condition. They predispose to TTS *in vivo* by promoting apical hypokinesia and basal hypercontractility. This occurs by reducing sensitivity to adrenaline in the apex and increasing maximal response to adrenaline in the base through alterations in G-protein signalling and calcium handling.

Declaration of originality

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Publications

Sattler S., **Couch L.S.** & Harding S.E. Takotsubo Syndrome – the latest addition to the expanding family of immune-mediated diseases? JACC: Basic to Translational Science. *In press.*

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Conference proceedings and awards

President's Awards for Excellence in Research 2018

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Abbreviations

α AR	Alpha adrenergic receptor
α_1 AR	Alpha 1 adrenergic receptor
α_2 AR	Alpha 2 adrenergic receptor
β AR	Beta adrenergic receptor
β_1 AR	Beta-1 adrenergic receptor
β_2 AR	Beta-2 adrenergic receptor
β_3 AR	Beta-3 adrenergic receptor
3'UTR	3' untranslated region
3AB	3-aminobenzamide
AAV	Adeno-associated virus
AAV9	Adeno-associated virus serotype 9
ACE	Angiotensin-converting enzyme
ACS	Acute coronary syndrome
AGO	Argonaute proteins
ANS	Autonomic nervous system
AMI	Acute myocardial infarction
ANOVA	Analysis of variance

APD	Action potential duration
APD50	Action potential duration to 50% repolarisation
APD75	Action potential duration to 75% repolarisation
AR	Adrenergic receptor
ATP	Adenosine triphosphate
BAG	Bcl2-associated athanogene
BNP	Brain natriuretic peptide
BSA	Bovine serum albumin
C&H	Collagenase II and Hyaluronidase
Ca ²⁺	Calcium
CACNA1C	Gene name for LTCC $\alpha 1c$ subunit
CACNB1	Gene name for LTCC $\beta 1$ subunit
cAMP	Cyclic adenosine monophosphate
CBS	Centre for Biological Sciences
CICR	Calcium-induced calcium release
CT	Cycle threshold
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

E8	Essential 8 media
ECG	Electrocardiogram
ECMO	Extracorporeal membrane oxygenation
EF	Ejection fraction
EF1 α	Elongation factor 1-alpha
ETC	Electron transport chain
FCCP	Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
FR	Fractional release
G α i	Inhibitory G-protein
G α s	Stimulatory G-protein
G β	G-protein β subunit
G γ	G-protein γ subunit
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GIRK	G-protein coupled inward rectifying potassium
GNB1	Gene name for G-protein β subunit 1
GNG12	Gene name for G-protein γ subunit 12
GRK	G-protein coupled receptor kinase
HCN	Hyperpolarisation-activated cyclic nucleotide-gated

HF	Heart failure
HFA	Heart Failure Association
HRP	Horseradish peroxidase
IABP	Intra-aortic balloon pump
I_{CaL}	Calcium current
IP	Intraperitoneal
iPSC	Induced pluripotent stem cell
iPSC-CM	Induced pluripotent stem cell-derived cardiomyocyte
ITS	Insulin transferrin selenium
IV	Intravenous
kDa	KiloDalton
KH	Krebs-Henseleit
LED	Light emitting diode
LTCC	L-type calcium channel
LV	Left ventricle
LVAD	Left ventricular assist device
LVOTO	Left ventricular outflow tract obstruction
MAPK	Mitogen-activated protein kinase

MCSYS	Myocyte Contractility System
MEM	Minimum essential medium
MI	Myocardial infarction
miR	MicroRNA
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MCSYS	Myocyte Contractility Recording System
MQ	Milli-Q H ₂ O
NCX	Sodium-calcium exchanger
NO	Nitric oxide
NOS	Nitric oxide synthase
NSM	Neurogenic stunned myocardium
NSTEMI	Non-ST-elevation myocardial infarction
NT	Normal Tyrode's
NT-pro-BNP	N-terminal pro brain natriuretic peptide
OCR	Oxygen consumption rate
PARP-1	Poly [ADP-ribose] polymerase 1
PCI	Percutaneous coronary intervention

PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PLB	Phospholamban
PNS	Parasympathetic nervous system
Pre-miR	Precursor-miR
Pri-miR	Primary-miR
PTX	Pertussis toxin
PVDF	Polyvinylidene difluoride
RGS	Regulator of G-protein signalling
RNA	Ribonucleic acid
RT	Reverse transcription
RTqPCR	Quantitative reverse transcription polymerase chain reaction
RV	Right ventricle
RyR	Ryanodine receptor
SAH	Subarachnoid haemorrhage
SCD	Sudden cardiac death
SEM	Standard error of the mean

SERCA	Sarco-endoplasmic reticulum calcium ATPase
SNS	Sympathetic nervous system
SR	Sarcoplasmic reticulum
STEMI	ST segment elevation myocardial infarction
TBP	TATA-box binding protein
TBT-T	TBS Tween
Tn	Troponin
TTS	Takotsubo syndrome
VF	Ventricular fibrillation
VT	Ventricular tachycardia

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1 Introduction

1.1 Adrenergic system

The autonomic nervous system (ANS) is instrumental in modulating the physiological response to environmental demands, with the parasympathetic and sympathetic nervous systems (PNS and SNS respectively). This enables appropriate reactions to be mounted in situations of low and high stress respectively, combining to further the survival of an individual (Florea and Cohn, 2014). Specifically, in the cardiovascular system, both systems interact in opposing fashion to modulate cardiac chronotropy (heart rate), but inotropy (contractility) and lusitropy (relaxation) are controlled almost solely by the SNS (Lymperopoulos, Rengo and Koch, 2013).

1.1.1 Adrenergic receptors

Within the SNS, adrenergic receptors exist to receive either the primary adrenal medullary hormone (secreted from chromaffin cells) and central neurotransmitter, adrenaline, or the primary sympathetic neurotransmitter, noradrenaline (Woo *et al.*, 2015). Adrenergic receptors are G-protein coupled receptors, consisting of alpha (α) and beta (β) subtypes, which can modulate the electrophysiological and energetic properties of cardiomyocytes upon ligand activation by catecholamines.

The myocardium possesses receptors for α_1 and α_2 , as well as β_1 , β_2 and β_3 . Whilst the action of β_3 is better understood in terms of hypertrophy than in contractile signalling, the β_1 AR and the β_2 ARs can result in canonical stimulatory G-protein (Gas) signalling, leading to an increase in adenylate cyclase activity, cyclic AMP production and, consequently, protein kinase A (PKA) activity (Paur *et al.*, 2012; Woo *et al.*, 2015). This enhances phosphorylation of cellular machinery to effect changes in calcium handling and contractility (Woo *et al.*,

2015). However, this is at the expense of increased cell death subsequent to Ca^{2+} -overload and necrosis secondary to increased cAMP, as well as apoptosis due to PKA activation and Ca^{2+} /calmodulin kinase-dependent mechanisms (Zhu *et al.*, 2003). It is similarly detrimental at the organ level contributing to arrhythmia (Esler *et al.*, 1997; Steinberg *et al.*, 2002; Mohamed, Napolitano and Priori, 2007) and heart failure progression (Lohse, Engelhardt and Eschenhagen, 2003).

Contrary to the $\beta_1\text{AR}$, the $\beta_2\text{AR}$ is coupled to an additional signalling pathway, the inhibitory G-protein ($\text{G}_{\alpha i}$). As increasing amounts of signal transduction ensue through $\text{G}_{\alpha s}$, stimulus trafficking occurs, and a switch to $\text{G}_{\alpha i}$ results (Lyon *et al.*, 2008; Woo *et al.*, 2015). This counteracts the stimulatory pathway to cause negative inotropy subsequent to the direct suppression of $\text{G}_{\alpha s}$ -mediated phosphorylation, as well as a separate cAMP-independent negatively inotropic pathway (Paur *et al.*, 2012). Increased PKA activity as a result of βAR - $\text{G}_{\alpha s}$ signalling phosphorylates the $\beta_2\text{AR}$ and is necessary to prime the receptor for further G-protein coupled receptor kinase (GRK) phosphorylation. This occurs at higher agonist concentrations and GRK is recruited by $\text{G}\beta\gamma$. This results in internalisation of the $\beta_2\text{AR}$ and stimulus trafficking to $\beta_2\text{AR}$ - $\text{G}_{\alpha i}$ (Liu *et al.*, 2009).

As aforementioned, a cost to an increased contractile response resulting from $\text{G}_{\alpha s}$ signalling is a concomitant decrease in cell survival, which $\text{G}_{\alpha i}$ also counteracts. This $\beta_2\text{AR}$ mediated cardiodepression was originally demonstrated in a transgenic mouse model of $\beta_2\text{AR}$ overexpression (Hasseldine, Harper and Black, 2003; Heubach, Ravens and Kaumann, 2004). Whilst this process appears to be mechanically detrimental, it serves to be cardioprotective (Paur *et al.*, 2012). This protective component for the cardiomyocyte has been well described in both *in vitro* (Zhu *et al.*, 2001) and *in vivo* (Ahmet *et al.*, 2004; Bernstein *et al.*, 2005; Tong *et al.*, 2005) models when compared to the $\beta_1\text{AR}$, with $\text{G}_{\alpha i}$ knockout or inhibition with

pertussis toxin (PTX) treatment removing this protective component (Zhu *et al.*, 2001; Foerster *et al.*, 2003; DeGeorge *et al.*, 2008), as well as G α i overexpression preventing (Rau *et al.*, 2003) and knockout predisposing (Zuberi *et al.*, 2010; Ang, Opel and Tinker, 2012) to arrhythmia.

Regulators of G-protein signalling (RGS) control G α -mediated signalling by accelerating G α GTPase activity markedly (up to 2000-fold) to result in termination of signalling from G α and G $\beta\gamma$, and directly antagonise G α -mediated signal generation (Anger, Zhang and Mende, 2004). The vast weight of experimental evidence supports the action of RGS proteins on G α i and G α q subunits, with a lack of data suggesting interaction with G α s (Zhang and Mende, 2011; Magalhaes, Dunn and Ferguson, 2012).

The cAMP independent negative inotropic pathway associated with G α i activation is not yet well defined. Changes in myofilament sensitivity to Ca²⁺ due to p38 mitogen-activated protein kinase (MAPK) activation by G α i have been suggested to mediate the negative inotropy in takotsubo syndrome (Liao *et al.*, 2002; Paur *et al.*, 2012).

1.1.2 Adrenergic signalling and excitation contraction coupling

Excitation contraction (ec) coupling is the process by which the voltage activation is transformed into mechanical contraction. Upon depolarisation of the cardiomyocyte, which occurs secondary to sodium influx, sarcolemmal voltage gated L-type calcium channels (LTCC) open to give rise to the plateau phase of the action potential. Within transverse-tubules (T-tubules), most LTCC are in close apposition to ryanodine receptors (RyR), which open by a process called calcium-induced calcium release (CICR). Calcium that enters the cell via the LTCC comes into contact with RyRs that are present on the sarcoplasmic reticulum to trigger opening and a subsequent rapid increase in cytosolic calcium concentration. Calcium binds to troponin C (TnC) which is present on the myofilaments,

which in turn results in sarcomeric contraction by allowing for the interaction between actin and myosin. In order for relaxation to occur, cytosolic calcium concentration is reduced. Calcium is resequestered into the SR via the SR calcium ATPase (SERCA) and extruded from the cardiomyocyte via the forward-mode of the sodium-calcium exchanger (NCX). Slow mechanisms of calcium decay exist, such as the mitochondrial calcium uniporter and the sarcolemmal calcium ATPase, although these are not thought to regulate calcium handling on a beat-to-beat basis (Bers, 2002). This process is illustrated in Figure 1.1.

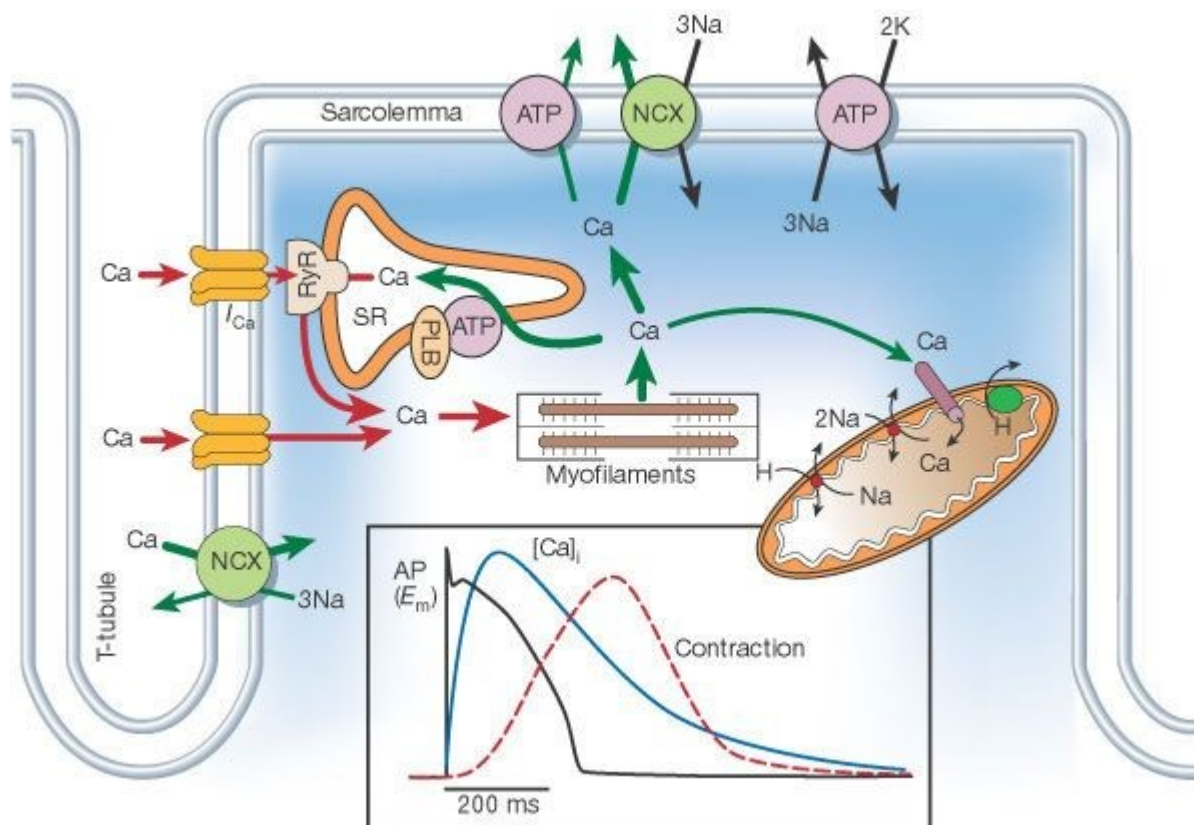


Figure 1.1: Cardiac excitation-contraction coupling

This figure is obtained from (Bers, 2002) wherein the process of ec coupling is illustrated where I_{Ca} represents the L-type calcium channel; NCX the sodium-calcium exchanger; ATP shows ATPases; PLB is phospholamban; SR is the sarcoplasmic reticulum; the pink cylinder represents the mitochondrial uniporter.

The adrenergic system is able to modulate Ca^{2+} coupling to alter the kinetics and force of cardiomyocyte contraction. Activation of $G_{\alpha s}$ as previously described results in increased cAMP and subsequent activation of PKA. PKA is able to directly phosphorylate numerous proteins, including LTCC, RyR, phospholamban (PLB), troponin I (TnI) and myosin binding protein C (Bers, 2002). Cardiac lusitropy predominantly occurs as a result of phosphorylation of PLB, which prevents inhibition of SERCA function to increase SR calcium reuptake. Phosphorylation of TnI also contributes to the lusitropic effect by reducing the sensitivity of the myofilaments to calcium, increasing the rate of dissociation and thus calcium decay. Cardiac inotropy is achieved through phosphorylation of LTCC and RyR which potently increases the gain in CICR and increases the cytosolic calcium concentration (Bers, 2002).

Cardiac chronotropy is also controlled within the sino-atrial node by the adrenergic system (Fu *et al.*, 2006). G-protein coupled inward rectifying potassium (GIRK) channels are activated by $G_{\beta\gamma}$ subunits released from $G_{\alpha i}$, the hyperpolarisation-activated cyclic nucleotide-gated (HCN) channel and LTCC is inhibited by $G_{\alpha o}$, and $G_{\alpha i}$ also regulates the LTCC (Sowell *et al.*, 1997; Valenzuela *et al.*, 1997; Ye *et al.*, 1999). cAMP is also able to directly bind to the HCN channel to cause a positive chronotropic effect (Greene *et al.*, 2012). This process is controlled by RGS proteins as described in Section 1.1.1 (Saitoh *et al.*, 1997; Hollinger and Hepler, 2002).

1.1.3 Organisation of the adrenergic system in cardiomyocytes

Organisation of the adrenergic system is different within the cardiomyocyte for the different β ARs. Indeed, the β_2 AR-mediated cAMP signalling is local while the β_1 AR-mediated cAMP signalling is global (Kuschel, Zhou, Cheng, *et al.*, 1999; Kuschel, Zhou, Spurgeon, *et al.*, 1999). Caveolin 3 has been shown to play a crucial role in the control of localisation of β_2 ARs and their cAMP mediated signalling within caveolae and T-tubules in adult

cardiomyocytes (Rybin *et al.*, 2000; Calaghan and White, 2006; Nikolaev *et al.*, 2010; Wright, Nikolaev, *et al.*, 2014). This is in contrast to β_1 ARs, which appear to be distributed evenly within cardiomyocyte fractions (Rybin *et al.*, 2000). Compartmentalising the adrenergic system in this way allows the common second messenger, cAMP, to perform a variety of selective functions (Woo *et al.*, 2015).

Indeed, these distinct localisations are associated with spatiotemporal differences in cAMP generation (Nikolaev *et al.*, 2006), PKA activation (Soto *et al.*, 2009) and target phosphorylation (Kuschel, Zhou, Spurgeon, *et al.*, 1999). This results in β_1 AR-mediated G α s signal generation being able to result in the phosphorylation of many proteins associated with the regulation of cardiomyocyte contractility, such as the LTCC (Hulme *et al.*, 2006), the RyR (Marx *et al.*, 2000), PLB and TnI (Sulakhe and Vo, 1995). The compartmentation applied to the β_2 AR results in β_2 AR-G α s being unable to phosphorylate these targets (Xiao *et al.*, 1999; Calaghan, Kozera and White, 2008).

Differences in the adrenergic system exist (summarised in

Table 1.1 below) depending upon the origin of the cardiomyocyte. The mammalian ventricle has a higher density of sympathetic nerve endings at the ventricular base (Kawano, Okada and Yano, 2003), and a higher density of β ARs in the apex (Mori *et al.*, 1993; Brouri *et al.*, 2004; Paur *et al.*, 2012). Furthermore, apical cardiomyocytes have been shown to exhibit a significantly larger overall β AR responses in mammalian models *in vivo* (Lathers, Levin and Spivey, 1986; Mantravadi *et al.*, 2007; Heather *et al.*, 2009) and β_2 AR response *in vitro* than basal cardiomyocytes (Paur *et al.*, 2012; Wright *et al.*, 2018). Indeed, Wright *et al.* recently demonstrated that this is due to a higher degree of microdomain organisation in basal cardiomyocytes with greater T-tubular and caveolar densities (Wright *et al.*, 2018). Local membrane microdomain responses in basal cardiomyocytes and whole hearts had significantly smaller and shorter-lived β_2 AR and cAMP signals (Wright *et al.*, 2018). cAMP responses and contractile response to β_2 AR stimulation were equilibrated by removal of phosphodiesterase (PDE) 4 activity, or disruption organisation of caveolar by removal of cholesterol or genetic deletion of Cav 3 (Wright *et al.*, 2018).

Table 1.1: Summary of apicobasal differences

Current known differences between apex and base *in vitro* and *in vivo*. β AR: beta-adrenergic receptor.

Characteristic	Apex	Base	References
β AR density	Higher	Lower	(Mori <i>et al.</i> , 1993; Brouri <i>et al.</i> , 2004; Paur <i>et al.</i> , 2012)
Density of sympathetic nerve endings	Lower	Higher	(Kawano, Okada and Yano, 2003)
<i>In vivo</i> β AR response	Larger	Smaller	(Lathers, Levin and Spivey, 1986; Mantravadi <i>et al.</i> , 2007; Heather <i>et al.</i> , 2009)
<i>In vitro</i> β_2 AR response	Larger, longer-lived	Smaller, shorter-lived	(Paur <i>et al.</i> , 2012; Wright <i>et al.</i> , 2018)
T-tubular and caveolar densities	Lower	Greater	(Wright <i>et al.</i> , 2018)
Phosphodiesterase activity	Lower	Higher	(Wright <i>et al.</i> , 2018)
Myocardial wall thickness	Thinner	Thicker	-
Computed fibre strain and external work	Lower	Higher	(Usyk, Mazhari and McCulloch, 2000)
Action potential duration	Shorter	Longer	(Szentadrassy <i>et al.</i> , 2005)
Phase-1 repolarisation	Larger	Smaller	(Szentadrassy <i>et al.</i> , 2005)
Transient outward K ⁺ current and delayed rectifier K ⁺ current amplitude	Larger	Smaller	(Szentadrassy <i>et al.</i> , 2005)

1.2 Takotsubo syndrome

Takotsubo syndrome (TTS), colloquially known as broken heart syndrome, is a severe but reversible type of acute heart failure that predominantly affects post-menopausal women in situations of extreme stress (Templin *et al.*, 2015; Lyon *et al.*, 2016; Ghadri *et al.*, 2018). The consequent surge in plasma catecholamines (Wittstein *et al.*, 2005) results in the characteristic wall motion abnormality, with akinesis of the apex of the ventricle and hyperkinesis of the basal segments (Medeiros *et al.*, 2014) as seen in Figure 1.2. This profound contractile deficit can lead to serious complications, including cardiogenic shock, thrombi formation, ventricular wall rupture, pulmonary oedema and arrhythmia. This results in a significant mortality rate of 4-5%, which is comparable to acute coronary syndrome (ACS) (Singh, Carson, Shah, *et al.*, 2014; Templin *et al.*, 2015).

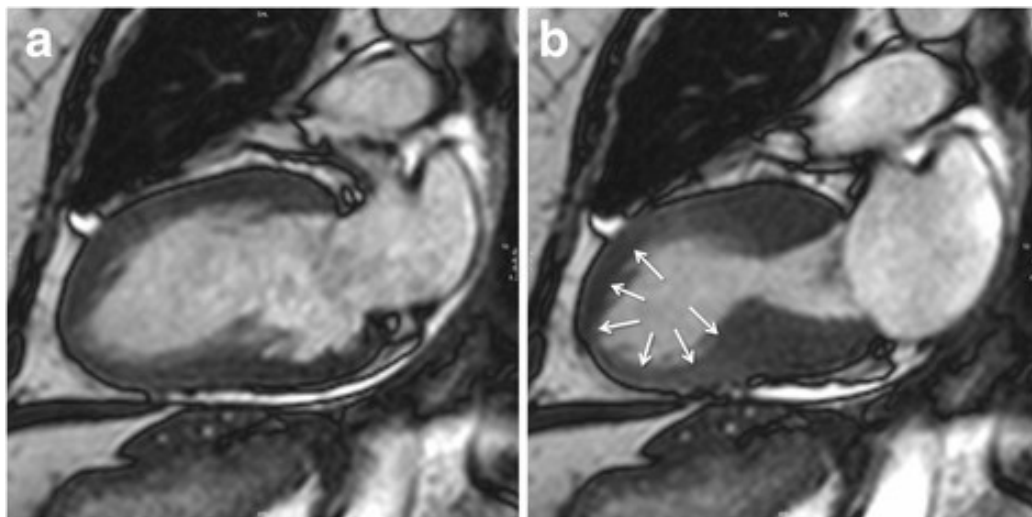


Figure 1.2: Representative magnetic resonance imaging (MRI) of TTS

Representative cardiac 2-chamber MRI image of the LV in (A) end-diastole and (B) end-systole in a TTS patient showing apical akinesia. Image obtained from (Plácido *et al.*, 2017) in June 2018.

TTS was first identified in 1990 in Japan (Sato *et al.*, 1990), and has become of increased interest since 2004, when increased incidence was noted following a major earthquake in the Niigata Prefecture in Japan (Sato *et al.*, 2006). The pathognomonic shape of TTS was compared to a ‘takotsubo’ or Japanese octopus pot (Sato *et al.*, 1990).

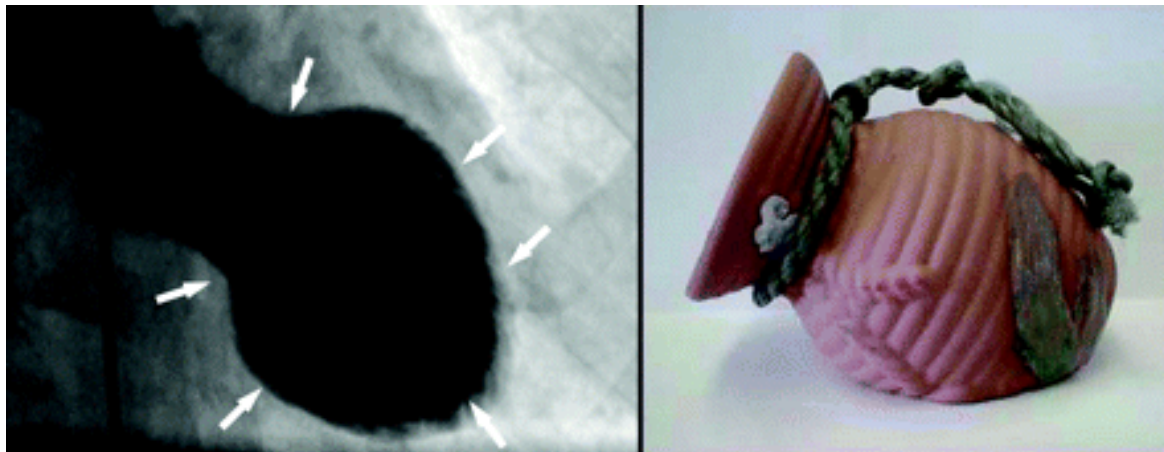


Figure 1.3: ‘Takotsubo’, the Japanese octopus pot

Representative images of apical akinesia in a TTS patient (**Left**) with a comparison to a Japanese octopus pot (**Right**). Image obtained from (Sharkey, Lesser and Maron, 2011) in June 2018, who originally obtained with permission from Dr Satoshi Kurisu, Hiroshima, Japan.

1.2.1 Nomenclature

Since the original naming of ‘takotsubo cardiomyopathy’ by Sato *et al.*, numerous alternatives have been proposed (Sharkey *et al.*, 2011). These include stress or stress-induced cardiomyopathy (Maron *et al.*, 2006), apical ballooning syndrome (Tsuchihashi *et al.*, 2001), ampullary-shaped cardiomyopathy (Kawai *et al.*, 2000; Owa *et al.*, 2001), and ‘broken heart syndrome’ owing to the occurrence after bereavement (Mukherjee, Sünkel-Laing and Dewhurst, 2013). The term takotsubo syndrome has been increasingly used and is sensibly becoming the consensus. Indeed, Lyon *et al.* surmise that the term ‘takotsubo’ is historically engrained in medical knowledge and registries (Sato *et al.*, 1990; Lyon *et al.*, 2016). Indeed,

they argue that since TTS is diagnosed based on numerous clinical observations, there is consensus that it fulfils the criteria of a syndrome (Lyon *et al.*, 2016). Furthermore, they state that the term ‘cardiomyopathy’ is inaccurate, as this refers to a primary disease of the myocardium of genetic or unknown origin, and TTS does not appear to be a primary muscle disorder, with no common genetic changes having been identified (Lyon *et al.*, 2016). Given the recoverability and low rate of major adverse cardiac events in the long-term aetiology of TTS patients, they conclude that TTS is distinct from other primary cardiomyopathies (Lyon *et al.*, 2016). ‘Takotsubo syndrome’ therefore seems to accurately describe all the disease elements within TTS and should be accepted.

1.2.2 Patient presentation

Patients initially present with symptoms and signs similar to acute coronary syndrome (ACS), with chest pain, ST-segment elevation on electrocardiogram (ECG) and a rise in troponin levels within the blood. Patients also commonly exhibit dyspnoea, and a minority present with syncope (Templin *et al.*, 2015). Coronary angiography is performed rapidly to diagnose and treat presumed acute myocardial infarction (MI), and once culprit coronary artery disease is excluded, TTS can be confirmed. For this reason, TTS has been historically missed, or considered late in the diagnostic process.

Use of biomarkers with improved sensitivity could aid in the early identification of TTS. TTS patients exhibit minor increases in creatine kinase-MB and cardiac troponin when compared to STEMI, reflecting lower amount of myocardial damage despite the apparent diffuse ventricular dysfunction (Akashi, Nef and Lyon, 2015). Resultant from the extreme ventricular dilatation, TTS patients display significant elevation of brain natriuretic peptide (BNP), or its by-product, NT-pro-BNP (Omeland *et al.*, 2002; Madhavan *et al.*, 2009; Fröhlich *et al.*, 2012). Accordingly, the comparative change between markers of myocardial necrosis and

ventricular dilatation could be used to differentiate TTS from ACS early in the diagnostic process (Madhavan *et al.*, 2009), with the highest sensitivity and specificity when using the ratio of NT-pro-BNP to troponin T (Fröhlich *et al.*, 2012). Indeed, NT-pro-BNP alone has recently been included in the updated diagnostic criteria for TTS from the European Society of Cardiology (Lyon *et al.*, 2016), with admission NT-pro-BNP levels being shown to be an independent predictor of 30-day major adverse clinical events and long-term mortality (Stiermaier *et al.*, 2018).

Recently, a profile of novel circulating microRNAs (miRs) has been identified that enables differentiation of TTS from both healthy controls, and STEMI. Indeed, miR-16 and miR-26a are significantly increased in TTS *versus* STEMI and control, and miR-1 and miR-133a are increased modestly in TTS *versus* control, and greatly in STEMI *versus* TTS and control as shown in Figure 1.4 (Jaguszewski *et al.*, 2014). When used in combination, this signature of miRs enabled for differentiation of TTS from STEMI with 96.77% sensitivity and 70.37% specificity (Jaguszewski *et al.*, 2014). miR-1 and miR-133a are enriched within the myocardium (Wystub *et al.*, 2013), so likely reflect the myocardial damage that occurs in STEMI as with cardiac troponin and creatine kinase-MB. As such, a combination of miR-16 and miR-26a, along with NT-pro-BNP and troponin T could result in the highly selective identification of TTS patients. Although, much is required to improve utilisation of novel non-coding RNA for use as clinical biomarkers (Gonzalo-calvo *et al.*, 2018).

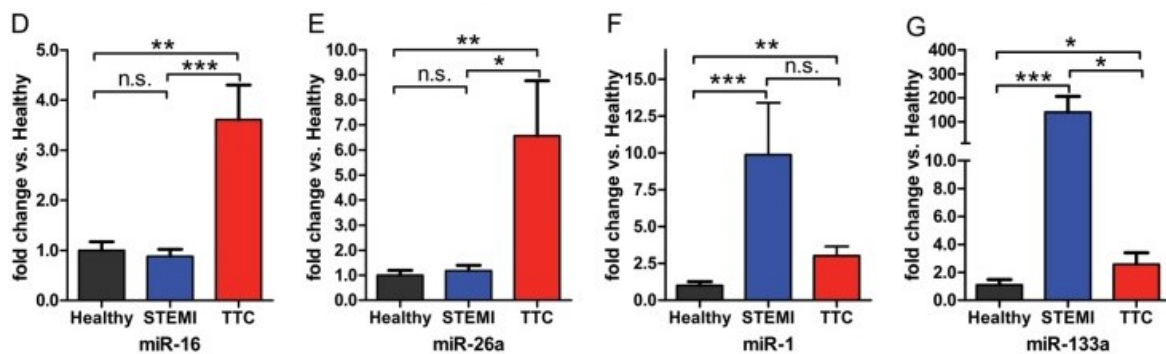


Figure 1.4: Circulating microRNAs in takotsubo syndrome

Comparison of the serum levels of miR-16, miR-26a, miR-1 and miR-133a between healthy control, and patients with STEMI (ST-segment elevation myocardial infarction) and TTS (takotsubo syndrome). Obtained from (Jaguszewski *et al.*, 2014) in June 2018.

In the majority of cases either a physical or emotional trigger can be identified, with a slight preponderance towards physical, with emotional triggers more common in women and physical stressors more common precipitants in men (Templin *et al.*, 2015). Recently, TTS has even been noted after happy events, such as weddings (Ghadri *et al.*, 2016).

TTS can occur in three common variants, but by far the commonest is the apical variant from which its name was derived. LV regional wall motion abnormality is present with apical and possible mid-LV hypokinesia and basal hypercontractility. An inverted-TTS is possible, with basal hypokinesia and apical hypercontractility, and also a mid-LV variant with apical and basal hypercontractility (Lyon *et al.*, 2016). Less common forms include biventricular, right ventricular and global and focal atypical variants (Lyon *et al.*, 2016). In their large cohort of patients, Templin *et al.* observed TTS variants to be 81.7% apical, 14.6% mid-LV, 2.2% basal and 1.5% focal (Templin *et al.*, 2015).

1.2.3 Epidemiology

Recent estimates suggest 2 to 3% of suspected ACS are eventually diagnosed as TTS (Redfors *et al.*, 2015). It has been calculated that the incidence of TTS is approximately 100 new cases per 1 million population per annum in the United States (Deshmukh *et al.*, 2012). Of women presenting with ACS, up to 10% are ultimately diagnosed as having TTS (Deshmukh *et al.*, 2012). This is probably a vast underestimate and the real incidence of TTS is likely to be much higher. Whilst TTS is becoming more widely recognised with increased awareness and access to early coronary angiography, atypical variants are often missed and pre-hospital sudden cardiac death resulting from TTS is overlooked (Lyon *et al.*, 2016; Templin, Napp and Ghadri, 2016).

Templin *et al.* found that 89.8% of TTS patients are women, with 79.1% of all patients being post-menopausal women (Templin *et al.*, 2015), although the reason behind this is not understood. Interestingly, there is a summer peak in incidence of TTS, as opposed to MI which is highest during the winter months (Manfredini *et al.*, 2016). Expectedly, the increase in incidence of TTS has recently been correlated with the rise in temperature that occurs in summer (Novo *et al.*, 2017).

1.2.4 Diagnosis

Until recently, the Mayo Clinic diagnostic criteria for TTS was the most widely used criteria for guidance of the identification of TTS patients (Prasad, Lerman and Rihal, 2008). This consisted of the expected contractility changes beyond the distribution of a single coronary, the absence of culprit coronary obstruction, ECG abnormalities and occurring in the absence of pheochromocytoma and myocarditis (Figure 1.5). The original criteria were amended to accept the inclusion of TTS secondary to pheochromocytoma, since there is a clear causal role of catecholamines in TTS.

Table II. Proposed Mayo Clinic criteria for ABS

1. Transient hypokinesis, akinesis, or dyskinesis of the left ventricular mid segments with or without apical involvement; the regional wall motion abnormalities extend beyond a single epicardial vascular distribution; a stressful trigger is often, but not always present. *
2. Absence of obstructive coronary disease or angiographic evidence of acute plaque rupture. †
3. New electrocardiographic abnormalities (either ST-segment elevation and/or T-wave inversion) or modest elevation in cardiac troponin.
4. Absence of:
Pheochromocytoma
Myocarditis

In both of the above circumstances, the diagnosis of ABS should be made with caution, and a clear stressful precipitating trigger must be sought.

* There are rare exceptions to these criteria such as those patients in whom the regional wall motion abnormality is limited to a single coronary territory.

† It is possible that a patient with obstructive coronary atherosclerosis may also develop ABS. However, this is very rare in our experience and in the published literature, perhaps because such cases are misdiagnosed as an acute coronary syndrome.

Figure 1.5: Mayo Clinic TTS diagnostic criteria

The original diagnostic criteria proposed by the Mayo Clinic for takotsubo syndrome, called ABS (apical ballooning syndrome) in this figure. Obtained from (Prasad, Lerman and Rihal, 2008) in June 2018.

The InterTAK Diagnostic Criteria (Figure 1.6, below) have since been proposed by the European Society of Cardiology, updated to encompass the greater understanding of TTS now possessed (Ghadri *et al.*, 2018). Whilst there is a distinct epidemiology of TTS patients, with a preponderance for post-menopausal women and clear associations such as following a stressful event, since these are not universal, so are contained within the diagnostic criteria but not required for TTS diagnosis.

Table I International Takotsubo Diagnostic Criteria (InterTAK Diagnostic Criteria)

1. Patients show transient^a left ventricular dysfunction (hypokinesia, akinesia, or dyskinesia) presenting as apical ballooning or midventricular, basal, or focal wall motion abnormalities. Right ventricular involvement can be present. Besides these regional wall motion patterns, transitions between all types can exist. The regional wall motion abnormality usually extends beyond a single epicardial vascular distribution; however, rare cases can exist where the regional wall motion abnormality is present in the subtended myocardial territory of a single coronary artery (focal TTS).^b
2. An emotional, physical, or combined trigger can precede the takotsubo syndrome event, but this is not obligatory.
3. Neurologic disorders (e.g. subarachnoid haemorrhage, stroke/transient ischaemic attack, or seizures) as well as pheochromocytoma may serve as triggers for takotsubo syndrome.
4. New ECG abnormalities are present (ST-segment elevation, ST-segment depression, T-wave inversion, and QTc prolongation); however, rare cases exist without any ECG changes.
5. Levels of cardiac biomarkers (troponin and creatine kinase) are moderately elevated in most cases; significant elevation of brain natriuretic peptide is common.
6. Significant coronary artery disease is not a contradiction in takotsubo syndrome.
7. Patients have no evidence of infectious myocarditis.^b
8. Postmenopausal women are predominantly affected.

^aWall motion abnormalities may remain for a prolonged period of time or documentation of recovery may not be possible. For example, death before evidence of recovery is captured.

^bCardiac magnetic resonance imaging is recommended to exclude infectious myocarditis and diagnosis confirmation of takotsubo syndrome.

Figure 1.6: InterTAK diagnostic criteria for TTS

Latest recommended diagnostic criteria for takotsubo syndrome proposed by InterTAK. Obtained from (Ghadri *et al.*, 2018) in November 2018.

If a patient presents with symptoms of acute chest pain of cardiac origin (angina), breathlessness, and palpitations due to sinus tachycardia or arrhythmia, urgent clinical evaluation should be carried out, along with a resting 12-lead ECG (Lyon *et al.*, 2016). In severe cases of TTS, pre-syncope or syncope may be present due to ventricular tachyarrhythmias, severe left ventricular outflow tract obstruction (LVOTO), or cardiogenic shock. Acute catecholamine and hypertensive surges may result in sensation of pressure waves from the chest to the neck and into the head, frequently associated with diaphoresis and heightened anxiety (Lyon *et al.*, 2016).

An ACS treatment pathway should be initiated, with coronary angiography where appropriate (Steg *et al.*, 2012). In stable cases where patients are pain free, or echocardiography shows typical TTS features, coronary computed tomography angiography can be considered (Otalvaro, Zambrano and Fishman, 2011; Lyon *et al.*, 2016). Following this, if TTS is

suspected, early cardiac imaging and characteristic cardiac biomarkers help exclude MI and stratify patients (Lyon *et al.*, 2016). With borderline cases, cardiac magnetic resonance (CMR) imaging with late gadolinium enhancement may help (Haghi *et al.*, 2006; Eitel *et al.*, 2011; Lyon *et al.*, 2016). This allows for better risk stratification and can better detect LV apical thrombi (Lyon *et al.*, 2016).

1.2.5 Prognosis

Whilst TTS was originally considered to carry a favourable prognosis (Elesber *et al.*, 2007), it is now appreciated that patients experience significant mortality in the acute incidence (Deshmukh *et al.*, 2012; Templin *et al.*, 2015; Tornvall *et al.*, 2016), and in the long-term (Sharkey *et al.*, 2010; Schultz *et al.*, 2012; Redfors *et al.*, 2015; Templin *et al.*, 2015; Ghadri *et al.*, 2018). A meta-analysis of studies from Germany and Austria (Schneider *et al.*, 2013), Italy (Citro *et al.*, 2014), the USA (Brinjkji, El-Sayed and Salka, 2012) and Japan (Isogai *et al.*, 2014) found in-hospital mortality to equal 4.5% (Singh, Carson, Shah, *et al.*, 2014), which is similar to STEMI. In the InterTAK registry, long-term mortality was recently shown to be comparable to patients with ACS (Ghadri *et al.*, 2018). Whilst it could be postulated that in-hospital onset of TTS would carry better prognosis than out of hospital onset, in fact it is significantly worse owing to the more frequent associations with comorbidities (Isogai *et al.*, 2014).

Beyond the initial contractile deficit, the long-term sequelae appear to involve cardiac inflammation. Indeed, endomyocardial biopsy shows mononuclear infiltrates and contraction-band necrosis (Wittstein *et al.*, 2005) and slowly resolving global myocardial oedema is present on MRI (Neil *et al.*, 2012). As this subsides, a process of global microscopic fibrosis develops in its place, detected as early as 4 months (Schwarz *et al.*, 2017). Therefore, it is

important to understand the processes linking the acute changes in contractility to the downstream inflammatory changes which worsen long-term prognosis.

Data pertaining to risk of TTS recurrence is limited, but has recently been summarised by Akashi *et al.*, who noted recurrence rate ranged from 0 to 22%, dependent on the size of the series studied and duration of follow-up (Akashi, Nef and Lyon, 2015). Singh *et al.* performed a meta-analysis, and reported the annual recurrence rate to be approximately 1.5%, with the cumulative incidence rising from 1.2% at 6 months to nearly 5% at 6 years (Singh, Carson, Usmani, *et al.*, 2014). Interestingly, the recurrence rate was higher than in younger patients aged less than 50 years (Patel, Chokka, *et al.*, 2013).

1.2.6 Treatment

Currently, there are no evidence-based treatments for TTS. The HFA have recently published recommendations for the management of TTS patients based on expert consensus (Lyon *et al.*, 2016). To summarise, patients should be risk stratified based on their proposed system (Figure 1.7), which includes important several important clinical variables including age, ejection fraction (EF) and presence of ventricular thrombi (Lyon *et al.*, 2016).

Beta blockers have been shown to be beneficial in pre-clinical studies (Izumi *et al.*, 2009; Paur *et al.*, 2012), and should be considered if there is a mild or greater reduction in LVEF and the patient is haemodynamically stable (Lyon *et al.*, 2016). If haemodynamically significant LVOTO is present, beta-blockers or selective alpha 1 agonists should be considered (Lyon *et al.*, 2016). It should be noted that beta blockers that confer a bias for β_2 AR have been shown to worsen mortality in a pre-clinical model (Paur *et al.*, 2012), since the cardioprotective effect of β_2 AR-Gai signalling is removed. β_2 AR agonists have been shown to confer greater risk in TTS patients (Tornvall *et al.*, 2016), as have other inotropes (Redmond *et al.*, 2013), as the catecholamine-induced TTS may be worsened (Paur *et al.*,

2012; Shao, Redfors, Scharin Täng, *et al.*, 2013; Shao, Redfors, Ståhlman, *et al.*, 2013; Lyon *et al.*, 2016).

Risk factor	Higher risk	Lower risk
MAJOR RISK FACTORS		
Age	≥75 years	See minor risk factors ^a
Systolic BP	<110 mmHg	≥110 mmHg
Clinical pulmonary oedema ^b	Present	Absent
Unexplained syncope, VT or VF	Present	Absent
LVEF	<35%	See minor risk factors ^a
LVOTO	≥40 mmHg	Absent or <40 mmHg
Mitral regurgitation ^c	Present	Absent
Apical thrombus	Present	Absent
New VSD or contained LV wall rupture	Present	Absent
MINOR RISK FACTORS		
Age	70–75 years	<70 years
ECG		
QTc	≥500 ms	<500 ms
Pathological Q waves	Present	Absent
Persistent ST elevation ^d	Present	Absent
LVEF	35–45%	≥45%
Physical stressor	Present	Absent
Natriuretic peptides		
BNP	≥600 pg/mL	<600 pg/mL
NT-proBNP	≥2000 pg/mL	NT-proBNP <2000 pg/mL
Bystander obstructive CAD	Present	Absent
Biventricular involvement	Present	Absent

BP, blood pressure; LVOTO, left ventricular outflow tract obstruction; VF, ventricular fibrillation; VSD, ventricular septal defect; VT, ventricular tachycardia.

^aSee minor criteria regarding LVEF in the absence of major criteria.

^bLower zone (basal) pulmonary rales on clinical examination or evidence on chest X-ray.

^cModerate or severe mitral regurgitation.

^d≥3 days.

Figure 1.7: Heart Failure Association risk stratification in Takotsubo syndrome

Patient risk stratification criteria for takotsubo syndrome proposed by the Heart Failure Association. Obtained from (Lyon *et al.*, 2016) in June 2018.

Lyon et al. suggest that other vasoactive drugs, including Angiotensin converting enzyme (ACE) inhibitors, should be avoided if cardiac output is normal, since peripheral vascular resistance may be low in some TTS patients (Lyon *et al.*, 2016).

They highlight that the predominant management of TTS patients should be by a specialist and is supportive. Higher risk patients should be in a coronary care unit or high-dependency unit with continuous monitoring (including echocardiography) and access to resuscitation equipment for at least 72 hours after presentation (Lyon *et al.*, 2016), as this is when risk from life-threatening complications is greatest (Schneider *et al.*, 2014).

In severe cases of cardiogenic shock with progressive organ dysfunction, mechanical support with LV assist devices (LVADs) and extracorporeal membrane oxygenation (ECMO) can be considered for bridge-to-recovery (Zegdi *et al.*, 2008; Bonacchi *et al.*, 2009; Donker *et al.*, 2012; Lazzeri *et al.*, 2013; Lyon *et al.*, 2016). Lyon et al. advise that intra-aortic balloon counterpulsation (IABP) should be avoided, as evidence from IABP-SHOCK II trial suggests dynamic LVOTO could be worsened (Lyon *et al.*, 2016).

Pre-clinical data has shown levosimendan, a catecholamine-sparing inotrope, to be beneficial (Paur *et al.*, 2012), but expert opinion is mixed due to limited clinical experience and lack of validation by randomised control trial (Padayachee, 2007; De Santis *et al.*, 2008; Antonini *et al.*, 2010; Karvouniaris *et al.*, 2012; Santoro *et al.*, 2013). In the absence of mechanical support, levosimendan may be the preferred inotrope in patients with cardiogenic shock and multiorgan failure (Lyon *et al.*, 2016).

TTS patients are vulnerable to thrombosis, since high concentrations of adrenaline are prothrombotic (Cecchi *et al.*, 2013), and stasis of blood is increased in the hypokinetic ventricle. This leads to thromboembolism being a complication in approximately 4% of TTS patients. TTS patients may initially be on dual-antiplatelet therapy plus heparin for STEMI,

although this can be withdrawn and oral anticoagulation therapy administered if intraventricular thrombus is detected (Lyon *et al.*, 2016). The role of prophylactic oral anticoagulation has not been assessed in TTS patients, although could be considered in patients with increased clotting risk such as from profound ventricular akinetic segments or with atrial fibrillation (Lyon *et al.*, 2016).

Recently the anaesthetic isoflurane has been shown to protect against TTS in a preclinical rodent model (Oras, Redfors, Ali, Alkhoury, *et al.*, 2017; Oras, Redfors, Ali, Lundgren, *et al.*, 2017), although this has not been assessed in human.

After recovery, all patients should be reassessed at 3 to 6 months to confirm the resolution of the echocardiographic and ECG changes (Lyon *et al.*, 2016). Medication administered for ACS or TTS may be removed in the absence of other indications (Lyon *et al.*, 2016). Patients with persistent symptoms require further treatment and investigation, as ongoing cardiac abnormalities may arise (Lyon *et al.*, 2016).

At present, no strategy to prevent TTS recurrence has been demonstrated. Despite the mechanistic involvement of catecholamines in TTS, a meta-analysis found beta-blockers, as well as other medication had no impact of the risk of recurrence (Santoro *et al.*, 2014).

Nonetheless, Lyon *et al.* advise that beta-blockers may still be applicable in some patients with elevated sympathetic tone, ongoing cardiac symptoms or persistent anxiety (Lyon *et al.*, 2016). Indeed, the ability to deal with stressful triggers could also be targeted with psychological counselling or cognitive behavioural therapy (Lyon *et al.*, 2016). Whilst the efficacy of this in preventing recurrence has not been determined, this seems sensible owing to the frequent association of TTS patients with psychiatric disorders (Templin *et al.*, 2015).

1.2.7 Pathophysiology

Whilst the association of TTS with catecholamines is well evidenced, there is no established pathophysiological mechanism.

Vascular dysfunction

Upon identification of TTS in 1990, Sato *et al.* proposed that the apical dysfunction was secondary to multivessel coronary vasospasm (Sato *et al.*, 1990; Dote *et al.*, 1991), more recently, this has been linked to microvascular coronary dysfunction (Patel, Lerman, *et al.*, 2013). This could contribute to the phenotype of acute ischaemic stunning (Kato *et al.*, 2017). The prevalence of vascular dysfunction seems variable in TTS patients, and is often not present (Tsuchihashi *et al.*, 2001; Akashi, Nef and Lyon, 2015). Endothelin has been proposed to mediate this vasospasm as is increased in TTS, although this is to the same level as in STEMI where this does not occur (Jaguszewski *et al.*, 2014). The surge in adrenaline is likely to lead to endothelial dysfunction (Naegelé *et al.*, 2016) that could sensitise to vasospasm upon provocation (Akashi, Nef and Lyon, 2015). As such, there is no causal evidence for the role of vasospasm in the generation of TTS, and this may just be an epiphenomenon of the catecholamine surge (Akashi, Nef and Lyon, 2015; Kato *et al.*, 2017). Indeed, catecholamines such as adrenaline or dobutamine (Abraham *et al.*, 2009) would vasodilate coronary arteries, and preclinical models inducing TTS with adrenaline or isoprenaline and without endothelin have specifically caused apical dysfunction (Paur *et al.*, 2012; Shao, Redfors, Scharin Täng, *et al.*, 2013) in the absence of myocardial perfusion abnormality (Redfors, Shao, *et al.*, 2014). Additionally, the localisation of contractility changes in many TTS variants would not be explained by multivessel vasospasm, and endomyocardial biopsies show no sign of ischaemic changes, stunned or hibernating

myocardium as proposed (Nef *et al.*, 2007; Kato *et al.*, 2017). When considering these factors, coronary vasospasm is unlikely to be the pathogenic mechanism in TTS.

Owing to the similarities in presentation between ACS and TTS, it has been suggested that TTS is a form of microvascular or aborted ACS (Lüscher and Templin, 2016). However, intravascular ultrasound studies have not noted plaque rupture, endothelial breach or intracoronary thrombus in TTS (Haghi *et al.*, 2010; Delgado *et al.*, 2011) and no changes in the frequency of wrap-around left anterior descending coronary artery anatomy (Hoyt *et al.*, 2010). As previously discussed, circulating miR profile has also been demonstrated to be different in TTS than STEMI, with higher miR-16 and miR-26a in TTS, and lower miR-1 and miR-133a (Jaguszewski *et al.*, 2014). Along with the differences in other biomarkers, such as troponin and NT-pro-BNP, this suggests a difference in the pathogenesis of the two conditions. Also, as aforementioned the epidemiology of TTS and ACS are different, with differences in patient demographic and seasonal and diurnal presentation pattern. Most pertinently, the hypokinetic region in TTS extends over the region of more than any single coronary artery. Therefore, there is strong evidence to show that TTS and ACS are pathogenically distinct (Wright, Tranter, *et al.*, 2014).

Catecholaminergic myocardial stunning

It is well evidenced that catecholamines play a causative role in TTS. Adrenaline levels have been reported to be 10 to 20 times normal, and also higher than in STEMI (Wittstein *et al.*, 2005). TTS has also been demonstrated to occur secondary to medical conditions with elated catecholamine levels such as pheochromocytoma (Y-Hassan, 2016), acute subarachnoid haemorrhage (Naredi *et al.*, 2000) and acute thyrotoxicosis (Eliades *et al.*, 2014).

Furthermore, iatrogenic TTS in stress dobutamine echocardiography or after adrenaline administration has been frequently reported (Abraham *et al.*, 2009). Pre-clinical models also

provide good evidence for this catecholamine based hypothesis, as TTS has been robustly induced in rodent and primate models with adrenaline (Izumi *et al.*, 2009; Paur *et al.*, 2012).

The central role of catecholamines has been demonstrated by the dependence of the anatomical deficit in contractility varying with different catecholamines in an apparently afterload dependent matter, wherein peripheral vasodilation was observed to be necessary for apical akinesia (Redfors, Ali, *et al.*, 2014). Indeed Paur *et al.* observed an initial hypertensive phase following adrenaline administration, and the acute heart failure only developed after an abrupt fall in blood pressure below normal after approximately 5 minutes (Paur *et al.*, 2012). The differences in peripheral vasoactive catecholamines has been implicated in the complication of LVOTO.

An elegant hypothesis that builds on the well-evidenced involvement of catecholamines and explains the apicobasal gradient in ventricular dysfunction centres around β ARs. Indeed, whilst the β_1 AR only signals via the canonical stimulatory $G_{\alpha s}$ pathway, the pleiotropic β_2 AR can signal via $G_{\alpha s}$ or the inhibitory $G_{\alpha i}$ (Hasseldine, Harper and Black, 2003; Heubach, Ravens and Kaumann, 2004). Physiologically, this limits the toxic effects of $G_{\alpha s}$ activity (Zhu *et al.*, 2003) by shifting receptor coupling to $G_{\alpha i}$, a process known as stimulus trafficking or biased agonism (Heubach, Ravens and Kaumann, 2004). This opposes the contractile effect of $G_{\alpha s}$, and is directly cardiodepressive (Heubach *et al.*, 2003). This system is spatially organised across the mammalian ventricle with sympathetic nerve endings having the highest density in the ventricular base (Kawano, Okada and Yano, 2003), and β ARs having the highest density in the apex as seen in Figure 1.8 (Mori *et al.*, 1993; Brouiri *et al.*, 2004; Paur *et al.*, 2012). Indeed, noradrenaline released from sympathetic nerve terminals has a higher affinity for β_1 AR, and circulating adrenaline has a higher affinity for β_2 AR. Humans also have a higher concentration of β_2 AR than other mammals, with 4 β_1 AR to 1 β_2 AR (Port

and Bristow, 2001). Furthermore, apical cardiomyocytes have been shown to exhibit a significantly larger overall β AR in mammalian models *in vivo* (Lathers, Levin and Spivey, 1986; Mantravadi *et al.*, 2007; Heather *et al.*, 2009) and β_2 AR response *in vitro* than basal cardiomyocytes (Paur *et al.*, 2012; Wright *et al.*, 2018). Together, circulating adrenaline would more profoundly affect the apex of the ventricle in extreme stress.

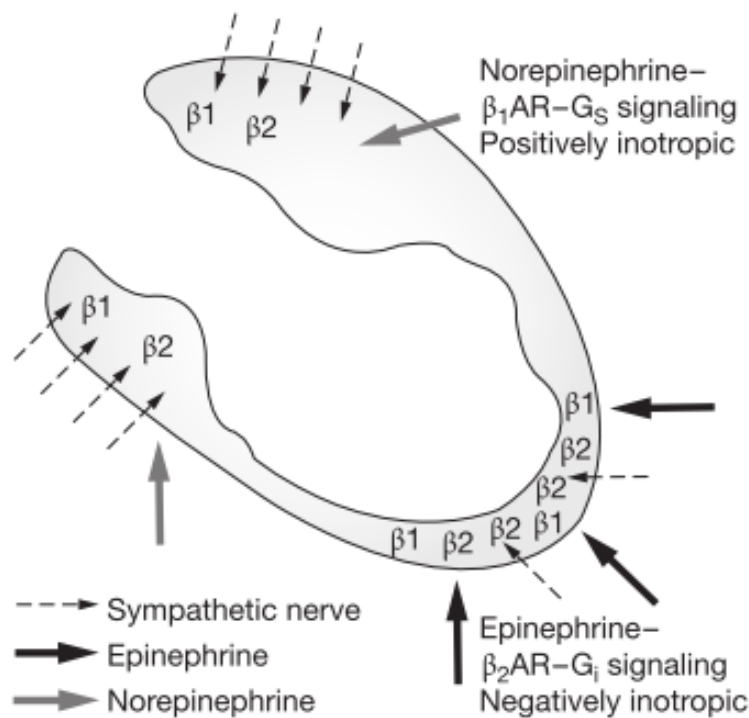


Figure 1.8: Schematic representation of the regional differences in adrenergic localisation

Schematic representation of the left ventricle, with neuronal innervation and adrenergic receptors shown.

Obtained from (Lyon *et al.*, 2008) in June 2018.

TTS has been recapitulated *in vivo* in preclinical rodent models by bolus injection of adrenaline, or its analogue isoproterenol (Izumi *et al.*, 2009; Paur *et al.*, 2012; Shao, Redfors, Scharin Täng, *et al.*, 2013). Paur *et al.* demonstrate the crucial role of β_2 AR- G_{ai} coupling in the generation of TTS, since TTS was prevented with inhibition of G_{ai} with PTX (Paur *et al.*, 2012). However, β_2 AR- G_{ai} has also been demonstrated to be markedly cardioprotective in

TTS, since inhibition with β_2 AR antagonists or PTX reduced apical hypokinesis, but also increase mortality by approximately 70% (Shao, Redfors, Scharin Täng, *et al.*, 2013).

Endomyocardial biopsy studies from TTS patients strengthen the evidence for the mechanistic involvement of β_2 AR-G α_i , as they show activation of downstream pro-survival pathways PI3K and Akt which are normalised after follow-up and not present in ischemia (Nef *et al.*, 2009). Indeed, increased levels of GRK and β -arrestin have recently been identified in TTS (Nakano *et al.*, 2018), and these are required for β_2 AR-G α_i stimulus trafficking to occur.

Oestrogen deprivation

Given the preponderance of TTS for post-menopausal women, the central involvement of oestrogen withdrawal has logically been proposed. Oestrogen confers cardioprotection via a multitude of mechanisms, including sympatholytic (Patten *et al.*, 2004; Ling, Komesaroff and Sudhir, 2006). This is of importance when considering basal circulating adrenaline is lower in women than in men (Davidson *et al.*, 1984), as are urinary cortisol, adrenaline and noradrenaline, which rise with age (Deane, Chummun and Prashad, 2002). Interestingly, hormone replacement therapy reduced the levels of these hormones, suggesting a regulation by oestrogen (Deane, Chummun and Prashad, 2002).

Indeed, adrenergic dependent gene expression changes are observed in a rodent model of emotional stress simulating 'stress cardiomyopathy' by conscious immobilisation (Ueyama, Yoshida and Senba, 1999; Ueyama *et al.*, 2003), and are prevented by oestrogen supplementation (Ueyama *et al.*, 2007). Indeed, oophorectomy increases the expression of β_1 ARs, which is prevented with oestrogen supplementation (Chu *et al.*, 2006). Furthermore, chronic oestrogen exposure reduces the enhanced expression of β_1 ARs in the myocardium that occurs secondary to catecholamines and ischemia-reperfusion injury (Kam *et al.*, 2004).

The impact that hormone replacement therapy has on the incidence and recurrence of TTS is not clear, although this offers an interesting avenue for investigation and in the future prevention of TTS.

Observationally, women demonstrate greater vascular β_2 AR sensitivity (Kneale *et al.*, 2000), which suggests that oestrogen is able to potentiate β_2 AR signalling. Furthermore, oestrogen has been demonstrated to signal by downstream prosurvival β_2 AR-G α_i coupled PI3K and Akt pathways (Deschamps and Murphy, 2009) which would confer β_2 AR-G α_i cardioprotective effects in cardiomyocytes (Lyon *et al.*, 2008), as shown to be upregulated in TTS. This balance of cardiodepressive but cardioprotective effects of β_2 AR-G α_i with the inotropic but toxic β_1 AR-G α_s could confer the difference between sudden cardiac death *versus* TTS subsequent to catecholamines.

Brain-heart axis

Given the implied importance of the ANS in TTS, and the close association with neuropsychiatric disorders, there seems to be a clear interaction between brain and heart in TTS. A recent MRI study has assessed the role of brain regions controlling the ANS and involved in the regulation of emotions and integration with the limbic system (Hiestand *et al.*, 2018). This was undertaken approximately a year after presentation with TTS, and they found reduced cortical thickness in the insula and cingulate cortex in TTS, as well as reduced connectivity within the limbic system and ANS-specific network constituting the left amygdala, both hippocampi, left para-hippocampal gyrus, left superior temporal pole, and right putamen (Hiestand *et al.*, 2018). Importantly, both the parasympathetic and sympathetic arms of the ANS have previously been shown to be altered, suggesting the balance may be more important than the sympathetic hypersensitivity as previously proposed (Klein *et al.*, 2017). Whilst this is not proof of a causal relationship, as it is not prospective and could be

associated with the catecholamine surge, it does preliminarily suggest processing of emotions and subsequent response from the ANS to stress may be profoundly different in TTS patients. This could explain the ‘gain’ in the hypothalamic-pituitary axis that has previously been suggested in TTS (Wittstein *et al.*, 2005; Lyon *et al.*, 2016).

The anatomical differences within the brains of TTS patients is particularly interesting due to the close association of TTS with neuropsychiatric disorders (Templin *et al.*, 2015).

Furthermore, the miRs recently identified to be increased in TTS (Jaguszewski *et al.*, 2014) have also been shown to be altered in mental stress and depression. Indeed miR-16 has been shown to be increased in stress and depression (Bai *et al.*, 2012; Katsuura *et al.*, 2012), and miR-26a to be reduced following treatment of depression (Radu Enatescu *et al.*, 2016).

Further links exist between the brain and heart in TTS, with interesting parallels existing between neurogenic stunned myocardium (NSM) and TTS (Pilgrim and Wyss, 2008; Bielecka-Dabrowa *et al.*, 2010; Guglin and Novotorova, 2011; Inamasu *et al.*, 2014). Both are possible consequences of subarachnoid haemorrhage (SAH) with sympathetic over-activity and exhibit characteristic echocardiographic and ECG findings. A comparison between NSM and TTS patients was carried out with comparable echocardiographic findings, although higher catecholamines levels in NSM (Inamasu *et al.*, 2014). This was likely due to the greater average stress in NSM as it was secondary to SAH, whereas TTS patients had various severities of triggers. There seems to be minor differences in presentation (Ancona *et al.*, 2016), although it is possible NSM is a specific subset of TTS. Indeed, echocardiography often observes global hypokinesia in NSM secondary to catecholamine surge with characteristic ECG changes as in TTS (Bybee *et al.*, 2004; Ko, 2014; Biso *et al.*, 2017) with similar diagnostic criteria (Bulsara *et al.*, 2003). The conditions have previously been compared in great detail to conflicting opinions (Guglin and Novotorova, 2011; Inamasu *et*

al., 2014; Biso *et al.*, 2017). It now seems likely that NSM is a subset of atypical TTS which is specifically secondary to catastrophic neurological conditions such as SAH.

Long-term aetiology

Beyond the initial contractile deficit, the long-term sequelae appear to involve cardiac inflammation. This is alongside gene changes in metabolism pathways, shifting to lipid based metabolism (Nef *et al.*, 2008). Endomyocardial biopsy shows mononuclear infiltrates and contraction-band necrosis (Wittstein *et al.*, 2005), and slowly resolving global myocardial oedema is present on MRI (Neil *et al.*, 2012). As this subsides, a process of global microscopic fibrosis develops in its place, detected as early as 4 months (Schwarz *et al.*, 2017). Therefore, it is important to understand the processes linking the acute changes in contractility to the downstream inflammatory changes which worsen long-term prognosis.

Altered nitric oxide (NO) signalling has previously been demonstrated in TTS patients (Nguyen *et al.*, 2013), a point that is particularly interesting given that NO synthase (NOS) can couple to the β_2 AR (Ferro *et al.*, 2004). Pilot immunohistological studies of LV myocardium from TTS patients show evidence of nitrosative stress (Surikow *et al.*, 2015) which can impair cardiac energetics. In a preclinical rodent model, inflammatory changes within the myocardium have been demonstrated 24 hours following isoprenaline, including increases in CD68⁺ macrophages and levels of inflammatory markers (Surikow *et al.*, 2018). They hypothesise that PARP-1 (Poly [ADP-ribose] polymerase 1) might be the link from nitrosative stress to the decline in systolic function, because of its effects on myocardial energetics. Pre-treatment of the PARP-1 inhibitor 3-aminobenzamide (3AB) limited the reduction in the apical radial strain and fractional shortening (Surikow *et al.*, 2018). However, there were no changes in the inflammatory markers, indicating failure to prevent inflammation, or the rebound increase in oxidative stress (Cieřlar-Pobuda, Saenko and

Rzeszowska-Wolny, 2012). Inflammation and oedema seem to underlie the chronic changes present within TTS (Couch and Harding, 2018).

Synthesising the pathophysiology of TTS

The various clinical and preclinical studies have demonstrated that the pathophysiology of TTS is complex and varied. Indeed, pharmacologically, catecholamines exert a multitude of physiological and pathological effects on many cell types. As such, it seems extremely likely that the catecholamine surge exerts numerous actions, including on the vasculature and myocardium. It seems differences in emotional processing may prime the ANS to result in the catecholamine surge after stress, which are potentiated by oestrogen withdrawal. Circulating adrenaline then exerts direct cardiodepressive effects on the myocardium in an apicobasal gradient according to the organisation of β AR across the myocardium. The phenotypic hypokinesia seems modulated by vascular tone and changes in afterload. Following the initial insult and recovery, the catecholamine storm results in inflammatory changes that cause long-term contractile dysfunction. Despite this assertion, it remains unclear how these factors interact, how patients are predisposed to develop TTS, and how similar changes in catecholamines particularly result in SCD in men.

1.2.8 Genetic alterations in TTS

Several efforts have been made to identify genetic mutations in patients with TTS, however relatively few have been found. It is possible that specific genetic mutations could confer adrenergic perturbations that would predispose to TTS. A family predilection has been observed in a few cases of TTS (Pison, De Vusser and Mullens, 2004; Kumar, Holmes and Prasad, 2010; Musumeci *et al.*, 2013; Ikutomi *et al.*, 2014), although presence of specific genetic mutations has not been identified. Functional polymorphisms have been proposed and

identified in isolated examples for α_1 AR, β_1 AR, β_2 AR, GRK5 and oestrogen receptors (Limongelli *et al.*, 2016).

Interestingly, comparison of TTS induced pluripotent stem cell-derived cardiomyocyte (iPSC-CM) patient cell lines supports the involvement of the β AR in TTS but did not identify any genetic differences. They observed increased β_1 AR, β_2 AR and cAMP responses, increased calcium transients and kinetics, increased intracellular lipid accumulation and reduced mitochondrial function (Borchert *et al.*, 2017). Engineered Heart Tissue (EHT), 3-dimensional, force-generating strips of iPSC-CMs (Schaaf *et al.*, 2011; Mannhardt *et al.*, 2016), made from these TTS iPSC-CMs had reduced baseline force generation compared with controls, higher sensitivity to catecholamines and reduced desensitisation following 24 hours of catecholamines (Borchert *et al.*, 2017). Whilst this may have been expected to be evidence of genetic changes in TTS, no mutations were identified on whole exome sequencing (Borchert *et al.*, 2017), leaving the possibility of epigenetic changes before or after the catecholamine storm in TTS.

Chen *et al.* investigated the epigenetic changes induced by chronic isoprenaline treatment in the context of chronic heart failure in mice and demonstrated marked genetic differences in DNA methylation that are associated with disease progression in heart failure (Chen *et al.*, 2016). This could explain the results obtained by Borchert *et al.* presented above (Borchert *et al.*, 2017).

Non-coding RNA is another source of variation between patients, that has been shown to be important in cardiovascular disease progression outside of TTS. There is one report of a mutation in Bcl2-associated athanogene 3 (BAG3) 3'-UTR which is a component of the chaperone-assisted autophagy pathway, that prevents miR-371a-5p binding and increases BAG3 in cardiomyocytes following adrenaline (d'Avenia *et al.*, 2015). However, this report

shows an atypical role for miR regulation, with increased protein expression, and does not relate how changes in BAG3 could be of any relevance in TTS.

The novel biomarkers identified by Jaguszewski et al. consisted of increased levels of miR-16 and miR-26a in TTS compared to STEMI and control as previously discussed (Jaguszewski *et al.*, 2014). This is suggestive of a distinct pathogenesis and could represent novel mechanistic factors predisposing patients to develop TTS.

1.3 MicroRNAs

MicroRNAs (miRs) are small non-coding sequences of nucleotides, approximately 22 base pairs in length, that post-transcriptionally regulate cellular function by degrading or functionally silencing messenger RNA (mRNA) (Bartel, 2004; Ha and Kim, 2014). Indeed, miRs have been demonstrated to regulate instrumental processes within the cell such as proliferation, differentiation and apoptosis (Bartel, 2004), are the dominant class of small RNAs in most somatic tissues (Ha and Kim, 2014), and are becoming of increased interest in research into cardiovascular physiology and disease (Thum and Condorelli, 2015).

1.3.1 Biogenesis

miRs are transcribed by RNA polymerase II (Pol II), and miRs with identical sequences at nucleotide 2 to 8 belong to the same ‘miR family’ (Bartel, 2004). Sister genes are indicated with lettered suffices, and if the same miR is produced from different loci, that is denoted by a numerical suffix. Each gene locus produces a 3’ strand and a 5’ strand, with one being named the ‘guide’ strand based on increased prevalence and biological activity, and the other termed the ‘passenger’ strand, denoted miR* (Ha and Kim, 2014). However, the passenger strands are not inert, and can exert biological effects (Bang *et al.*, 2014).

The majority of miRs in human are encoded in intronic regions of other non-coding or protein coding transcripts, termed a polycistronic transcription unit when several miR loci are in close proximity (Lee *et al.*, 2002). miR transcription can be regulated by transcription factors such as p53 and MYC (Kim, Han and Siomi, 2009; Krol, Loedige and Filipowicz, 2010), or be under epigenetic control by DNA methylation and histone modification (Davis-Dusenbery and Hata, 2010).

miRs are transcribed as primary miR sequences (pri-miRs) and the long primary transcript (usually over 1kb) has a local hairpin structure where miRNA sequences are embedded, as displayed in Figure 1.9 (Lee *et al.*, 2002). This is cleaved by the nuclear RNase III Drosha, which crops the stem-loop of the pri-miR to form a precursor miR (pre-miR), a hairpin structure of approximately 65bp (Lee *et al.*, 2003). Drosha does this with DGCR8, an essential cofactor, which forms the Microprocessor complex (Denli *et al.*, 2004; Gregory *et al.*, 2004; Han *et al.*, 2004; Landthaler, Yalcin and Tuschl, 2004).

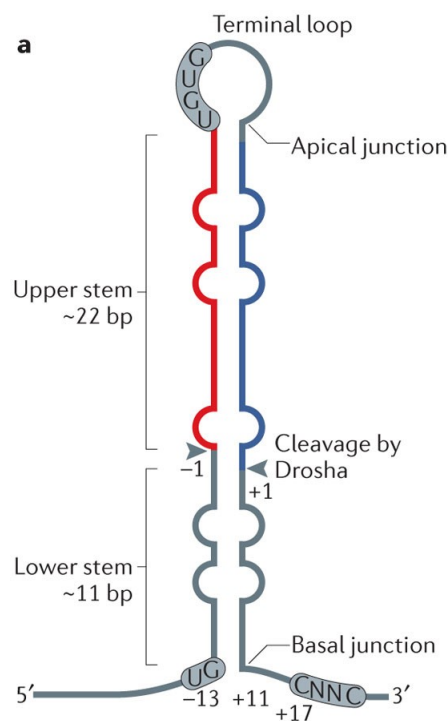


Figure 1.9: Schematic representation of primary miRs

Schematic of primary microRNA with red and blue arbitrarily represent guide and passenger strands. Taken from (Ha and Kim, 2014) in June 2018.

The synthesised pre-miR is exported from the nucleus into the cytoplasm in complex with exportin 5 and RAN.GTP (Yi *et al.*, 2003; Bohnsack, Czaplinski and Gorlich, 2004; Lund *et al.*, 2004), with release of the pre-miR upon GTP hydrolysis (Ha and Kim, 2014). Pre-miR is

then cleaved by another RNase III, Dicer, to yield a small RNA duplex (Bernstein *et al.*, 2001; Grishok *et al.*, 2001; Hutvagner *et al.*, 2001; Ketting *et al.*, 2001; Knight and Bass, 2001).

The small RNA duplex is loaded into an RNA-induced silencing complex (RISC) formed of argonaute proteins (AGO) (Hammond *et al.*, 2001; Mourelatos *et al.*, 2002), upon which it is unwound (Kawamata and Tomari, 2010). This removes the passenger strand to form the mature RISC, which is able to exert its functional effects (Ha and Kim, 2014). The entire biogenesis of miRs is illustrated in Figure 1.10.

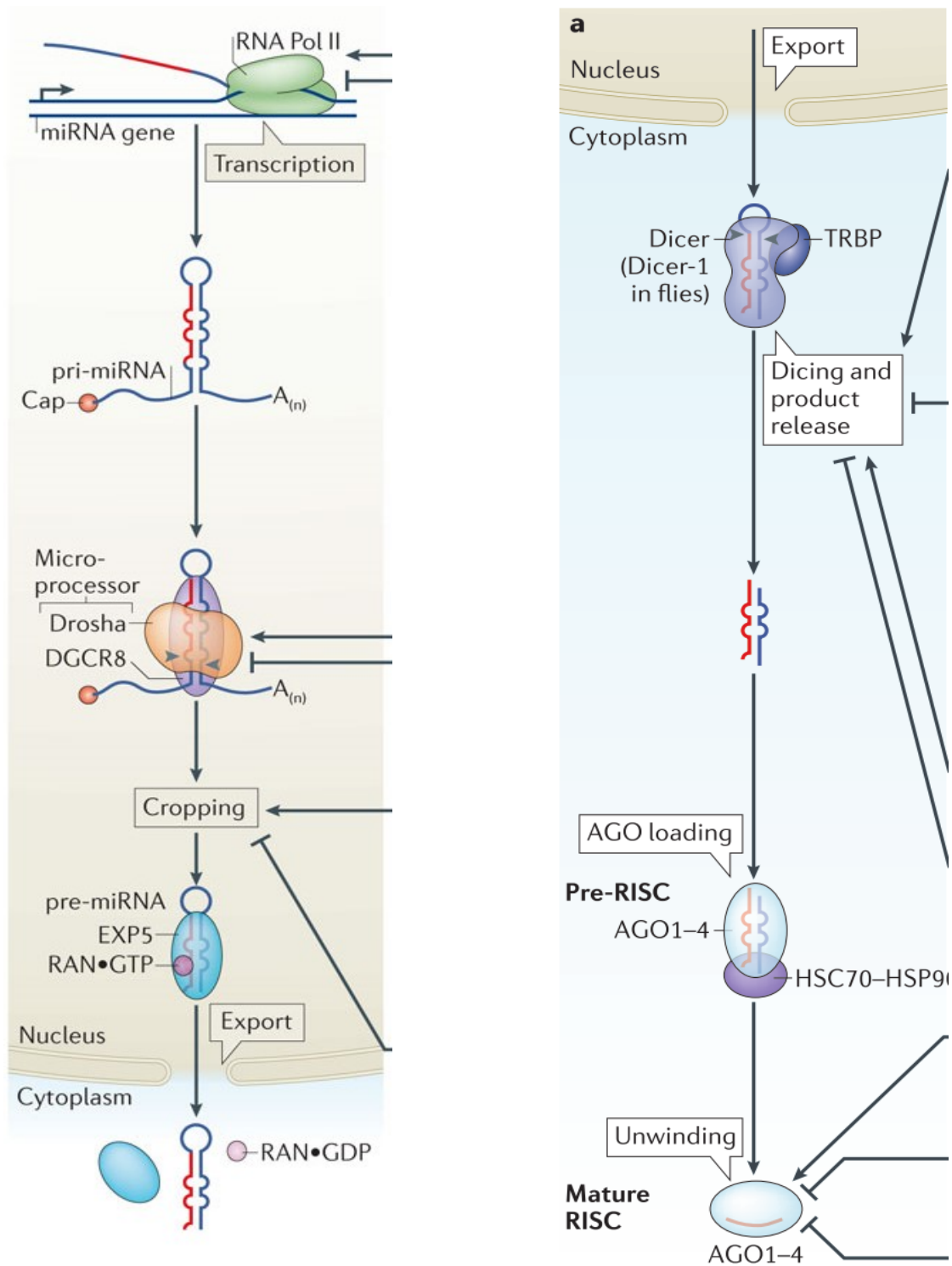


Figure 1.10: miR biogenesis

Nuclear (**left**) and cytoplasmic (**right**) processing of microRNAs. Obtained from (Ha and Kim, 2014) in June 2018.

1.3.2 Mechanism of action

Most human protein-coding genes are under the control of miRs, since more than 60% contain one or more conserved miR-binding sites, with non-conserved sites in numerous others (Friedman *et al.*, 2008).

Upon RISC formation, miRs are able to regulate protein expression. miRs act as a guide to bind to messenger RNA (mRNA) sequences of complementary base pairing. These binding sites are usually located within the 3' untranslated region (3'UTR) of the mRNA (Bartel, 2004). The 5' domain at the terminus of the miR that spans from nucleotide position 2 to 7 is crucial for target recognition and is known as the 'miRNA seed' (Ha and Kim, 2014). AGO proteins recruit factors that induce translational repression, mRNA deadenylation and mRNA decay as seen in Figure 1.11 (Huntzinger and Izaurralde, 2011). This classically results in a reduction in protein expression of the target mRNA, with a single miR being able to bind to multiple mRNAs (Bartel, 2004).

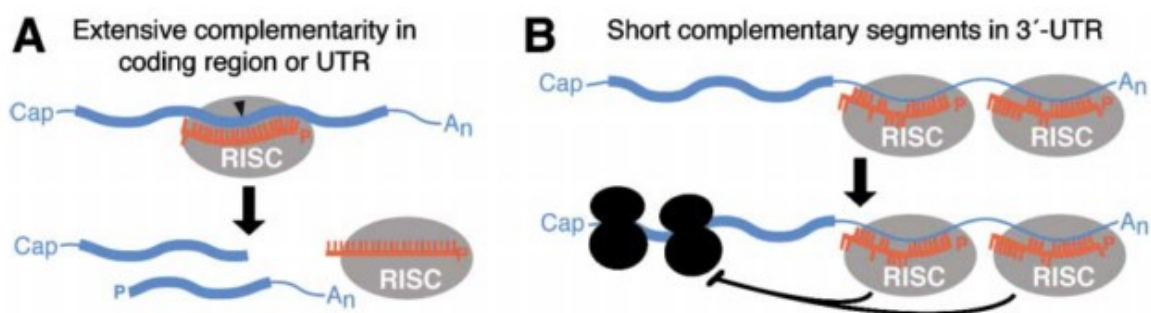


Figure 1.11: Mechanism of action of miRs

A Cleavage of mRNA by RISC complex. **B** Translational repression by RISC complex. Blue strands represent messenger RNA bound to microRNA in red along with the RNA-induced silencing complex (RISC) in grey. Black circles schematically represent messenger RNA silencing. Obtained from (Bartel, 2004) in June 2018.

1.3.3 miRs in cardiovascular disease

Understanding the effects of alterations in miR expression enable the mechanisms of dysregulation in disease to be investigated. This is evidenced by the importance of miRs in heart failure which has previously been demonstrated. Reduction in the cardiotropic miR-1 in heart failure is associated with reduced SERCA expression (Kumarswamy *et al.*, 2012). Additionally, reducing miR-1 expression in healthy adult rat cardiomyocytes *in vitro* has been shown to replicate the changes in calcium handling seen in heart failure (Kumarswamy *et al.*, 2012). Normalising miR-1 levels in an *in vivo* heart failure model by adeno-associated virus 9 (AAV9) transfection resulted in reductions in cardiac hypertrophy, fibrosis, apoptosis, MAPK signalling, and improvements in calcium handling (Karakikes *et al.*, 2013).

miRs have also been demonstrated in numerous other cardiac diseases, with miR-21 increased and miR-29 decreased in cardiac fibrosis (van Rooij *et al.*, 2008; Patrick *et al.*, 2010), and miR-208 being required for cardiac hypertrophy whereas miR-133 reduces it (Carè *et al.*, 2007; Van Rooij *et al.*, 2007).

Similarly, interactions with contractility and the β -adrenergic system have been reported. Pilot data in the Harding laboratory have linked miR-1 to changes in the β_2 AR response, with upregulation restoring contractile response to isoprenaline in heart failure cardiomyocytes by a β_2 AR specific mechanism (Mills, unpublished). miR-133 is able modulate the adrenergic system, counteracting the deleterious apoptotic effects caused by chronic β_1 AR stimulation (Castaldi *et al.*, 2014), and miR-30 effects the alterations in the adrenergic system that occurs with doxorubicin treatment (Roca-Alonso *et al.*, 2015).

As introduced in Section 1.2.2, a novel profile of miRs has been identified in TTS than enables for differentiation from healthy control and STEMI, which it closely resembles at patient presentation. This consists of elevated miR-16 and miR-26a in TTS *versus* STEMI,

and elevated miR-1 and miR-133a in STEMI *versus* TTS (Jaguszewski *et al.*, 2014). While this miR profile can be used for TTS identification, it is not known whether these have a mechanistic role in TTS.

miR-16 and miR-26a have been shown to be stress and depression-associated (Bai *et al.*, 2012; Katsuura *et al.*, 2012), and previously been shown to be involved in cell cycle regulation (Lezina *et al.*, 2013). Interestingly, miR-16 has been linked to reduced contraction in cardiomyocytes differentiated from patient-derived induced pluripotent stem cells (iPSC-CM), an *in vitro* system available within the laboratory.

1.4 Hypothesis

miR-16 and miR-26a play a mechanistic role to predispose to or exacerbate the cardiodepression seen in TTS.

1.5 Aims

1. Investigate how miR-16 and miR-26a alter cardiomyocyte contractility *in vitro* and how this is associated with changes in calcium handling and responses to catecholamines
2. Determine the effect of miR-16 and miR-26a in a functionally relevant human cardiomyocyte model
3. Assess whether manipulation of miRs in rat *in vivo* with AAV9 infection can predispose to, or exacerbate induction of TTS
4. Investigate targets of miR-16 and miR-26a using *in silico* and laboratory methods, and validate their mechanism of action

2 Materials and methods

2.1 Animals used

All procedures were carried out in compliance with the standards for the care and use of animal subjects as stated in the Guide of the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996) and the requirements of the UK Home Office (ASPA 1986– Amendments Regulations 2012) incorporating the EU directive 2010/63/EU. Protocols were approved by the Animal Care and Use Committee of Imperial College London.

2.1.1 Sprague-Dawley rats

Male, wild type, Sprague-Dawley rats (*rattus norvegicus*) were obtained from Charles River Laboratories (Harlow, UK) and maintained in the Centre for Biological Services at Imperial College London on standard rat chow eaten *ad libitum*. Rats were housed at four to five animals per cage and exposed to a 12-hour light-dark cycle at 21°C. Rats typically weighed between 150-250g for cardiomyocyte isolation and either 250-350g or 350-450g for use in the *in vivo* induction of TTS, with the latter having undergone adeno-associated virus (AAV) infection (see section 2.7.4).

2.1.2 Schedule 1 method

For cell isolation, rats were killed in accordance to the Human Killing Register via Schedule 1 method subsequent to completing a Society of Biology approved Home Office modules 1-4 course and obtaining a personal licence. Animals were briefly anaesthetised using 5% isoflurane in an anaesthetic induction box. Primary method of killing involved cervical dislocation before confirmation of death with decapitation.

2.2 Solutions

Reagents for these prepared solutions were obtained from Sigma-Aldrich unless otherwise stated.

2.2.1 Krebs-Henseleit solution

Krebs-Henseleit (KH) solution contains:

119mM NaCl (VWR Chemicals), 4.7mM KCl (VWR Chemicals), 0.94mM MgSO₄ (VWR Chemicals), 1.2mM KH₂PO₄ (Scientific Laboratory Supplies), 25mM NaHCO₃ (VWR Chemicals) and 11.5mM glucose (VWR Chemicals)

1mM CaCl₂ (Fluka) was added when using isolated adult rat ventricular cardiomyocytes, whereas 4mM was used with isolated donor or failing human cardiomyocytes. To maintain pH and oxygenation, KH is continually bubbled through with 95% O₂, 5% CO₂.

2.2.2 Low calcium solution

Low calcium (Ca²⁺) solution is used to dissociate cardiomyocytes electrically, closing gap junctions during isolation, and contains:

12-15μM CaCl₂, 120mM NaCl, 5.4mM KCl, 5mM MgSO₄, 5mM pyruvate (VWR Chemicals), 20mM glucose, 20mM taurine, 10mM HEPES, 5mM nitriloacetic acid (NTA); bubbled with 100% O₂

2.2.3 Enzyme buffer

Enzyme buffer was used to dissolve the enzymes used in cardiomyocyte isolation, and contains:

12-15 μ M CaCl₂ plus an added 150 μ M CaCl₂, 120mM NaCl, 5.4mM KCl, 5mM MgSO₄, 5mM pyruvate, 20mM glucose, 20mM taurine, 10mM HEPES

2.2.4 Normal Tyrode's solution

Normal Tyrode's (NT) solution was used with epi-fluorescence, as does not require CO₂ to maintain pH, and contains:

140mM NaCl, 4.5mM KCl, 10mM Glucose, 10mM HEPES, 1mM MgCl₂, 1mM CaCl₂; pH 7.4

When investigating the function of the NCX, Na⁺ was replaced with Li⁺, and Ca²⁺ removed from the solution and the Ca²⁺ chelator EGTA (AppliChem Panreac, ITW companies) was added:

140mM LiCl, 4.5mM KCl, 10mM Glucose, 10mM HEPES, 1mM MgCl₂, 10mM EGTA; pH 7.4

2.2.5 Adult rat cardiomyocyte maintenance media

Media used to culture adult cardiomyocytes was based on M199 containing Earle's salts, L-Glutamine, 25mM HEPES and L-amino acids (Gibco), supplemented with:

1% ITS (insulin, transferrin, selenium, Gibco), 5% Penicillin / Streptomycin, 0.2% bovine serum albumin (BSA), 100 μ M Ascorbic Acid, 5.8mM Creatine, 5mM Taurine, 2mM L-Carnitine

This was filtered in a cell culture hood with a 0.22 μ m filter system (Gorning) to create sterile media.

2.2.6 Healthy human cardiomyocyte maintenance media

Minimum Essential Medium (MEM) Eagle with Hanks' salts, L-glutamine and non-essential amino acids with the following added:

9mM NaHCO₃, 1% L- Glutamine (ThermoFisher Scientific), 1% Penicillin / Streptomycin

This was filtered in a cell culture hood with a 0.22µm filter system (Gorning) to create sterile media. This media was based upon media generated by Li et al., which was first used to improve adenovirus-mediated gene transfer (Li *et al.*, 2003). This was found to work particularly well with human cardiomyocytes and was thus used.

2.2.7 Induced pluripotent stem cell-derived cardiomyocyte media

Media used for generation and maintenance of iPSC-CMs included the following:

RB+: RPMI 1640 (ThermoFisher Scientific) and 1x B27 with insulin (ThermoFisher Scientific)

RB-: RPMI 1640 and 1x B27 without insulin

Metabolic selection media: RPMI 1640 without glucose (ThermoFisher Scientific) and 1x B27 with insulin

Cell dissociation solution (CDS): 40% RPMI 1640, 40% enzyme-free cell dissociation buffer (ThermoFisher Scientific), and 20% Trypsin-EDTA 0.05% (ThermoFisher Scientific)

2.2.8 Seahorse maintenance media

Bicarbonate-free Seahorse assay media was made using:

DMEM Base, 10mM glucose, 2mM Glutamax-1 (ThermoFisher Scientific), pH 7.4

This was filtered in a cell culture hood with a 0.22µm filter system (Goring) to create sterile media.

2.2.9 Lamelli buffer

Lamelli buffer was used as a loading dye for western blots, and contains the following by volume:

20% SDS (20% w/v solution, AppliChem Panreac, ITW companies), 20% glycerol (BHD Biochemical), 12% 1M Tris HCl (Trizma® HCl solution), 10% β-mercaptoethanol, 10% bromophenol blue (0.2% w/v solution), and 28% ddH₂O (Milli-Q, MQ)

2.2.10 TBS-Tween

10X TBS-Tween was prepared as followed for use in western blot, and diluted to 1X before use which contains:

20mM Tris base and 137mM NaCl adjusted to pH 7.8 in MQ, with 0.1% Tween 20 (VWR International)

For use in blocking solutions and antibody dilution, 5% BSA was added.

2.2.11 Loading dye

Loading dye was used for sample dilution and staining in DNA gel electrophoresis, and contains:

10mM Tris HCl, 60% glycerol, 60mM EDTA, 0.3% bromophenol blue, made up in RNase free H₂O

2.3 Adult cardiomyocyte studies

2.3.1 Isolation of adult rat apical cardiomyocytes

Following killing by Schedule 1, incisions were made along the subcostal margin and the diaphragm was dissected away to expose the thoracic cavity. Viscera were removed and submerged in ice-cold KH buffer (Section 2.2.1). The heart was lightly massaged to remove blood, placed in fresh ice-cold KH, and dissected from lungs, thyroid and pericardial fat. The aorta was cannulated and coronary arteries retrogradely perfused via Langendorff method with KH (37°C). Beating was permitted for 1 to 2 minutes to clear blood from the coronary circulation, before the perfusate was switched to low calcium (Ca^{2+}) solution (Section 2.2.2) to rapidly cease contraction. After 5 minutes, solution was changed to enzyme buffer (Section 2.2.3) containing Collagenase II and Hyaluronidase (C&H, 1mg/mL and 0.6mg/mL respectively), for 10 minutes of enzymatic degradation of extracellular matrix. The heart was cut down and placed in fresh C&H and atria and right ventricle (RV) dissected away from the LV. The apex was defined as the lower third of the LV, whereas the base was the upper third. Apex and base were divided and minced into fresh C&H. This was mechanically shaken for 5 minutes (35°C), and supernatant was filtered, and C&H replaced. The shaking was continued for 30 minutes before replacing C&H again. The resulting mixture of cardiomyocytes and C&H was centrifuged at 600rpm for 1 min to yield a pellet of cardiomyocytes at the bottom of the tube. Supernatant was discarded and replaced with enzyme buffer to result in calcium tolerant rod-shaped cardiomyocytes. Mr Peter O’Gara performed the Schedule 1 killing and cardiomyocyte isolation.

2.3.2 Isolation of human ventricular myocytes

Once human tissue was received post-transplantation or after failure of donation, a 500mg piece of myocardium was removed and dissected away from adherent fat and connective tissue. This was minced into low Ca^{2+} solution (Section 2.2.2) and transferred to a tube with 10mL low Ca^{2+} and agitated in a water bath for 3 minutes (35°C ; 100% O_2), upon which the solution was discarded and sample retained, and repeated 3 times. The tissue was then placed in 10mL enzyme buffer (Section 2.2.3) containing Protease XIV (0.36mg/ml) and shaken for 25 minutes (35°C). This supernatant contained fibroblasts and was retained for others. The tissue was introduced into 10mL enzyme buffer with Collagenase V (1mg/ml) and shaken for further 10 to 15 minutes (35°C). The solution was filtered and replaced a further 2 times. This supernatant was retained and produced digestions 1 to 3 in which cardiomyocytes are contained. Each filtrate was centrifuged for 2 to 3 minutes at 600rpm to produce a visible pellet. Supernatant was retained as it contained fibroblasts, and the pellet resuspended in enzyme buffer to yield a solution containing human ventricular myocytes. Human cardiomyocyte isolation was performed either by Jose Sanchez, Peter Wright or myself.

2.3.3 IonOptix Myocyte Contractility Recording System (MCSYS)

Cardiomyocyte contractility measurements were obtained using an IonOptix MCSYS equipped with a Myocam-S camera scanning at 240Hz (IonOptix Corp.) fixed to a Nikon TE-200 inverted microscope to assess the fractional shortening (percentage change in cell length) of individual cardiomyocytes.

A single drop of isolated cardiomyocytes was left to settle in a glass coverslip bottomed myocyte perfusion bath for 5 minutes, before being perfused with KH (37°C for rat and 32°C for human, Section 2.2.1) at 1 to 2mL/min with a peristaltic pump. Myocytes were

simultaneously field stimulated with platinum electrodes contained within the bath connected to an electrical stimulator (at 20-50V, 0.5Hz, 0.5ms for rat and 50V, 0.2Hz, 5ms for human). Whilst lower than the *in vivo* beating frequency in human and rat, these frequencies were used *in vitro* due to the reductionist nature of the model and lack of elastic recoil during diastole. As such, at high stimulated frequencies, cardiomyocytes in isolation tend to hypercontract despite the representative temperature used.

A stabilisation period of 15 minutes was allowed before any measurements were taken. A schematic for this system can be seen in Figure 2.1 (below). Cardiomyocytes were only recorded if the following criteria were met:

- Rod-shaped
- Maximum of 1 calcium wave every 30 seconds
- Stably contracting and relaxing throughout recording with absence of alternans

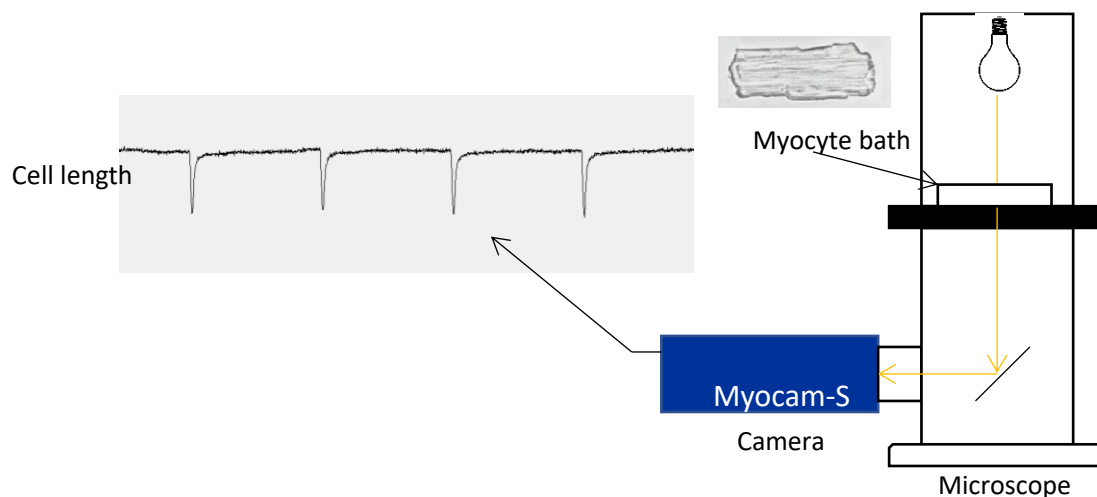


Figure 2.1: Schematic drawing of MCSYS

Schematic of the Ionoptix myocyte contractility system, where cardiomyocyte is shown to be placed into the perfusion bath for subsequent tracking of cell length with the Myocam-S as shown.

2.3.4 Calcium transient epi-fluorescence

Changes in cytoplasmic Ca^{2+} were measured using the fluorophore Fluo-4-AM (Invitrogen) suspended in DMSO (Sigma) with 3% Pluronic Acid (Biotium). Cells were incubated in 1mL Dulbecco's Modified Eagle Medium (DMEM, Gibco) with 4 μ M Fluo-4 AM for 20 minutes (37°C). De-esterification of the dye was then permitted by changing to fresh media for a further 20 minutes (37°C).

The cells were imaged using a 40x oil emersion objective (Olympus) in a dark room and perfused with NT (37°C, Section 2.2.4) and stimulated at 1Hz (20 $\pm$ 10V, 5ms duration). Fluo-4-AM was excited using an LED with an excitation wavelength of 470nm, with emission being passed through a 560 $\pm$ 35nm filter. This was recorded by a NeuroCMOS camera (Redshirt) sampling at 0.5KHz, averaging with a temporal bin of 2, to produce a final frame rate of 250/s. Further information is stated in Section 2.5.3, as epi-fluorescence was carried out alongside caffeine application.

2.3.5 Metabolic profiling

Cellular energetic status was analysed using Seahorse XFp Extracellular Flux Analyzer (Aligent) by measuring oxygen consumption rate (OCR) of 3000 transfected adult rat apical cardiomyocytes cultured in a Seahorse XFp Miniplate (Aligent) in Seahorse media (Section 2.2.8). This enabled assessment of oxidative phosphorylation within the mitochondria, and how this changed with application of various drugs as per the manufacturer's protocol (Agilent Technologies, 2010). At first, basal respiration was measured in the cells before application of oligomycin. This has the effect of inhibiting ATP synthase and hence ATP production, leaving leak of protons across the cristae and non-mitochondrial respiration to account for the remaining OCR. FCCP (Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone) was then applied. FCCP is an ionophore that is used to disperse the proton

gradient, and hence drive the mitochondria to a maximal rate of respiration. Finally, antimycin A and rotenone are used to inhibit complexes I and III of the electron transport chain to result in complete cessation of the production of the proton gradient, leaving only non-mitochondrial respiration. OCR was standardised to protein level to obtain OCR/ μ g (Figure 2.2).

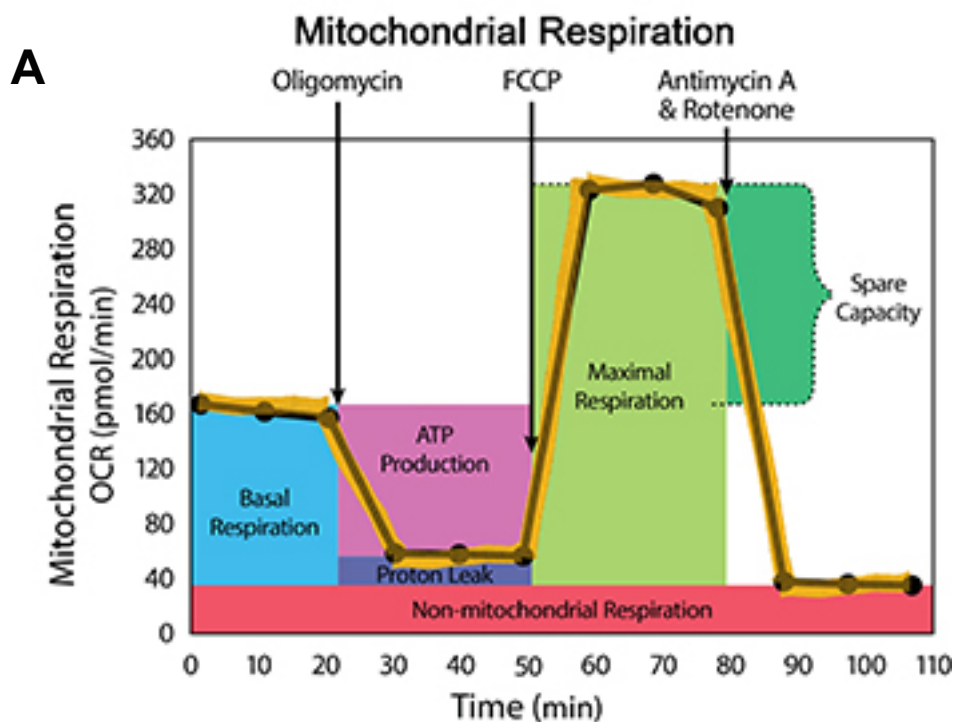


Figure 2.2: Representative trace obtained for an induced mitochondrial stress test

Representative trace of the mitochondrial stress test protocol shown with varying OCR (oxygen consumption rate) after application of the various drugs.

2.3.6 Calcium current patch clamp

Calcium current patch clamp was conducted by Eef Dries, a post-doc with extensive patch clamping expertise in Professor Cesare Terracciano's Cellular Electrophysiology group.

The whole-cell configuration of the patch-clamp technique was used to record calcium currents (I_{CaL}). Patch pipettes were pulled from borosilicate glass capillary tubes (Harvard Apparatus, 1.5 OD x 0.86 ID) using a horizontal pipette puller (P-97, Sutter Instruments). The pipette resistances were in the range of 2.5-3 M Ω . I_{CaL} was monitored using a patch-clamp amplifier (Axopatch 200A, Molecular Devices) interfaced with a digitizer (Digidata 700A, Molecular Devices) connected to a computer. Data acquisition and analysis were performed using pClamp software. All experiments were performed at 37°C. Myocytes were constantly perfused with modified Tyrode solution: TEACl 120mM, CsCl 10mM, CaCl₂ 2mM, MgCl₂ 1mM, HEPES 10mM, glucose 10mM, pH 7.4 (adjusted with CsOH). Patch pipettes were filled with: Cs methanesulfonate 90mM, CsCl 20mM, HEPES 10mM, Mg-ATP 4mM, Tris-GTP 0.4mM, CaCl₂ 3mM, EGTA 10mM, pH 7.2 (adjusted with CsOH).

I_{CaL} was elicited by 400 ms duration test pulses from a holding potential of -80mV to +50mV in 10mV increments at a rate of 1Hz. A conditioning pulse (from -80mV to -40mV for 200ms) was used to inactivate any residual cardiac sodium current before each test pulse. I_{CaL} was normalised to cell capacitance and the resultant current density was plotted against the test potentials to yield the current-voltage relationship.

A standard two-pulse protocol was used to monitor the recovery of I_{CaL} from fast inactivation. A 500ms conditioning pulse from -80mV to 0mV was followed by a 600ms test pulse from -80mV to 0mV in varying intervals from 0ms to 600ms in 50ms increments. The holding potential was set at -80mV. Current amplitude elicited by test pulses were normalised to the peak current elicited by the conditioning pulse. The normalised currents were plotted against

each time interval and fitted with a single exponential function that yielded the time constant for recovery from fast inactivation.

The steady-state inactivation curves were obtained using a standard protocol, in which 500ms conditioning pulses from a holding potential of -80mV to +20mV in 10mV increments were followed by a 600ms test pulse to 0mV. Current amplitude was normalized to the peak current (I/I_{max}), and plotted against each conditioning potential.

2.4 Induced pluripotent stem cell-derived cardiomyocyte studies

2.4.1 Culture of human induced pluripotent stem cells

The commercial control line, IMR90-4 was purchased from WiCell Research Institute, and derived from lung fibroblasts via lentiviral reprogramming (Yu *et al.*, 2007). The following protocol to form iPSC-CM was performed by Thusharika Kodagoda. Cryovials of cells were stored in N₂ (l) and thawed by immersion in a water bath (37°C) then diluted 1 in 10 in Essential 8 media (E8, ThermoFisher Scientific) supplemented with 10µM Y-27632 (Selleckchem), a ROCK inhibitor. This was done in a dropwise fashion to prevent osmotic shock. The suspensions of human iPSCs were always handled with wide-orifice 1000µL pipette tips (Starlab) to avoid shearing the cells and breaking colonies into single cells. The diluted cell suspensions were centrifuged at 200g for 4 minutes and the resultant pellet resuspended in E8 with 10µM Y-27632. ROCK inhibitor was supplemented only at thawing or passaging of hiPSC. The contents from the singular thawed vial were plated into a 6-well plate (Becton Dickson) coated in growth factor reduced Matrigel (Corning), which was formed by diluting Matrigel (1 in 50) in KnockOut DMEM (ThermoFisher Scientific). IMR90 cell cultures were maintained in a 170-300 Galaxy R CO₂ humidified incubator (RS Biotech) at 37°C, 5% CO₂ and 21% O₂.

E8 media was replaced daily and IMR90s passaged every 4 days. hiPSCs were dissociated by incubating in Versene (ThermoFisher Scientific), an EDTA solution, at 37°C for 5 minutes before being resuspended in E8 with Y-27632. This was split to a ratio of 1:16 into new Matrigel coated 6-well plates.

2.4.2 iPSC-CM generation

hiPSCs were differentiated into cardiomyocytes (iPSC-CMs) with small molecule modulation of the Wnt/ β -catenin signalling pathway (Lian *et al.*, 2013; Burrridge *et al.*, 2014). At day 4 post-passaging (termed day 0), media was changed to RB- (Section 2.2.7) containing 6 μ M CHIR-99021 (Tocris), a GSK-3 inhibitor, for 48 hours. Following this, RB- was replaced to remove CHIR-99021 for a further 24 hours. On day 3, this was changed to RB- with 2.5 μ M Wnt-C59 (Tocris), a Wnt inhibitor. RB- media was then replaced every two days and beating hiPSC-CMs were visible from day 8. Glucose starvation was carried out at day 11 of differentiation for a further four days to obtain a pure hiPSC-CM population as previously demonstrated (Sharma *et al.*, 2015), by changing to a metabolic selection media (Section 2.2.7) that did not contain glucose.

2.4.3 iPSC-CM maintenance

Following metabolic selection, hiPSC-CMs were dissociated in CDS (Section 2.2.7) for 8 minutes (37°C). The resultant cell suspension was triturated with a wide-orifice 1000 μ L pipette tip and diluted four-fold in RPMI 1640 with 10% FBS (ThermoFisher Scientific) and centrifuged at 200g for 4 minutes.

hiPSC-CMs were counted in an automated fashion using a LUNA-FL™ Dual Fluorescence Cell Counter (Logos Biosystems) stained with Acridine Orange/ Propidium Iodide Stain (Logos Biosystems). This yielded cells that were stained either green or red, depending if they were live or dead respectively, that could be quantified automatically. This was done in accordance with the manufacturer's protocol, by mixing 9 μ L cell suspension with 1 μ L fluorescent dye.

6-well plates were coated in 1:100 diluted bovine fibronectin (Sigma-Aldrich) in DPBS (Gibco) by incubating for 2 hours at 37°C. hiPSC-CMs were resuspended in RB+ (Section 2.2.7) supplemented with 10% FBS and 10µM ROCK inhibitor and plated at 1 million per well. Media was changed to RB+ only after 24 hours, and cells maintained in RB+ until day 26, changing RB+ every two days, before being dissociated and re-plated at 30k in the centre of a glass-bottomed MatTek dishes (MatTek corporation) coated in fibronectin. These were then transfected at day 28 to 30 as described in Section 2.6.1, and used for final experimentation at day 30 to 32.

2.4.4 IonOptix Myocyte Contractility Recording System (MCSYS)

The Ionoptix MCSYS was used to measure beating rate of iPSC-CMs. A dish of iPSC-CMs were placed on the microscope stage and perfused with KH (37°C, Section 2.2.1) for 15 minutes. An area of the dish located that displayed a sharp difference in contrast in the monolayer, and this was tracked using the Ionoptix MCSYS as previously discussed (Section 2.3.3). This enabled the spontaneous beating rate of iPSC-CMs to be manually counted. A β_2 AR response was induced as described in Section 2.5.1, and beating rate was calculated at minute 0 (baseline), 1, 2, 3, 4, 5, 10, 15 and 20. This enabled the peak response to β_2 AR stimulation to be measured within 5 minutes, and the subsequent phase of desensitisation to be measured between 10 and 20 minutes.

2.4.5 Calcium transient epi-fluorescence

iPSC-CMs were loaded with Fluo-4 AM as described in Section 2.3.4. For iPSC-CM, Ca^{2+} transients were recorded at four different 400µm² areas for 5s and spatially averaged. The first four transients were then analysed using a script for MATLAB R2017a (MathWorks) designed by Kitanan Worrapong to obtain time to peak (time from stimulation to peak),

normalised fluorescent amplitude (ratio of the fluorescence at peak against fluorescence at baseline, F/F_0), time to 50% decay and time to 80% decay (time from peak to 50% and 80% decay of the transient amplitude respectively).

2.4.6 Membrane potential epi-fluorescence

iPSC-CMs were loaded with FluoVolt (ThermoFisher Scientific), a voltage-sensitive membrane dye, for 15 minutes (37°C). An LED with an excitation wavelength 470nm was used, with emission being passed through a 560 ± 35 nm filter, sampling at 1KHz.

Four areas of approximately $400\mu\text{m}^2$ recorded for 5s to obtain action potential recordings, field-stimulated at 1Hz or 2Hz (20 ± 10 V, 5ms duration). These were analysed manually using PClamp 10 software (Molecular Devices) to obtain values for the rise time, and action potential duration to 50% and 90% repolarisation (APD50 and APD90).

2.5 Cardiac pharmacological protocols

2.5.1 β_2 AR response

Cardiomyocyte baseline contractility was recorded with the Ionoptix MCSYS (Section 2.3.3) in presence of KH (Section 2.2.1) for 5 minutes before the selective β_1 AR antagonist CGP-20712A (300nM) was perfused across the cells for 10 minutes. The non-specific β AR agonist isoprenaline (1 μ M) was then applied to elicit a specific β_2 AR response as the presence of CGP-20712A was maintained. This was maintained for 10 to 15 minutes to allow for a maximal increase in contractility to occur, and then washed with KH to return contractility to baseline.

This procedure was also carried out for hiPSC-CMs, but beating rate was quantified in the place of contractility as described in Section 2.4.4.

2.5.2 Adrenaline concentration-response curves

To assess cardiomyocyte sensitivity to adrenaline, increasing concentrations of adrenaline were perfused across the isolated cardiomyocytes, and contractility measured. A stable baseline was established as previously mentioned (Section 2.3.3), before 3×10^{-10} M adrenaline was applied. A period of 5 to 10 minutes was permitted to allow for any changes in contractility to occur. Adrenaline was then increased by half a log-10 in molarity in this fashion until a maximum of 3×10^{-6} M was reached. Cardiomyocytes were washed out with KH (Section 2.2.1) if they became arrhythmic or no longer increased in contractility with increasing concentrations of adrenaline.

2.5.3 Caffeine micropipette application

Caffeine was locally applied to cells to increase RyR open probability, and hence elicited a transient that is a measure of sarcoplasmic reticulum (SR) Ca^{2+} content as shown in Figure 2.3 (below) (O'Neill, Donoso and Eisner, 1990). A glass micropipette was loaded with a NT solution (Section 2.2.4) containing caffeine (40mM, Sigma), and positioned manually above a single adult rat apical cardiomyocyte for subsequent local application via positive pressure. A 10ms recording was obtained, allowing for three normally elicited Ca^{2+} transients, before stimulation was turned off and caffeine was applied (Figure 2.3).

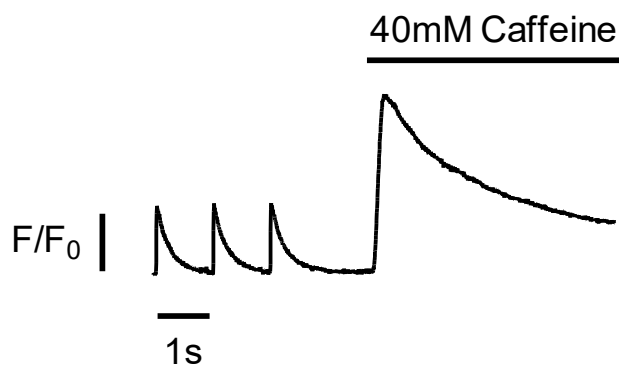


Figure 2.3: Caffeine-induced calcium transient

Representative caffeine-induced calcium transient obtained with calcium epi-fluorescence. Shown is the relative change in the amplitude of the fluorescence (F/F_0) over time with 3 stimulated calcium transients followed by a caffeine-induced calcium transient.

The perfusate was then replaced with zero Na^+ , zero Ca^{2+} solution in order to inhibit NCX, leaving only slow mechanisms of calcium extrusion intact. A caffeine-elicited transient was then obtained.

Analysis of Ca^{2+} transient decay was conducted according to the well-established method used by Maier et al. to work out the relative contributions of SR Ca^{2+} uptake, NCX and the slow mechanisms, the sarcolemmal Ca^{2+} ATPase and the mitochondrial Ca^{2+} uniport system (Bers, 2001; Maier *et al.*, 2003), expressed below (where k (s^{-1}) is the rate of decay):

$$k_{\text{Total}} = k_{\text{SR}} + k_{\text{NCX}} + k_{\text{Slow}}$$

Caffeine application removed contribution from SR Ca^{2+} uptake, leaving calcium extrusion being dependent on NCX and slow mechanisms. The contribution of SR Ca^{2+} uptake was back-calculated from:

$$k_{\text{SR}} = k_{\text{Total}} - (k_{\text{NCX}} + k_{\text{Slow}})$$

This was repeated in zero Na^+ , zero Ca^{2+} solution with EGTA (a Ca^{2+} chelator) to inhibit NCX, and caffeine was once again applied to obtain k_{Slow} , enabling k_{NCX} to be calculated:

$$k_{\text{NCX}} = k_{\text{Total}} - k_{\text{SR}} - k_{\text{Slow}}$$

Data was analysed manually using PClamp 10 software (Molecular Devices) with the following parameters manually calculated:

- SR Ca^{2+} transient amplitude (F/F_0)
- Ca^{2+} transient amplitude (F/F_0)
- Fractional release (FR) ($\frac{\text{Ca}^{2+} \text{ transient amplitude}}{\text{Caffeine-induced transient amplitude}}$)
- Decay constant (τ^{-1}), transients were fitted with a mono-exponential function to obtain τ (tau), the exponential time constant: $f(t) = \sum A_i e^{-t/\tau_i} + C$ $n_i=1$
- Time to peak (s)
- Time to 50% calcium transient decay (s)
- Time to 80% calcium transient decay (s)

2.6 Molecular biology techniques

2.6.1 MicroRNA transfection

Lipofectamine 3000 was chosen for transfection of miRs as was demonstrated to be the least toxic and most efficient (data not shown). Cardiomyocytes were transfected as per the manufacturer's protocol (ThermoFisher Scientific, 2016) with the following miRs at 100nM as previously used within our laboratory:

- Pre-miR negative control #2 (Ambion)
- Hsa-pre-miR-16 (Ambion)
- Hsa-pre-miR-26a (Ambion)
- Anti-miR negative control #1 (Ambion)
- Hsa-anti-miR-16 (Ambion)
- Hsa-anti-miR-26a (Ambion)

Briefly, a master mix was made by combining OptiMEM (Gibco) serum free media with Lipofectamine 3000. A separate tube containing OptiMEM was then combined with each respective miR. Equal volumes of master mix were then added to each respective miR tube, and mixed and left for 15 minutes for liposomes to form. This is added to cells at 100nM miR according to the 'Scaling Up or Down Lipofectamine® 3000 Reagent Transfections' scaling document available from ThermoFisher Scientific (ThermoFisher, no date). Where miR-16 and miR-26a were co-transfected, 100nM of each miR was added to investigate if any synergism occurred.

For Ionoptix, 12,500 cardiomyocytes per well were transfected in suspension in a 24-well plate, whereas 25,000 were seeded with Laminin (0.5mg/mL, Trevigen) and transfected in a 12-well plate for protein and RNA isolation.

2.6.2 RNA isolation

RNA isolation from cells was carried out after transfection and culture as per Section 2.6.1. In short, media was removed and cells were washed with PBS (Gibco). TRIzol® Reagent (Invitrogen) was then applied and cells scraped, and RNA isolated as per the manufacturer's protocol (Thermo Fisher Scientific, 2016). In brief, chloroform (Sigma) was added and centrifuged to obtain an aqueous phase containing RNA. This phase was combined with isopropanol (Sigma) to precipitate RNA and centrifuged to form an RNA pellet. This was washed with ethanol and then resuspended in RNase-free water. Isolated RNA was stored at -80°C.

For serum, the miRNeasy Serum/Plasma (Qiagen) kit was used as per instruction. This is similar to the TRIzol® detailed above, but Qiazol Lysis Reagent (Qiagen) is used in place of TRIzol®, and columns are used to isolate RNA.

Isolation of RNA from tissue was carried out using the TRIzol® Reagent protocol as discussed above, but tissue 50mg to 150mg tissue was homogenised using Stainless Steel Beads (Qiagen) in an Eppendorf tube (Eppendorf) shaken by a TissueLyser LT (Qiagen).

2.6.3 Quantitative real-time PCR (RTqPCR)

RNA concentration was quantified using NanoDrop 2000 (ThermoScientific) before being diluted as appropriate and reverse transcription (RT) undertaken to produce cDNA using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) for miRs and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) for mRNA. For miR RT reactions, specific primers were used and shown below with RNU6B or U6 snRNA as a reference gene for cells or tissue, and Cel-miR-39 as the spike-in control for quantification in serum:

- RNU6B
- U6 snRNA
- Cel-miR-39
- Hsa-miR-16
- Hsa-miR-26a

RTqPCR was then carried out for miRs using the protocol in TaqMan® Small RNA Assays (Applied Biosystems) utilising TaqMan® Universal PCR Master Mix II, with UNG (Applied Biosystems) and TaqMan® Small RNA Assay (Applied Biosystems) primers for the miRs listed above. For mRNA, TaqMan™ Gene Expression Master Mix (Applied Biosystems) was used along with Taqman primers (Applied Biosystems), with GAPDH, TBP and 18S used as endogenous controls.

- CACNA1C
- CACNB1
- RGS4
- GNB1
- GNG12
- GAPDH
- TBP
- 18S

Technical duplicates were carried out at the qPCR amplification step. For mRNA PCR, the QIAgility robot (Qiagen) was used for high-throughput preparation of the 384-well PCR plates. For the majority of the reactions, a Quantistudio 12K Flex (Applied Biosystems, by Life Technologies) was used in a 384-well plate. Data was analysed using the comparative cycle threshold (CT) method (ΔC_t) as previously described (Schmittgen and Livak, 2008).

2.6.4 Protein isolation

After culture as per Section 2.6.1, media was removed, and cells were washed with ice-cold PBS. Cardiomyocytes were lysed using 100µL per well of ice-cold RIPA lysis buffer (Sigma Aldrich) and isolated at 4°C. Samples were centrifuged to clear the lysates, before protein containing lysates were obtained. Protein concentration was subsequently quantified using the Pierce™ BCA protein assay kit (Thermo Scientific).

For tissue, 100mg to 300mg was homogenised in RIPA buffer using the TissueLyser LT and Stainless Steel Beads (Section 2.6.2). Protein was then isolated and quantified as described above.

2.6.5 Tissue fixation and cryosection

Isolated hearts were divided as described in Section 2.7.2. Tissue was fixed in paraformaldehyde (PFA) for 24 hours, before being transferred to 30% sucrose (Sigma) solution to alter the osmotic potential of the tissue. Once the tissue sank in this solution, it was transferred to PBS. Tissue was then frozen in OCT (optimal cutting temperature) compound (ThermoFisher Scientific) with isopentane (Sigma) and stored in -80°C before use. Frozen hearts were then sectioned using a cryostat, cutting 14µm tissue sections.

2.6.6 Widefield microscopy

Once fixed and sectioned, the samples were mounted in Hard-set Vectashield (Vector Laboratories) and imaged on a Zeiss AxioObserver widefield microscope using a 20x 0.8 DIC Plan Apochroma objective (Zeiss). Samples were imaged with Zen Blue (Zeiss) software using acquisition settings for mCherry using a 555nm LED, imaging the whole heart slice. Images were analysed using Image J with the average of three 1000 by 1000 pixel areas taken per image.

2.6.7 Luciferase primer design

miR targets were predicted using the *in silico* method described in Section 2.8.1, and then primers were designed with Jan Fiedler to construct final luciferase plasmids that contained each respective gene of interest. Forward and reverse primers were designed to contain a 5'-*SpeI* and 3'-*HindIII* restriction sites, and complementary base pairing to enclose between 700 and 800 base pairs of the 3'UTR of the gene of interest. This enabled downstream amplification of the gene of interest, and inclusion into the pmiRReport vector. Primer3 was used to assist in primer design. The primer sequence designed was then checked in TargetScan and Ensembl to confirm the putative miR binding site was included. Restriction sites are enclosed as follows:

5'-*SpeI*: ACT AGT

3'-*HindIII*: AAG CTT

Designed primer sequences, along with putative 3'UTR binding sites are shown in Appendix 1.

2.6.8 Synthesis of plasmid

Each gene of interest was synthesised by a PCR based technique, combining the forward and reverse primers designed in Section 2.6.7 with global rat cDNA with HotStar Taq Mastermix (Qiagen). The PCR reaction was purified using a QIAquick PCR Purification Kit (Qiagen) and run via gel electrophoresis in loading dye (Section 2.2.11) using a 1.5% agarose (Roth company) gel containing 0.004% Midori Green (Nippon Genetics EUROPE GmbH) in 1X Tris-Acetate EDTA (TAE) buffer (Roth company) with a 100bp DNA ladder (New England Biosciences) to check for bands of expected length.

Upon confirmation of sequences of expected lengths, constructed gene inserts and pmiRReport vector were digested for 2 hours at 37°C with HindIII and SpeI to yield complementary overhangs for subsequent ligation. The digested products were purified again using the QIAquick PCR Purification Kit before commencement of ligation. 1 in 10 pmiRReport vector (Figure 2.5) was combined with the double digested insert and T4 DNA ligase (ThermoFisher Scientific) at room temperature for 10 minutes. The product from this ligation was transformed into competent *E. coli* via heat shock at 42°C for 90 seconds. 500µL LB-medium (Invitrogen) was added, and transformed bacteria incubated at 37°C for 30 to 45 minutes with gentle shaking. 100 to 200µL was spread onto LB-ampicillin culture plates for overnight incubation at 37°C.

The pmiRReport vector contains an ampicillin resistance gene (Figure 2.5), so only transformed bacterial colonies could grow. Colonies were picked and incubated in 5mL LB-ampicillin overnight at 37°C before isolation of expanded plasmids using the QIAprep Spin Miniprep Kit (Qiagen). Plasmids were digested as before with HindIII and SpeI and product run on gel electrophoresis for control reasons, to check to sequence of expected length, confirming incorporation of desired insert length. Positive clones were sent for sequencing at Eurofins-MWG, and the sequencing results compared with Emsembl 3'UTR. Matched sequencing formed successful production of luciferase vector containing desired 3'UTR (Figure 2.4) and could be used for downstream experimentation.

Score	Expect	Identities	Gaps	Strand
1376 bits(745)	0.0	766/775(99%)	5/775(0%)	Plus/Plus
Query 1	CCTCCTATTAATTTTGTG-ATTTTATTATTTGTTTTCCCGCAAAGCGGAAAAGGTTTT	59		
Sbjct 43	CCTCTAATTTATTTTGTGAATTTTATTATTTGTTTTCCCGCAAAGCGGAAAAGGTTTT	102		
Query 60	ATTTTGTAAATTATTGTGATTTCTGTGGCCCCAGCCTGGCCCAGCCCCAGGGAGGGGCC	119		
Sbjct 103	ATTTTGTAAATTATTGTGATTTCTGTGGCCCCAGCCTGGCCCAGCCCCAGGGAGGGGCC	162		
Query 120	TGCTTGCCCTCAGCTCCTGCTGCCACCAACCCAGCCACTGTCTAGCACCCCTGCCCCGCC	179		
Sbjct 163	TGCTTGCCCTCAGCTCCTGCTGCCACCAACCCAGCCACTGTCTAGCACCCCTGCCCCGCC	222		
Query 180	CCACCGCATCCCCCTAGGGTCCTTGTCTCCAGTATCTTCTACTGGGAAGAGGAGCAACC	239		
Sbjct 223	CCACCGCATCCCCCTAGGGTCCTTGTCTCCAGTATCTTCTACTGGGAAGAGGAGCAACC	282		
Query 240	CTCAGCAGCCCTCTTGCCCCCTCCATGGGGTGAAGCCACAGTCGGTGGGTGTGTCACTTCA	299		
Sbjct 283	CTCAGCAGCCCTCTTGCCCCCTCCATGGGGTGAAGCCACAGTCGGTGGGTGTGTCACTTCA	342		
Query 300	GGCTCTGTGGGTACACTCAGCCTGAGGCCAGCTGGGTGGCCCAGCACCCCTACCCTAG	359		
Sbjct 343	GGCTCTGTGGGTACACTCAGCCTGAGGCCAGCTGGGTGGCCCAGCACCCCTACCCTAG	402		
Query 360	GAAGGTGCAACACAGGGATACTACTTGAACCTTCTATCATAGTCCCATGCCACAGAGG	419		
Sbjct 403	GAAGGTGCAACACAGGGATACTACTTGAACCTTCTATCATAGTCCCATGCCACAGAGG	462		
Query 420	GACAGACCTCTCTGTCTGCCCTCTTGCCACTGGTGAGAGAAGGCCCTTGTCTCCCCAGA	479		
Sbjct 463	GACAGACCTCTCTGTCTGCCCTCTTGCCACTGGTGAGAGAAGGCCCTTGTCTCCCCAGA	522		
Query 480	CTTGGCGGTCTCCACAGTGACGGGCTCCCTGGG-CCTTGTTTAGGAAAAGCCTGTGTGT	538		
Sbjct 523	CTTGGCGGTCTCCACAGTGACGGGCTCC-TGGGTCTTGTTTAGGAAAAGCCTGTGTGT	581		
Query 539	GA-ACCTGCCTC-TCAGGAGGCTGCCATTGGCAGTGGCTGCTGCTGGCCTCCCTGCAAGT	596		
Sbjct 582	GACACCTGCCTCATCAGGAGGCTGCCATTGGCAGTGGCTGCTGCTGGCCTCCCTGCAAGT	641		
Query 597	CCCCCTTTCCCATTTGCGTGCCTGGACTCAGGAGCATAGAAAAACGACTGTAGATTGTTGG	656		
Sbjct 642	CCCCCTTTCCCATTTGCGTGCCTGGACTCAGGAGCATAGAAAAACGACTGTAGATTGTTGG	701		
Query 657	GCCACACTGGCCTAGCCTGAACCTAGATCCCTAACTCTGGGCTGGGTGAGGCTCATCTGC	716		
Sbjct 702	GCCACACTGGCCTAGCCTGAACCTAGATCCCTAACTCTGGGCTGGGTGAGGCTCATCTGC	761		
Query 717	CCAGGACCACAGTGGGACAGCCTGGGCAGGTGGGTGGCACAGCATAAGACCCAG	771		
Sbjct 762	CCAGGACCACAGTGGGACAGCCTGGGCAGGTGGGTGGCACAGCATAAGAACCCAG	816		

Figure 2.4: Example sequencing of synthesised plasmid

Representative image of the synthesised plasmid (Query) with the expected plasmid (Sbjct). Highlighted is the miR binding site. Shown here is ADRBK1 with miR-16 binding.

2.6.9 Luciferase assay

miRs (100nM), a synthesised pmiRReport-3'UTR plasmid and a β -galactosidase control plasmid (20ng each, Figure 2.5) were transfected into HEK293 cells at 200,000 per well using Lipofectamine 2000 (ThermoFisher Scientific). After 24 hours, cells were lysed. HEK293 were detached from the culture wells using PBS (4°C) for 10 minutes whilst on ice. Cells were collected, centrifuged at 300g for 5 minutes (4°C) and exchanged for 1X Lysis Buffer (Luciferase Assay System, Promega). Lysates were cleared by centrifugation (8000g, 5 minutes, 4°C), before lysate was added to luciferase substrate (Luciferase Assay System) in a white 96-well plate and measured after approximately 20 minutes using a Synergy HT multiplate reader (Biotek) for measurements over 10 minutes. This was normalised to β -galactosidase activity using the β -Galactosidase Enzyme Assay System (Promega), incubating for 10 to 30 minutes at 37°C as required. Dilutions for luciferase assay and β -Galactosidase normalisation were determined empirically before final measurement to ensure values were within the detection limits of the multiplate reader.

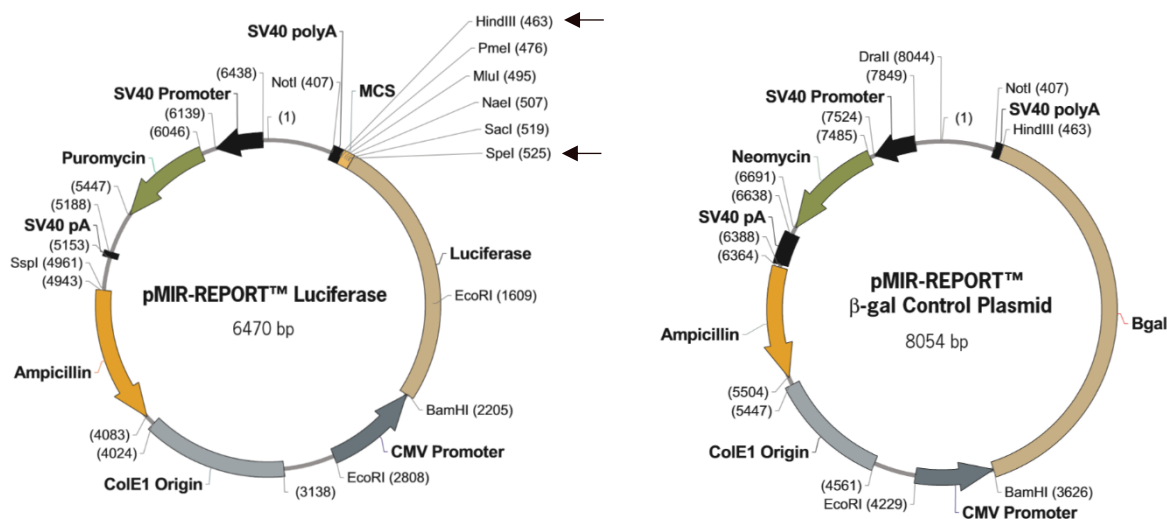


Figure 2.5: Luciferase primer design

Schematic representation of the pMIR-REPORT plasmids used for cloning.

2.6.10 Western blot

Samples were prepared to contain 10µg protein for *in vivo* samples and 5µg protein for *in vitro* samples per lane. This was reconstituted in MQ with 9.5µL Lamelli buffer (Section 2.2.9) to give a total volume of 30µL per well. Samples were boiled on a heat block at 90°C for 10 minutes before being loaded into each lane of a 17-well Bolt™ 4-12% Bis-Tris Plus Gels (Invitrogen) in a Mini Gel Tank (ThermoFisher Scientific) containing 1X Bolt™ MOPS SDS Running Buffer (Novex). Precision Plus Protein™ Dual Color Standards (BioRad) was used as a protein ladder in each outer well. These gels were run for 32 minutes at 200V and run in triplicate.

The gel was transferred to a polyvinylidene difluoride (PVDF) membrane using an iBlot™ 2 PVDF Transfer Stack (Novex) in an iBlot 2 Dry Blotting System (Invitrogen). The PVDF membrane was blocked in TBS-T with 5% BSA for 1 hour at room temperature whilst agitated. The membranes were cut to isolate molecular weight regions for LTCC α 1c subunit

(CACNA1C, 250kDa), Vinculin (124kDa) and RGS4 (23kDa) on one membrane, and Vinculin, LTCC β 1 subunit (CACNB1, 66kDa), G-protein β 1 subunit (GNB1, 37kDa), and G-protein γ 12 subunit (GNG12, 8kDa) on the second membrane. Each of these sections were incubated with their respective primary antibodies at 4°C overnight, using 1:200 rabbit anti-CACNA1C (Abcam, ab58552), 1:10,000 rabbit anti-vinculin (Abcam, ab129002), 1:500 rabbit anti-RGS4 (Abcam, ab9964), 1:500 rabbit anti-CACNB1 (Sigma, AV34953), 1:333 rabbit anti-GNB1 (ThermoFisher Scientific, PA1-725) and 1:500 rabbit anti-GNG12 (ThermoFisher Scientific, PA5-75620).

Membranes were washed for 15 minutes in TBS-T three times whilst being agitated, and then incubated with the 1:3000 anti-rabbit horse radish peroxidase (HRP) linked secondary antibody (Cell Signalling Technologies, 7074S) for 1 to 2 hours at room temperature whilst agitated. Membranes were washed a further three times for 15 minutes in TBS-T before being developed using Clarity™ Western ECL Blotting Substrates (BioRad) and imaged using a ChemiDoc imaging system (BioRad). Images were analysed using Image J, comparing the intensity of each band to a paired value for vinculin, used as the loading control.

2.7 *In vivo* investigations

2.7.1 Anaesthetic protocol

Sprague-Dawley rats were transferred into an anaesthetic induction chamber and anaesthetised using isoflurane (5%; 100% O₂ 5L/min). The animal was determined anaesthetised when the righting reflex was lost and confirmed by assessment of the pain withdrawal reflex. Anaesthetised state was maintained outside of the chamber using a Bain co-axial tube delivering isoflurane (1.5-2.5%; 1.5L/min 100% O₂), connected to a Fluovac scavenger (Harvard Instruments, USA) to collect and remove waste gases. Depth of anaesthesia was constantly monitored to provide both a surgical level of anaesthesia with absent pain reflexes, whilst using the minimal amount of isoflurane, since this has recently been demonstrated to protect against TTS (Oras, Redfors, Ali, Alkhoury, *et al.*, 2017; Oras, Redfors, Ali, Lundgren, *et al.*, 2017).

2.7.2 Echocardiography

Following anaesthesia, the thorax of the male Sprague-dawley rat was shaved using an electric hair trimmer and then transferred to a heated ultrasound table (Visualsonics). Hair removal cream was then applied to the shaved area for up to 5 minutes to yield a hairless thorax for good echocardiographic imaging. The cream was gently scraped off and the thorax of the rat washed thoroughly to remove any residual hair removal cream, before being secured to the ultrasound table with surgical tape. Body temperature was measured with a rectal thermometer and maintained to a physiological level (37°C) using a homeothermic temperature monitor connected to a heated blanket (Harvard Apparatus).

Ultrasound gel (Henleys Medical Supplies) was warmed and then applied to the thorax. An RMV-730 ultrasound probe (Visualsonics) was lowered onto the thorax with the probe

parallel to the craniocaudal axis. Using the Vevo 770 ultrasound system (Visualsonics), the position of the probe was optimised via B-mode scanning to find the heart, and laterally displaced to best visualise both apex and ascending aorta. The probe was then rotated to align with the axis of the heart, which is approximates to the axis between right forelimb and left hindlimb. This yielded an ‘on-axis’ parasternal long axis view of the left ventricle where the cavity could clearly be seen between apex and ascending aorta. At this point, the protocol for ‘catecholamine cardiomyopathy’ could be commenced as described in section 2.7.3. M-mode images could then be taken at apex, mid-left ventricle and basal segments of the left ventricle, which were defined as lower third, midpoint and upper third respectively.

Analysis was undertaken using the Vevo 770 software, which consisted of a left ventricular internal dimensions tool, where the inside of the ventricular wall was traced with concomitant exclusion of trabeculae. Three to five beats were analysed and then averaged, depending on how many beats could be measured in an uninterrupted fashion before breathing artefacts disrupted the signal. Fractional shortening was calculated as follows:

$$\text{Fractional shortening} = 100 * \frac{\text{End diastolic diameter} - \text{End systolic diameter}}{\text{End diastolic diameter}}$$

This was transformed to a percentage and the relative change from baseline was calculated. Ejection fraction was not measured, as this is only accurate from a 2-dimensional view when measured from the mid-LV, and the assumptions made about LV volume for this calculation is also incorrect in a disease model such as TTS, where the ventricular dimension varies between the regional zones of the LV. At the end of the procedure, rats were killed by a non-schedule 1 method by excision of the heart under anaesthesia according to the approved protocol. Hearts were divided into apex and base, and split for protein isolation, RNA isolation and fixation.

2.7.3 Catecholamine cardiomyopathy

Once the parasternal long-axis view had been established, a vertical superficial incision was made superior to the mid-point of the left clavicle using surgical scissors and pressure was applied with sterile swaps to result in cessation of any resultant bleeding. The skin was then separated from the underlying subcutaneous fat and fascia by blunt dissection, before cutting the fascia to expose the underlying musculature. This was continued in this fashion until the left external jugular vein was exposed. Cannulation of this was then attempted using a 26G cannula (Abbocath), being extremely careful to tether the vessel and enter at a shallow angle before withdrawal of the needle as soon as the needle tip and top of the plastic sheath had breached the vessel wall. If at any point this process failed, and the vessel collapsed, sterile gauze was placed over the vessel with application of pressure to promote haemostasis, and the procedure repeated on the right external jugular vein. Once venous access had been established, the cannula was flushed with sterile PBS (Gibco) and sealed. Proper access was confirmed at this point by lack of fluid accumulation in the surrounding tissue following injection.

Baseline echocardiographic images were taken at this point before immediate injection of the adrenaline (Hameln) bolus over approximately 10 seconds. For simple induction of TTS, 55ug/kg adrenaline was injected, whereas this was titrated down to 18ug/kg when TTS was induced in the AAV treated animals, since these rats were older and consequently heavier. This was altered, as when 55ug/kg was administered to rats weighing between 350 to 450g, an unacceptably high mortality was observed. This was likely due to the increased body fat percentage of these older rats and adrenaline not being fat-soluble, resulting in a proportionally higher dose given, as dosage was simply scaled according to body weight. Animals were maintained between 37 to 38°C using the homeothermic heated blanket, before

the hyperthermic effect of adrenaline was permitted. Central body temperature was permitted to rise to up to 39°C in this study, as we have recently demonstrated hyperthermia is required for reliable induction of TTS (Tranter, Couch *et al.*, in press).

2.7.4 Adeno-associated virus 9 infection via tail vein injection

AAV serotype 9 (AAV9) was chosen to upregulate the miRs *in vivo*, as this is the serotype which exhibits a cardiac tropism. An EF1 α (elongation factor 1-alpha) promoter was chosen, as this is a generalised promoter and would allow for action in multiple organs and cell-types, as the originating tissue of miR-16 and miR-26a in TTS patients is unknown (Jaguszewski *et al.*, 2014). This was enabled by the concomitant ability of AAV9 to cross the blood-brain barrier (DiMattia *et al.*, 2012).

Within the intronic regions of the promoter, we designed areas for the primary sequences for miR-16 and miR-26a (pri-miR-16 and pri-miR-26a) and chose an mCherry reporter to be driven by EF1 α , as shown in Figure 2.6 (below). Accordingly, AAV9-EF1 α -mCherry and AAV9- EF1 α -pri-miR-16 + pri-miR-26a-mCherry were designed and purchased (Vector Biolabs).

AAV9 was administered via tail vein injection 6 weeks prior to induction of TTS. Male Sprague-dawley rats weighing between 75-150g were placed into a heated chamber for 10 minutes to vasodilate peripheral vessels, before being lightly and briefly anaesthetised using the induction protocol described in section 2.7.1. Rats were placed on a heated mat, and tails were washed in warm water to clean the tail and maintain vasodilation. The tail veins were located on the lateral border of the tail on both right and left sides, and these were wiped with ethanol wipes for sterilisation prior to injection. Using a 30G needle, sterile PBS (Gibco) containing AAV9 was injected into the vein, starting peripherally to allow for subsequent

proximal injections should the first injection fail, or if the full volume was not completely ejected.

An initial dose response was carried out with AAV9-EF1 α -mCherry and AAV9- EF1 α -pri-miR-16 + pri-miR-26a-mCherry using a 0%, 12.5%, 25%, 50% and 100% of 5×10^{12} gene copies as the selected doses. Data from this dose response can be seen in Section 5.3.3.

Following this, the 50% dose of 2.5×10^{12} gene copies was selected, and 10 rats infected with each virus as shown in Figure 2.7 (below).

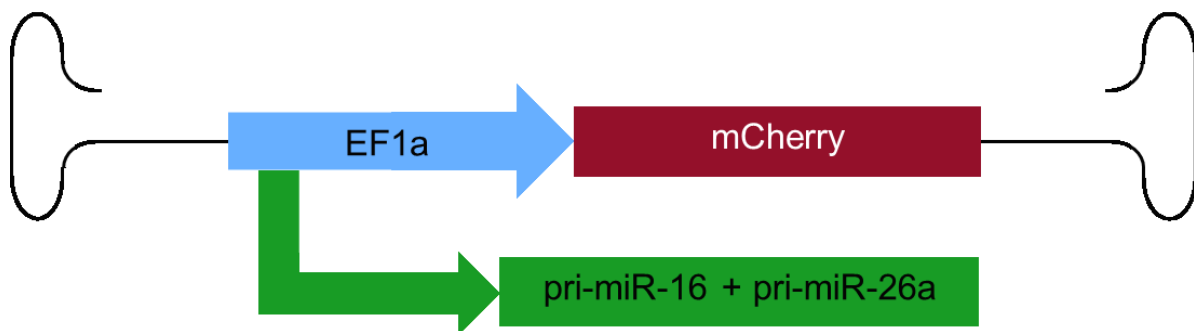


Figure 2.6: Schematic representation of AAV9 design chosen

Schematic of AAV9 (adeno-associated virus serotype 9) vector chosen for *in vivo* experimentation. EF1 α promoter is shown in blue, the mCherry reporter in red, and primary-miR-16 and primary-miR-26a in green within the intronic regions of the promoter.

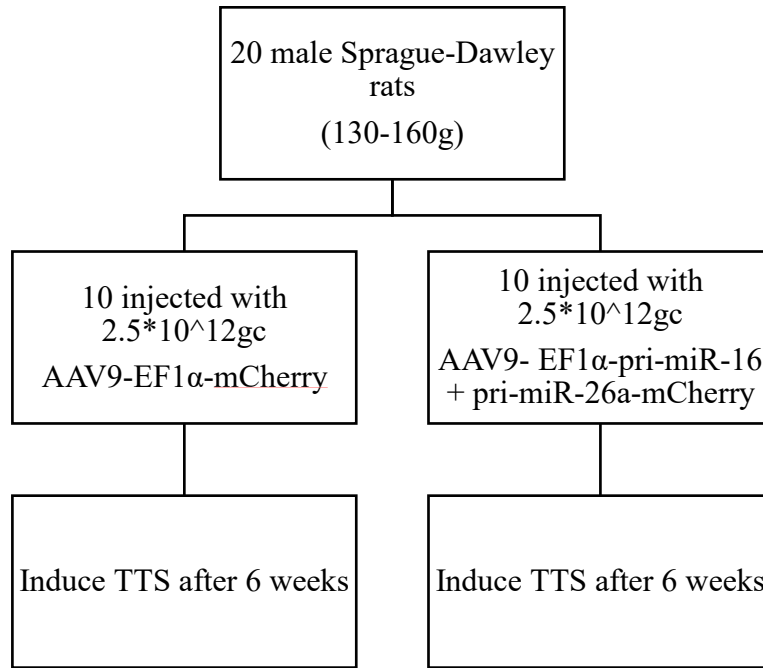


Figure 2.7: Schematic for AAV9 infection with subsequent TTS induction

Workflow of the *in vivo* induction of takotsubo syndrome with AAV9 upregulation of miR-16 and miR-26a.

2.7.5 Sampling of blood

Whilst the external jugular vein was cannulated as described in Section 2.7.3, blood could be sampled with a Vacutainer SST Advance blood tube. With TTS induction, blood was sampled at baseline, as well as at 20 minutes and 60 minutes post-adrenaline administration. The blood was allowed to remain at room temperature for up to an hour or stored in ice for longer time periods, before being processed as per the protocol outlined in the Qiagen miRNeasy Serum/Plasma isolation handbook (Qiagen, 2012). In short, the blood was centrifuged for 10 minutes at 1900g (4°C). This yielded serum which was isolated and centrifuged for a further 10 minutes at 16,000g (4°C) to remove additional cellular nucleic acids and cell debris.

2.7.6 Monitoring of AAV treated rats

After consulting a veterinarian from Imperial CBS, a behavioural monitoring plan was devised. The rat grimace scale (Sotocina *et al.*, 2011) was used to monitor whether the AAV treated rats experienced any pain daily during the 6-week monitoring period. The activity level of the rats was evaluated with the help of Hannah Jones, an animal technician in CBS, to grade the average activity level of a cage of animals treated with either AAV-control or AAV-miR. Low, medium and high levels of activity were assigned values of 0, 1 and 2.

AAV treated rats were weighed at baseline and then weekly using a bench-top set of digital scales within the CBS animal facility. This enabled the growth of AAV treated rats to be monitored and compared.

2.8 Bioinformatic analysis

2.8.1 *In silico* target prediction

To delineate which genes miR-16 and miR-26a can target, a workflow was designed to robustly locate genes associated with cardiomyocyte contractility. miRWalk 2.0 was used to predict target gene binding by analysing 3'UTR sequence homology by cross-comparing 12 different miR databases (Dweep and Gretz, 2015). These consisted of miRWalk, MicroT4, miRanda, miRBridge, miRDB, miRMap, PICTAR2, PITA, RNA22, RNAhybrid and Targetscan. This yielded a list of thousands of genes, but only genes located in 7 or more databases were included. This list was then stratified using Panther DB (Mi *et al.*, 2013, 2017) to obtain a list of genes associated with contractility.

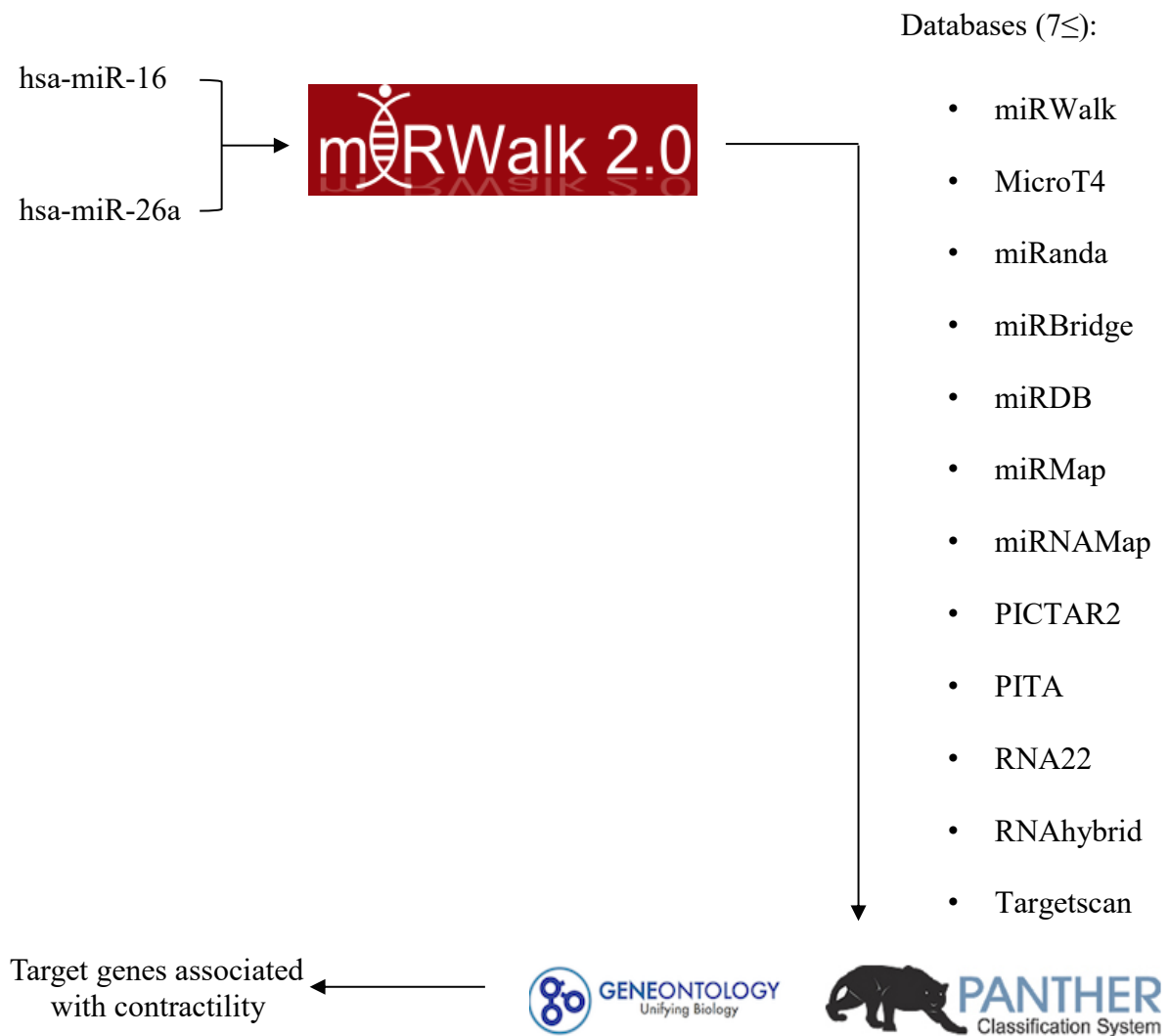


Figure 2.8: *In silico* target prediction workflow

Workflow used for the *in silico* target prediction of miR-16 and miR-26a. miRWalk 2.0 was used to cross-reference the targets contained in the 12 listed databases, and these were stratified using Panther DB.

2.9 Statistical analysis

Results were analysed in Prism5 software (GraphPad Software) and represented as mean $\pm$ SEM with an α critical value of 0.05 set for the level of significance ($p < 0.05$). To compare the relative changes between two data sets, Student's t-tests was used. Data was tested for normality throughout, and initial statistical comparisons were supported in Section 3.3.2 by hierarchical clustering, a statistical technique recently published for use in isolated cardiomyocyte studies (Sikkel *et al.*, 2017).

Where there were more than two statistical comparisons to be made, one-way analysis of variance (ANOVA) was used. Tukey's test was carried out for post-hoc analysis where there was significance present within a dataset. When comparing two groups with time, repeated measures ANOVA was conducted, and with two variables, two-way ANOVA was used. For these, subsequent Bonferroni's post-hoc test was carried out if required. Where the fitted curves of datasets were compared, a non-linear regression was carried out comparing agonist *versus* response. An F-test was used to compare the two fitted curves to test whether they were better fitted by a single curve (i.e. not significantly different) or by two curves (significantly different). Level of significance is presented throughout as follows: $\ast = p < 0.05$, $\ast\ast = p < 0.01$, $\ast\ast\ast = p < 0.001$.

Experiments were blinded wherever possible. In particular, *in vivo* upregulation of miRs in Chapter 5 was blinded at the point of administration and analysis. Therefore, induction of TTS was conducted in a blinded manor, and data was analysed by an independent expert echocardiographer who was also blinded to experimental conditions.

3 The effect of takotsubo syndrome associated miR-16 and miR-26a on adult rat ventricular cardiomyocytes *in vitro*

3.1 Introduction

Whilst TTS is becoming of increased interest in cardiovascular research, the mechanism that underlies the regional negative inotropic changes in TTS is still widely debated. Furthermore, the reason for the predisposition of post-menopausal women is entirely unknown.

Consequently, it is extremely important to investigate the mechanisms that could underlie both the preponderance and regionality of TTS. TTS-associated miR-16 and miR-26a represent novel biomarkers to distinguish TTS from both healthy patients, and those with STEMI (Jaguszewski *et al.*, 2014). This represents a key difference, since TTS resembles STEMI at presentation, with chest pain and ST-elevation on ECG being the most common symptoms and signs (Templin *et al.*, 2015). Whether these play a role in the mechanistic generation of TTS is unknown, however they could feasibly represent a novel explanation behind the predisposition of TTS. As such, we hypothesised that they could be novel factors predisposing to or exacerbating TTS.

Primary isolated adult cardiomyocyte physiology can be readily studied *in vitro* by assessment of a wide variety of functional parameters. This is a reduced system, where other cell types such as endothelial cells and fibroblasts are removed, and direct responses from cardiomyocytes can be measured. As such, this was utilised in order to investigate the preliminary effects of miR-16 and miR-26a on cardiomyocyte contractility.

3.2 Materials and methods

Specific details for the methods used in this chapter are detailed in Chapter 2.

3.2.1 miR transfection and quantification

Cardiomyocytes were transfected with 100nM miR over 48 hours using Lipofectamine 3000 as per the manufacturer's protocol. Pre-miR negative control #2 and Anti-miR negative control #1 were used as negative controls and are referred to as 'control P' and 'control A' respectively throughout. Hsa-pre-miR-16 and hsa-pre-miR-26a were used to increase levels of miR-16 and miR-26a, whereas hsa-anti-miR-16 and hsa-anti-miR-26a were used for downregulation.

miR expression was assessed by transfecting 25,000 cardiomyocytes in a 12 well plate, and RNA isolated using trizol (Section 2.6.2). cDNA was produced for each respective miR using the TaqMan™ MicroRNA Reverse Transcription Kit with the respective RT primers, and RTqPCR carried out using the primers outlined in Section 2.6.3. Analysis was carried out using the ΔC_t method.

3.2.2 IonOptix Myocyte Contractility Recording System (MCSYS)

12,500 cardiomyocytes were transfected in suspension in a 24 well plate, before being placed on the MCSYS as previously described (Section 2.3.3). Baseline contractility could be measured, and subsequent pharmacological protocols induced. Specifically, β_2 AR reponses were induced with 300nM CGP 20712A (selective β_1 AR inhibitor) and 1 μ M isoprenaline (non-selective β AR agonist). Adrenaline concentration-response curves were obtained using between 3×10^{-10} M and 3×10^{-6} M adrenaline increasing in half-log₁₀ doses.

3.2.3 Epi- fluorescence

Cardiomyocyte calcium transients were assessed using Fluo-4 AM, with concomitant application of caffeine as described in Section 2.5.3. 12,500 were seeded on a 3.5mm glass surface within a Mattek dish on laminin, before being transfected. This enabled calcium transient amplitude and a measure for SR calcium content to be obtained, as well as calcium transient kinetics (time to peak and 50% and 80% decay), and rates of SERCA, NCX and slow mechanisms to be calculated.

3.2.4 Metabolic assessment

Metabolic function of cardiomyocytes was measured using the Seahorse XFp Extracellular Flux Analyzer, where 3000 cardiomyocytes were transfected per well of a Seahorse XFp Miniplate. Oligomycin, FCCP, antimycin A and rotenone were applied as discussed in Section 2.3.5 to obtain OCR for various metabolic parameters. OCR was subsequently normalised to weight of protein (OCR/ μ g) as determined using a Pierce™ BCA protein assay kit.

3.2.5 Statistics

Data is displayed as mean $\pm$ SEM, and significance calculated with Student's t-test for the comparison of two groups, or one-way ANOVA with Tukey's post-hoc test for comparison of more than two groups. Where two variables were present, two-way ANOVA was used with Bonferroni's post-hoc test. Concentration-response curves were analysed by non-linear regression comparing agonist *versus* response. An F-test was used to compare the two fitted curves to test for significant differences. Significance is shown as appropriate as follows: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.3 Results

3.3.1 miR-16 and miR-26a expression *in vitro*

To determine the expression pattern of miR-16 and miR-26a, Gene Expression Omnibus was utilised for bioinformatic analysis (Barrett *et al.*, 2012). In mouse, miR-16 and miR-26a are lowly expressed in heart as seen in Figure 3.1A and B (Meng *et al.*, 2016). miR-16 is highest expressed in spleen, but also elevated in the gastrointestinal system, kidney, liver, lung and brain (Figure 3.1A). miR-26a is highest in lung and brain, and also high in kidney (Figure 3.1B). Comparison between baseline expression in apex and base of the heart was conducted in Figure 3.1C and D to show that there was no difference in expression levels for miR-16 or miR-26a respectively. However, the variability within this experiment is large and thus suggests this dataset is underpowered for this comparison. A power calculation should be done to guide numbers used.

Before functional experiments were commenced, transfection of cardiomyocytes was carried out to assess the extent of upregulation and downregulation obtainable with miR-16 and miR-26a pre-miR and anti-miR sequences respectively. In Figure 3.2, it can be seen that miR-16 and miR-26a were significantly increased (Figure 3.2A and C), and reduced (Figure 3.2B and D). This indicates that TTS-associated miRs can be reliably changed *in vitro*, and whilst an order of magnitude larger than the changes first noted *in vivo* (Jaguszewski *et al.*, 2014), these conditions were used for subsequent functional experiments to enable biological effects of miR-16 and miR-26a to be evaluated.

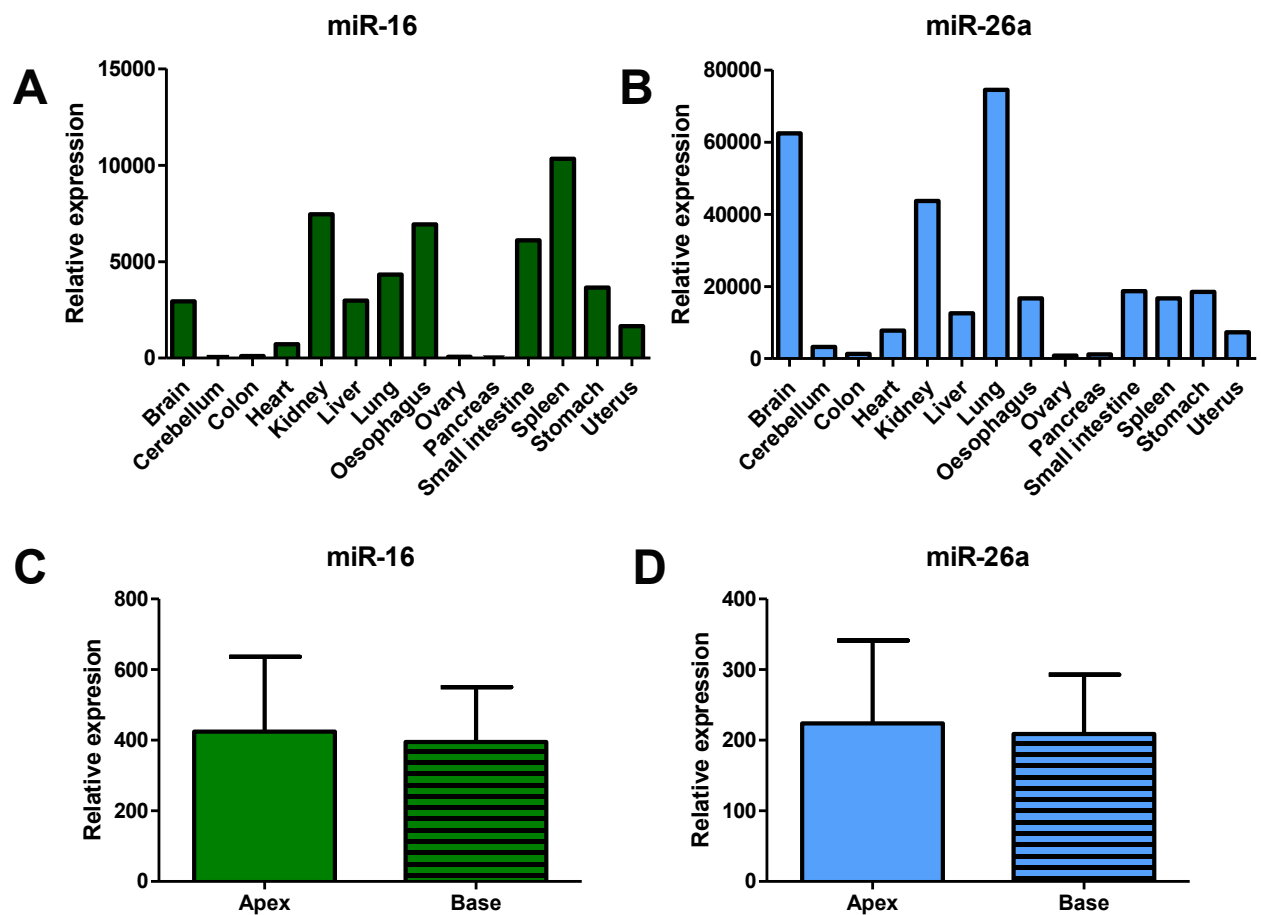


Figure 3.1: miR-16 and miR-26a baseline expression levels

Relative expression (obtained by normalisation to RNU6B) of miR-16 (**A**) and miR-26a (**B**) in tissues from mouse by bioinformatic analysis (Barrett *et al.*, 2012; Meng *et al.*, 2016) and expression in apex and base in rat isolated cardiomyocytes (**C** and **D** respectively, N=6). Data is displayed as mean $\pm$ SEM

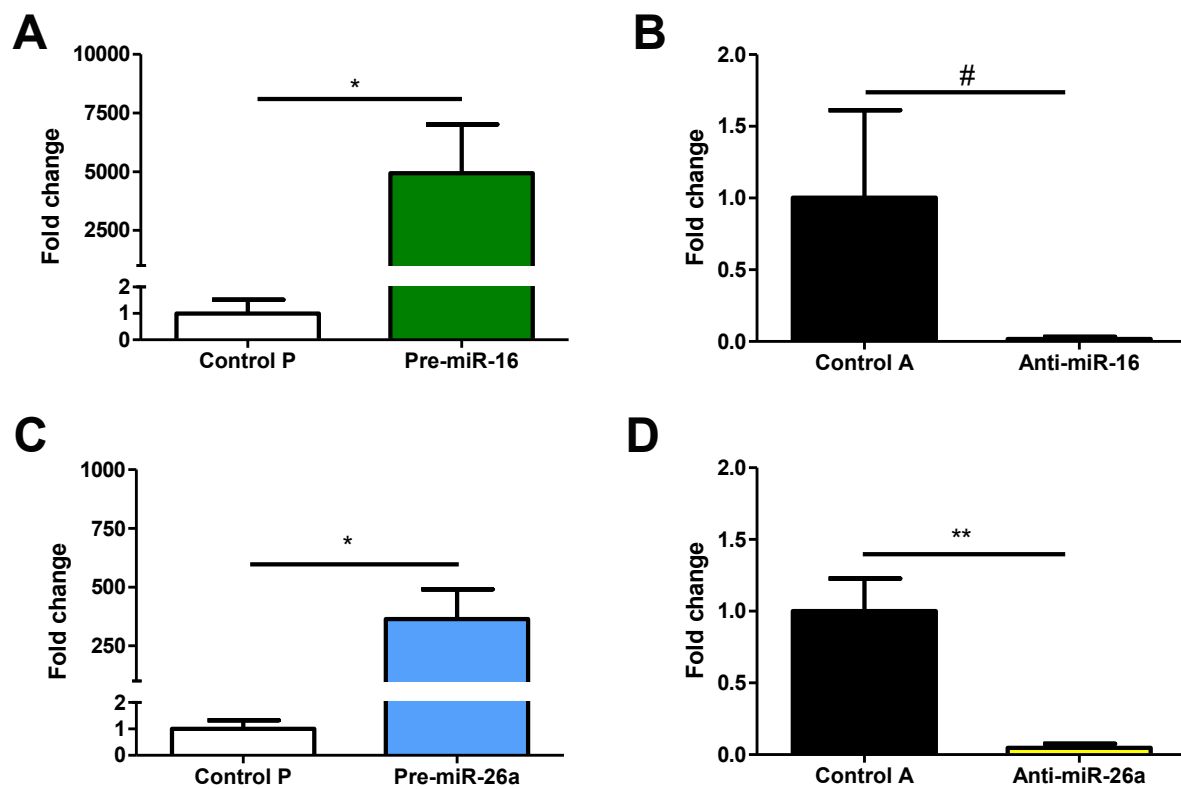


Figure 3.2: miR-16 and miR-26a can be upregulated and downregulated in cardiomyocytes *in vitro*

Relative expression of miR-16 and miR-26a modulated by *in vitro* transfection in adult rat apical cardiomyocytes. **A** miR-16 upregulation (N=4). **B** miR-16 downregulation (N=4). **C** miR-26a upregulation (N=3). **D** miR-26a downregulation (N=3). N represents the number of transfections. Data is displayed as mean $\pm$ SEM, with significance determined by Student's t-test and shown as follows: # = $p=0.055$, * = $p<0.05$, ** = $p<0.01$.

3.3.2 miR-16 and miR-26a reduce baseline contractility of adult rat apical cardiomyocytes, but not synergistically

Upregulation and downregulation of miR-16 and miR-26a in adult rat apical cardiomyocytes was carried out to see if emulating the miR profile seen in patients with TTS could produce any changes in myocyte contractility (Figure 3.3). Increased levels of both miR-16 (Figure 3.3A) and miR-26a (Figure 3.3C) significantly reduced baseline contraction when compared to control, with no changes in the time to peak, or time to 50% and 90% relaxation (Figure 3.4A, B and C respectively). This is represented in Figure 3.3F, where a representative contractile transient can be seen. This was not the case with downregulation, where anti-miR-16 and anti-miR-26a had no effect on baseline contraction (Figure 3.3B and D). As miR-16 and miR-26a are concomitantly increased *in vivo*, co-transfection was undertaken, however this produced no additional reduction in baseline contractility when compared to miR-16 and miR-26a separately (Figure 3.3E).

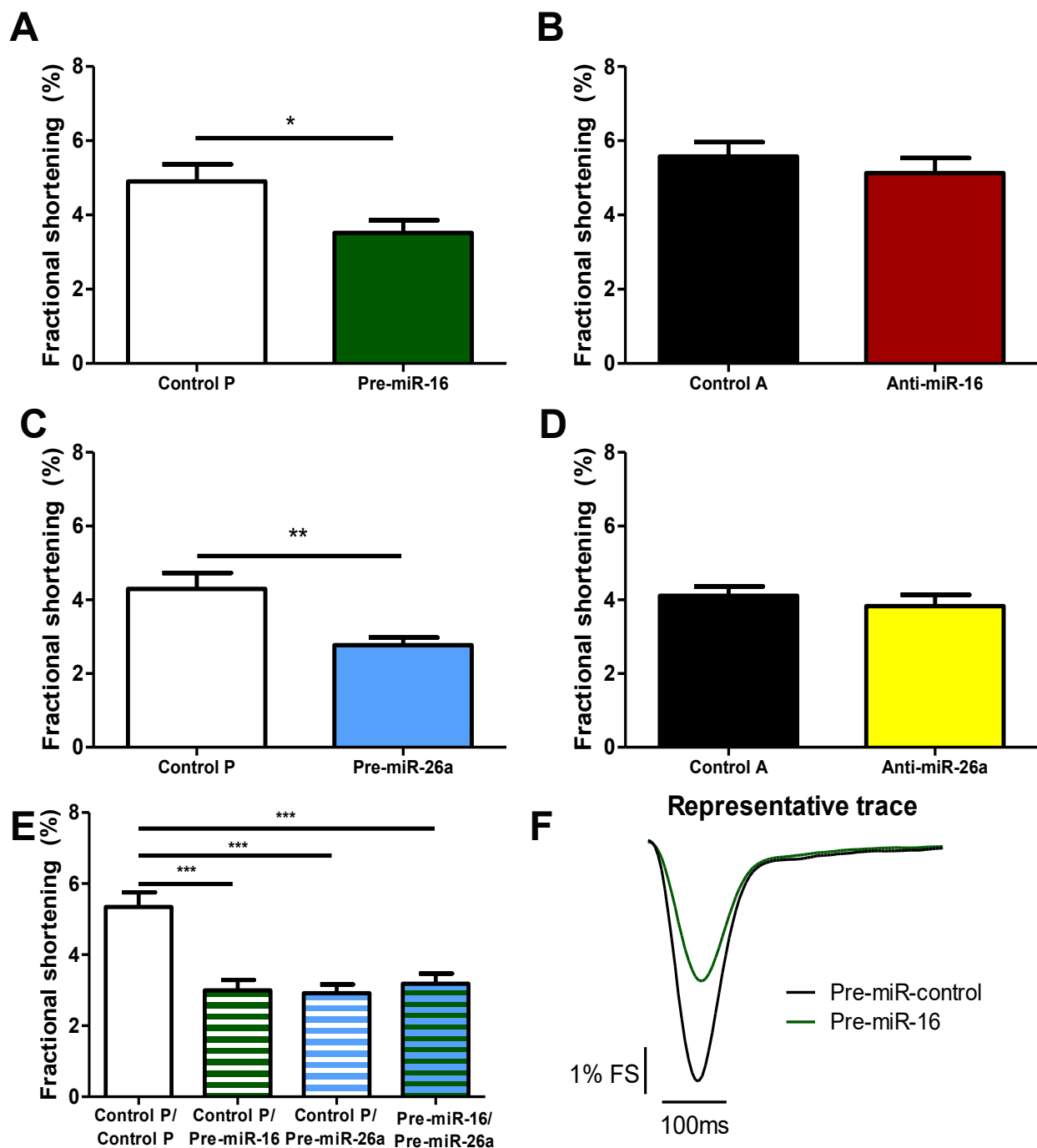


Figure 3.3: miR-16 and miR-26a reduce baseline contractility of apical cardiomyocytes, but do not synergise

Baseline percentage fractional shortening of adult rat apical cardiomyocytes shown with upregulation of miR-16 (A, n/N=30/6) and miR-26a (C, n/N=50/10), and downregulation of miR-16 (B, n/N=30/6) and miR-26a (D, n/N=50/10). E Co-transfection of miR-16 and miR-26a (n/N=60/6). Numbers are displayed as n/N, where 'n' is equal to the number of cardiomyocytes and 'N' represents number of rats. Data is displayed as

mean $\pm$ SEM, with significance determined using Student's t-test or one-way ANOVA with Tukey's post-hoc test and shown as follows: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

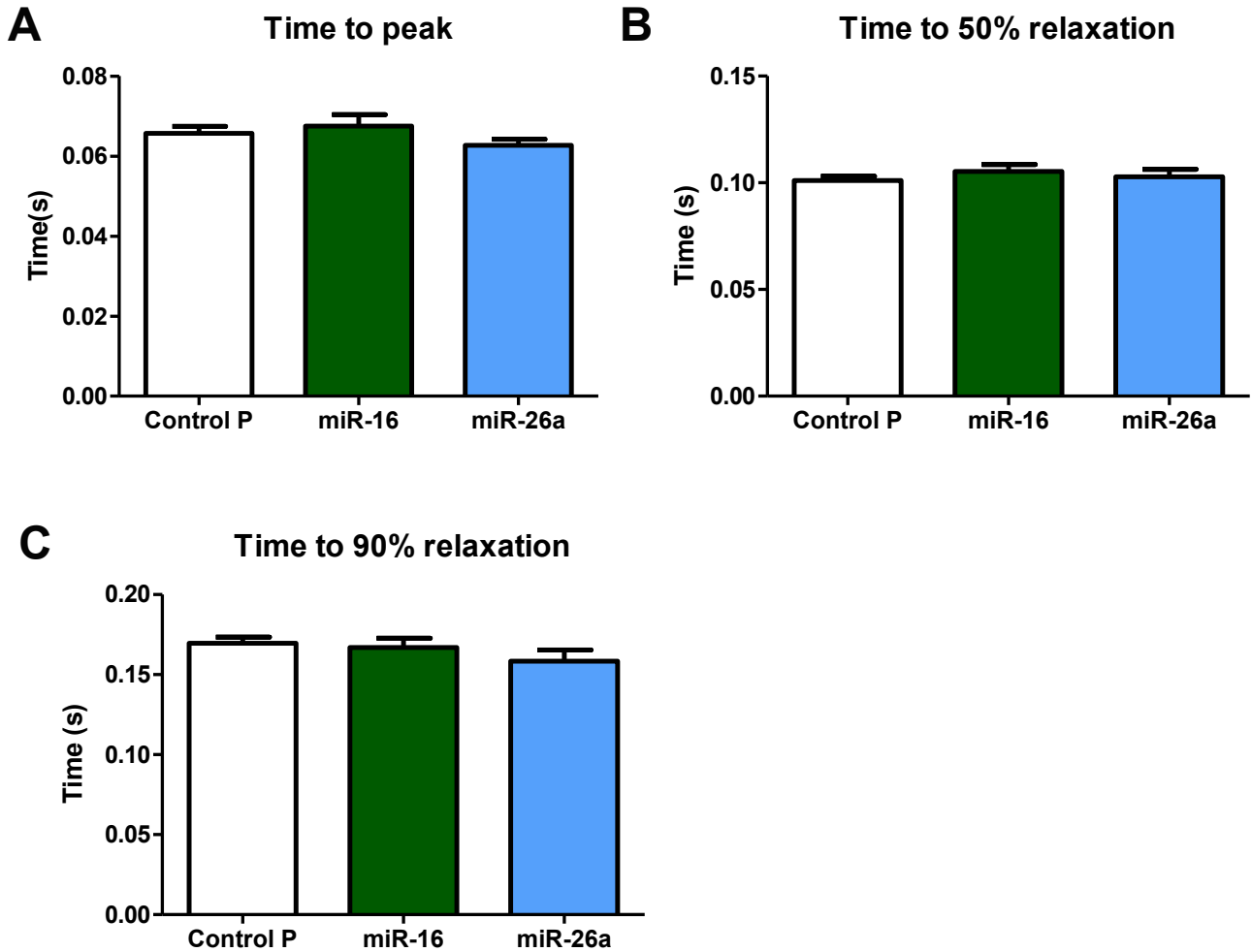


Figure 3.4: miR-16 and miR-26a do not alter kinetics of contraction

Baseline kinetics of contraction of adult rat apical cardiomyocytes with upregulation of miR-16 and miR-26a showing time to peak (A), time to 50% relaxation (B) and time to 90% relaxation (C). $n/N=30/6$ where 'n' is equal to the number of cardiomyocytes and 'N' represents number of rats. Data is displayed as mean $\pm$ SEM and compared using one-way ANOVA.

3.3.3 The negative inotropic effect of miR-16 and miR-26a occurs specifically in apical cardiomyocytes, and not basal

Given the observed reduction in baseline contractility in apical cardiomyocytes, the effect of miR-16 and miR-26a was compared between apical and basal cardiomyocytes in a paired experiment to investigate whether the apicobasal gradient in negative inotropy would be observed *in vitro*. This experiment was conducted by Rory Clayton under my direct supervision as part of his Cardiovascular Science BSc project, and is shown in Figure 3.5. Here it can be seen that miR-16 and miR-26a do not reduce baseline contractility of basal cardiomyocytes, as there is no difference from apical or basal control transfected myocytes. Indeed, there is a statistically significant difference between the apex and base of miR-16 and miR-26a transfected cardiomyocytes (both $p < 0.001$), as seen in TTS *in vivo*.

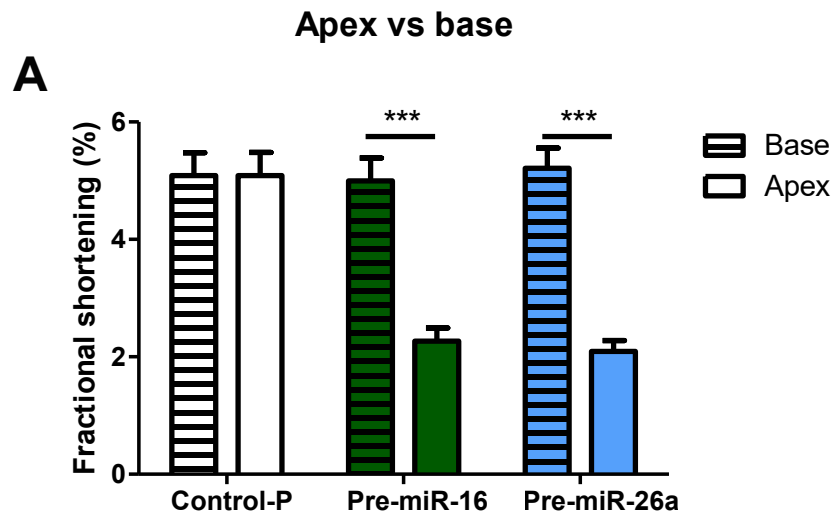


Figure 3.5: miR-16 and miR-26a reduce contractility of apical cardiomyocytes, but not basal

A Fractional shortening of apical and basal adult rat cardiomyocytes transfected with miR-16 and miR-26a (n/N=40/4). Data is displayed as mean $\pm$ SEM, with significance determined by two-way ANOVA with Bonferroni post-hoc test and shown as *** = $p < 0.001$.

3.3.4 miR-26a increases response to adrenaline in cardiomyocytes from the base, but not apex

Given the differential apicobasal response to adrenaline in TTS, adrenaline concentration-response curves were established for miR-16 and miR-26a (Figure 3.6), with statistical comparisons displayed in Table 3.1. In all cases, adrenaline had the expected positive inotropic effect.

In apical cardiomyocytes, miR-16 or miR-26a adrenaline concentration-response curves were unchanged from control in absolute (Figure 3.6A) or normalised responses (Figure 3.6C) by F-test. Fractional shortening trended towards a reduction on the bottom plateau for miR-26a but not miR-16, although this was not powered for this comparison (Figure 3.6A).

In basal cardiomyocytes, miR-26a was significantly different from control by F-test, and miR-16 was on the verge of significance (Figure 3.6B). Indeed, with miR-26a, the curve was displaced vertically, with a higher top plateau, although also a higher bottom plateau. After normalisation, there are no differences in logEC50 between miR-16 or miR-26a from control in the base (Figure 3.6D).

miR-16 and miR-26a were concomitantly increased (Figure 3.7), as this is the pattern in which they are altered *in vivo*, with statistical comparisons in Table 3.2. In the apex, adrenaline concentration-response curve of miR transfected cardiomyocytes was significantly different from control, and logEC50 was on the verge of being reduced (Figure 3.7E). After normalisation, there was a highly significant reduction in the sensitivity to adrenaline, as determined by the logEC50 (Figure 3.7G).

In the base, the maximal response to adrenaline was significantly increased with miRs, with a higher top plateau (Figure 3.7F) as in Figure 3.6B. There was no difference in sensitivity after normalisation in the base (Figure 3.7H).

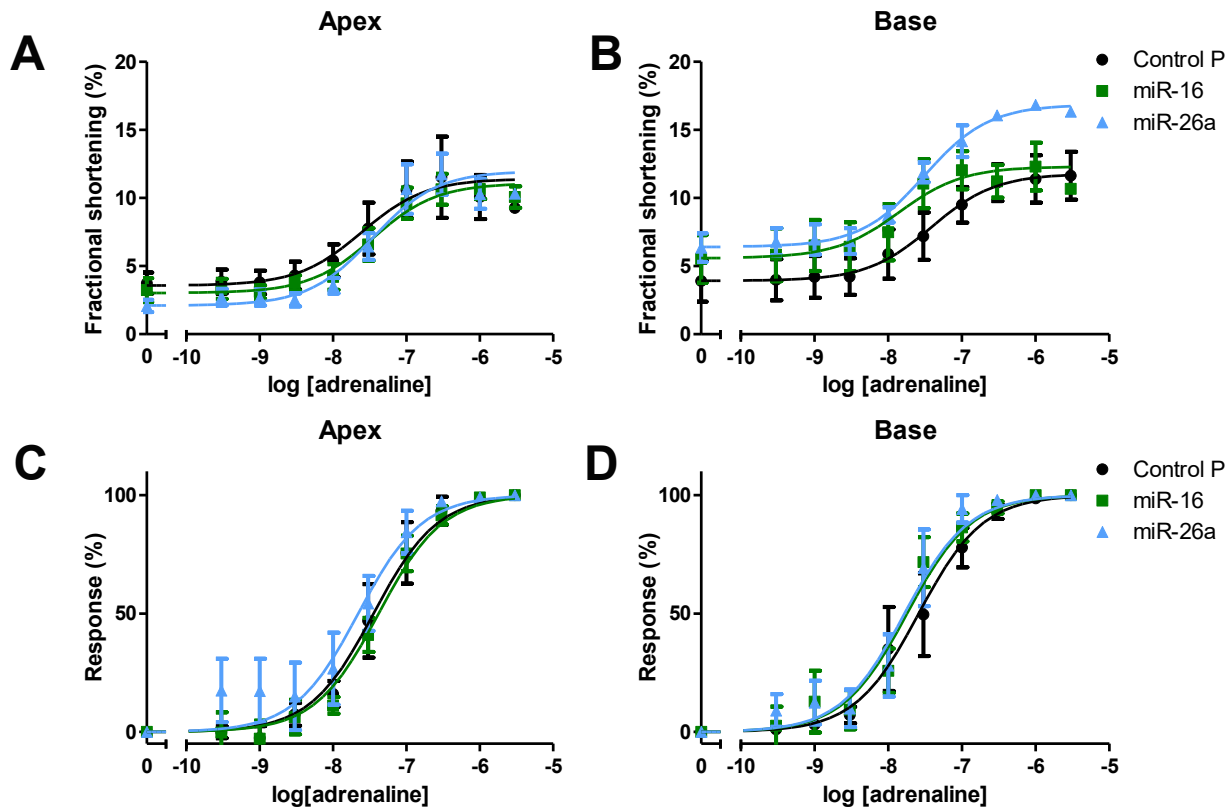


Figure 3.6: miR-26a increases response to adrenaline in basal cardiomyocytes

Adrenaline concentration-response curves of miR-16 and miR-26a transfected adult rat apical or basal cardiomyocytes. **A** Fractional shortening of apical cardiomyocytes with adrenaline. **B** Fractional shortening of basal cardiomyocytes with adrenaline. **C** Percentage response to adrenaline between maximum and baseline in apical cardiomyocytes. **D** Percentage response to adrenaline between maximum and baseline in basal cardiomyocytes. $n/N = 6/6$ throughout. Data is displayed as mean $\pm$ SEM, with significance determined by non-linear regression comparing agonist *versus* response by F-test and shown in Table 3.1.

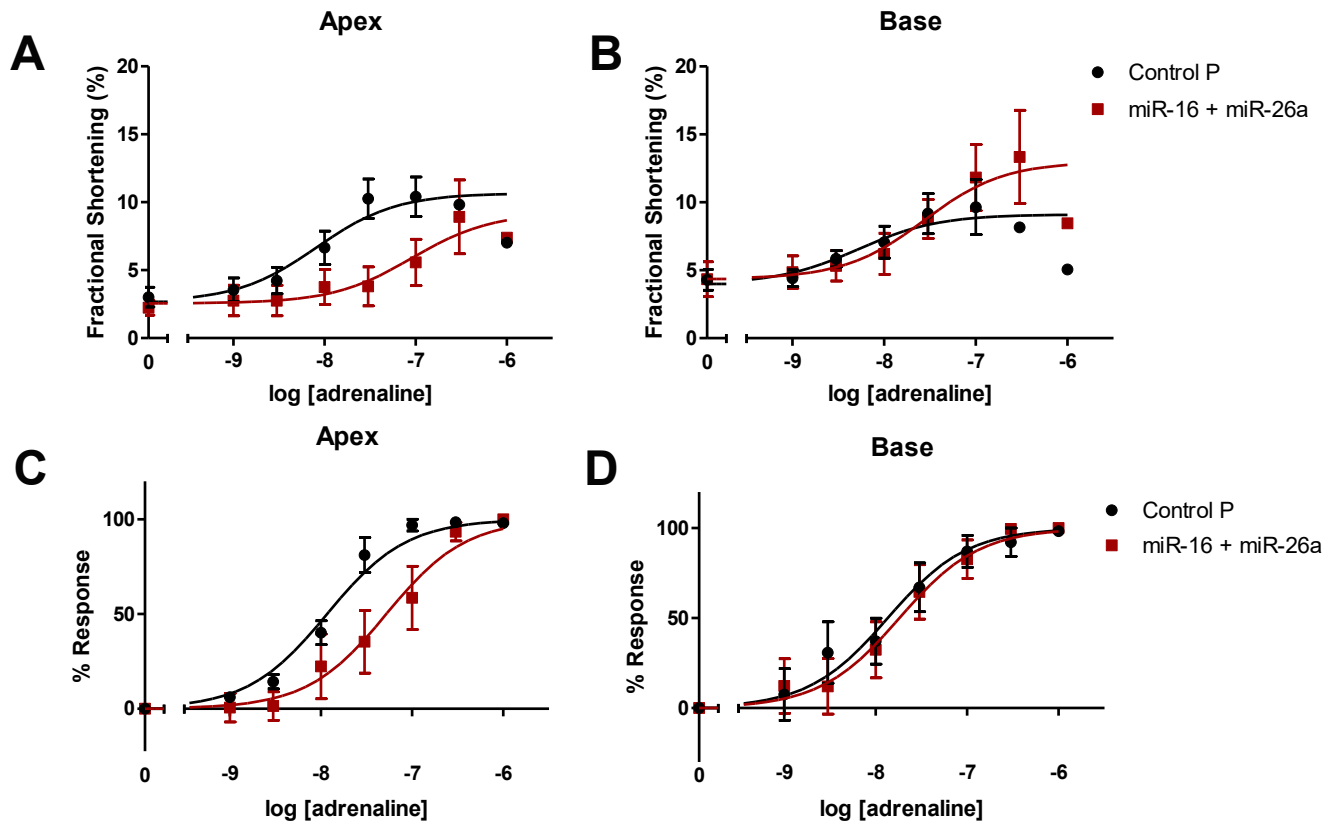


Figure 3.7: miRs in combination reduce sensitivity to adrenaline in the apex and increase maximum response to adrenaline in the base

Adrenaline concentration-response curves of miR-16 and miR-26a transfected adult rat apical or basal cardiomyocytes. **A** Fractional shortening of apical cardiomyocytes with adrenaline. **B** Fractional shortening of basal cardiomyocytes with adrenaline. **C** Percentage response to adrenaline between maximum and baseline in apical cardiomyocytes. **D** Percentage response to adrenaline between maximum and baseline in basal cardiomyocytes. $n/N = 6/6$ throughout. Data is displayed as mean $\pm$ SEM, with significance determined by non-linear regression comparing agonist *versus* response by F-test and shown in Table 3.1.

Table 3.1: Statistics for adrenaline dose-response curves shown in Figure 3.6

Adrenaline concentration-response curves for miR-16 and miR-26a treated cardiomyocytes analysed with non-linear regression comparing agonist *versus* response.

An F-test was used to compare the bottom and top plateaus and the logEC50 of the two fitted curves to test for significant differences with p values and confidence intervals (CI) shown.

Fractional Shortening (%)	Apex	Control P	CI	miR-16	CI	p value	miR-26a	CI	p value
	Bottom	3.57	2.090 to 5.051	3.026	2.119 to 3.933	0.5082	2.102	1.164 to 3.039	0.083
	Top	11.41	8.717 to 14.11	11.08	9.698 to 12.47	0.8073	11.97	10.23 to 13.72	0.7069
	LogEC50	-7.608	-8.238 to -6.978	-7.456	-7.818 to -7.093	0.6194	-7.464	-7.792 to -7.137	0.6208
	F-test					0.5265			0.1463
	Base	Control P	CI	miR-16	CI	p value	miR-26a	CI	p value
	Bottom	3.925	2.375 to 5.476	5.595	3.580 to 7.610	0.1817	6.414	5.459 to 7.369	0.0071
	Top	11.78	9.334 to 14.23	12.3	9.625 to 14.98	0.7727	16.85	14.41 to 19.29	0.0068
	LogEC50	-7.412	-8.038 to -6.787	-7.851	-8.725 to -6.977	0.3738	-7.508	-7.853 to -7.162	0.7834
	F-test					0.0562			P<0.0001
Response (%)									
	Apex	Control P	CI	miR-16	CI	p value	miR-26a	CI	p value
	LogEC50	-7.446	-7.602 to -7.290	-7.377	-7.500 to -7.253	0.4657	-7.655	-7.877 to -7.434	0.1186
	Base	Control P	CI	miR-16	CI	p value	miR-26a	CI	p value
	LogEC50	-7.585	-7.765 to -7.405	-7.743	-7.906 to -7.581	0.1879	-7.777	-7.957 to -7.597	0.1256

Table 3.2: Statistics for adrenaline dose-response curves shown in Figure 3.7

Adrenaline concentration-response curves for miR-16 and miR-26a treated cardiomyocytes in combination analysed with non-linear regression comparing agonist *versus* response. An F-test was used to compare the bottom and top plateaus and the logEC50 of the two fitted curves to test for significant differences with p values and confidence intervals (CI) shown.

Fractional Shortening (%)	Apex	Control P	CI	miR-16 + miR-26a	CI	p value	
	Bottom	2.674	0.8694 to 4.479	2.556	1.106 to 4.007	0.9167	
	Top	10.66	8.316 to 13.00	9.23	4.541 to 13.92	0.5678	
	LogEC50	-8.089	-8.660 to -7.517	-7.042	-8.034 to -6.051	0.06	
	F-test						0.0004
	Base	Control P	CI	miR-16 + miR-26a	CI	p value	
	Bottom	3.994	1.978 to 6.010	4.366	2.410 to 6.321	0.7846	
	Top	9.106	7.041 to 11.17	12.98	9.034 to 16.93	0.0467	
	LogEC50	-8.281	-9.172 to -7.389	-7.563	-8.307 to -6.820	0.1703	
	F-test						0.2579
Response (%)							
	Apex	Control P	CI	miR-16 + miR-26a	CI	p value	
	LogEC50	-7.932	-8.040 to -7.825	-7.282	-7.523 to -7.042	P<0.0001	
	Base	Control P	CI	miR-16 + miR-26a	CI	p value	
	LogEC50	-7.868	-8.109 to -7.628	-7.741	-7.986 to -7.495	0.4658	

3.3.5 miR-16 and miR-26a do not alter peak β_2 AR response, or change relative to baseline

Since response to adrenaline was altered by TTS-associated miRs in Section 3.3.4, we investigated whether the β_2 AR response was specifically changed, especially given the demonstrated importance in TTS (Paur *et al.*, 2012). β_2 AR responses were elicited as described in section 2.5.1. There was no difference in the peak contractility observed after induction of a β_2 AR response with upregulation or downregulation of miR-16 and miR-26a (Figure 3.8A and B respectively). Similarly, there were no differences with the fold or absolute changes from baseline contractility with miR-16 or miR-26a transfection, as shown in Figure 3.8C to Figure 3.8F.

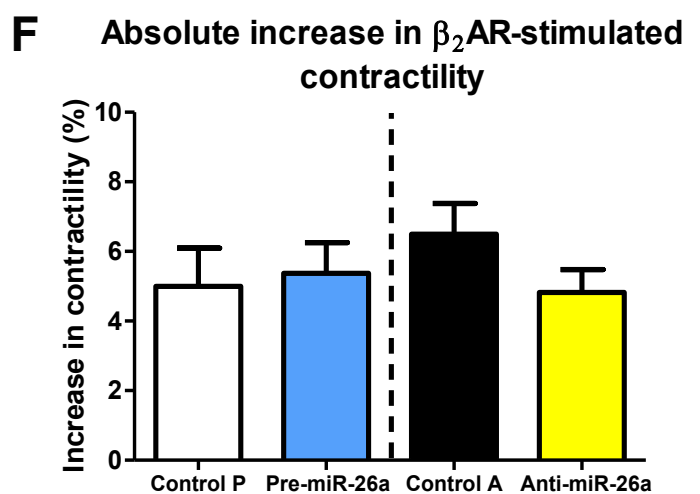
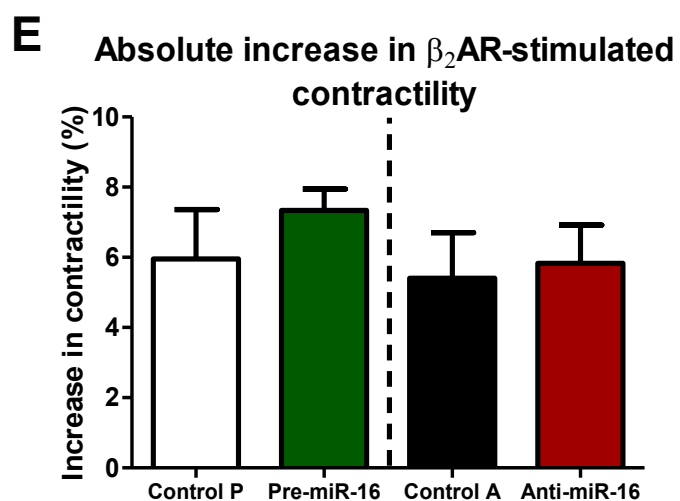
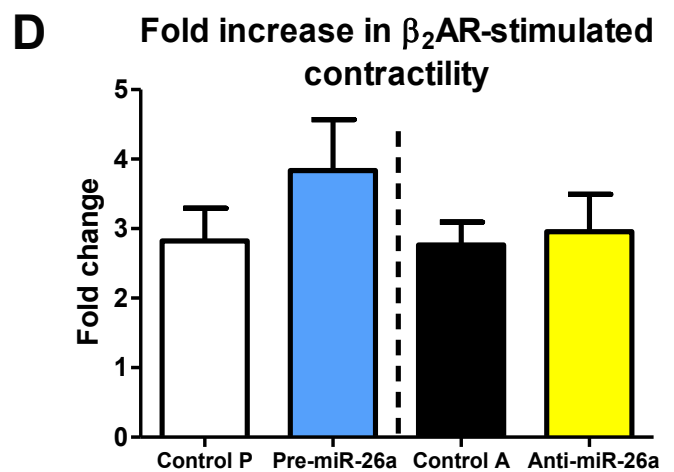
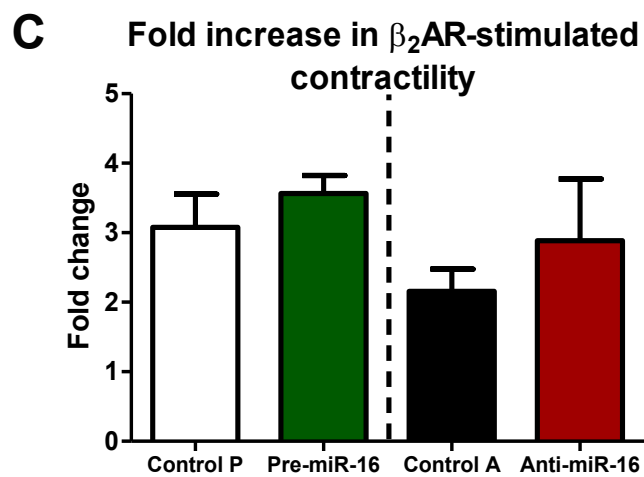
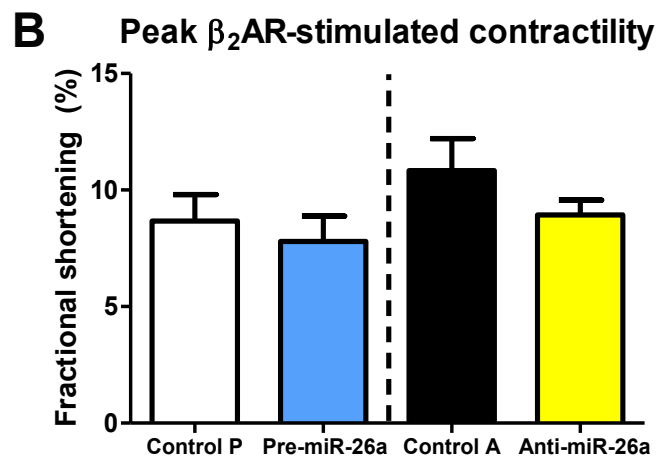
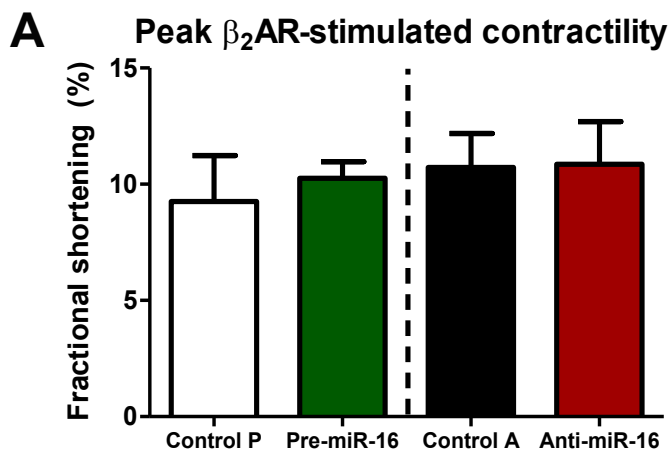


Figure 3.8: miR-16 and miR-26a do not alter cardiomyocyte β_2 AR response

β_2 AR response obtained by β_1 AR antagonism with CGP 20712A and applying the β AR agonist isoprenaline in adult rat apical cardiomyocytes treated with miR-16 and miR-26a pre-miRs and anti-miRs. **A** and **B** The peak contractile response following β_2 AR stimulation. **C** and **D** The fold increase in contractility after β_2 AR response. **E** and **F** The absolute increase in contractility following β_2 AR stimulation. n/N = 6/6 for miR-16 experiments (**A**, **C** and **E**) and 10/10 for miR-26a (**B**, **D** and **F**). Numbers are displayed as n/N, where 'n' is equal to the number of cardiomyocytes and 'N' represents number of rats. Data is displayed as mean $\pm$ SEM and compared using Student's t-test.

3.3.6 miR-16 and miR-26a also reduce calcium transient amplitude and sarcoplasmic reticulum calcium content, but not rates of decay

To further understand how the reduction in baseline contractility occurred, we investigated whether any alterations in calcium cycling were present. Specific elements of calcium transient handling proteins involved in calcium transient decay were estimated using the caffeine application protocol described in section 2.5.3 was used. Briefly, application of caffeine increases open probability of ryanodine receptors, to yield a caffeine-induced calcium transient that is a measure of SR calcium content. Simultaneously, this prevents the SR from refilling, removing contribution of SERCA from calcium transient decay. NCX and slow mechanisms of calcium transient decay remain, with contribution of NCX removed by replacing NT with NT containing zero sodium and zero calcium.

Figure 3.9A show that the calcium transient amplitude of miR-16 and miR-26a transfected cardiomyocytes was significantly reduced with representative traces shown in Figure 3.9B. This was seen along with a significant reduction in the caffeine-induced calcium transient amplitude for miR-16 and miR-26a, which is indicative of a reduced SR calcium content (Figure 3.9C). There was no difference in the fractional release, as shown in Figure 3.9D, which is a comparison between the calcium transient amplitude and the caffeine-induced calcium transient amplitude. This indicates that the reduction in calcium transient amplitude is likely due to the reduction in SR calcium content.

There was no difference in the time to peak (Figure 3.10A) or time to 50% decay (Figure 3.10B), but there was a significant reduction in time to 80% decay in the miR-16 transfected cardiomyocytes (Figure 3.10C). Overall, there was no difference in the overall rate of calcium transient decay (Figure 3.10D).

A reduced SR calcium content could be due to reduced function in SERCA, which re-sequesters calcium back into the SR, so this was particularly of interest. However, there was no significant difference in SERCA or NCX function (Figure 3.10E and F respectively), the two major contributors to calcium transient decay (Figure 3.10H). There was a significant increase in the rate of the slow mechanisms that contribute to calcium transient decay with miR-26a transfection (Figure 3.10G), although these are not thought to modulate calcium handling on a beat-to-beat basis (Bers, 2008).

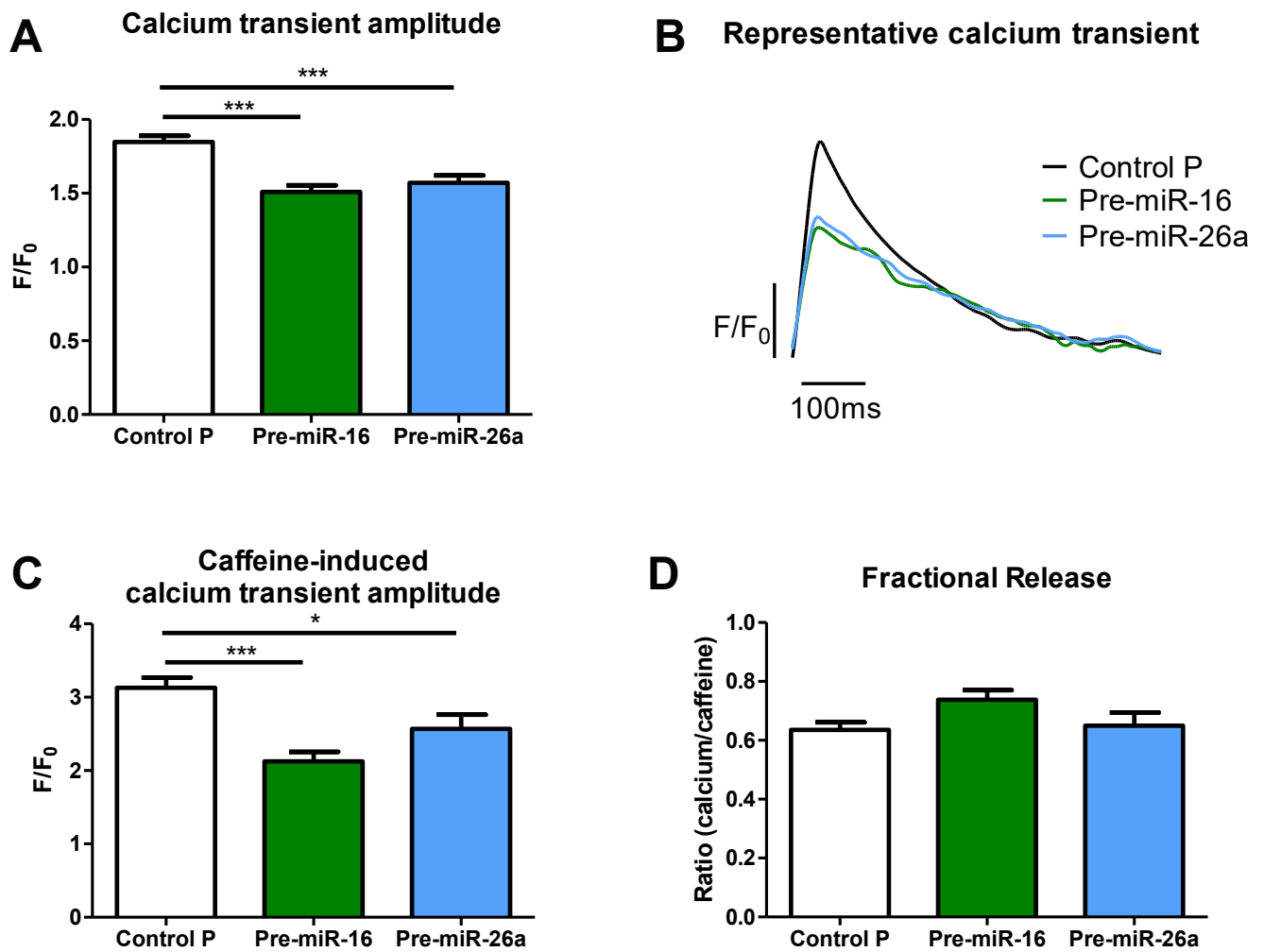


Figure 3.9: miR-16 and miR-26a reduce calcium transient amplitude and SR calcium content by equal proportions

Epi-fluorescence of miR-16 and miR-26a transfected adult rat apical cardiomyocytes. **A** Calcium transient amplitude of transfected cardiomyocytes (control $p = 66/4$, pre-miR-26 = $37/4$ and pre-miR-26a = $26/4$). **B** A representative baseline calcium transient obtained showing relative change in amplitude over time. **C** Caffeine-induced calcium transient amplitude used as a marker of SR calcium content (control $p = 32/4$, pre-miR-26 = $22/4$ and pre-miR-26a = $17/4$). **D** Fraction release of calcium, where this is the ratio between calcium transient amplitude and caffeine-induced calcium transient amplitude (control $p = 32/4$, pre-miR-26 = $22/4$ and pre-miR-26a = $17/4$). Data is displayed as mean $\pm$ SEM and differences determined by one-way ANOVA with Tukey's post-hoc tests, and significance shown as follows: * = $p < 0.05$, *** = $p < 0.001$.

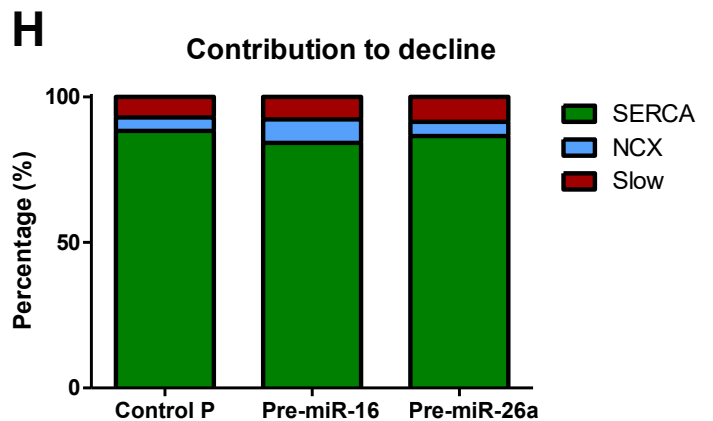
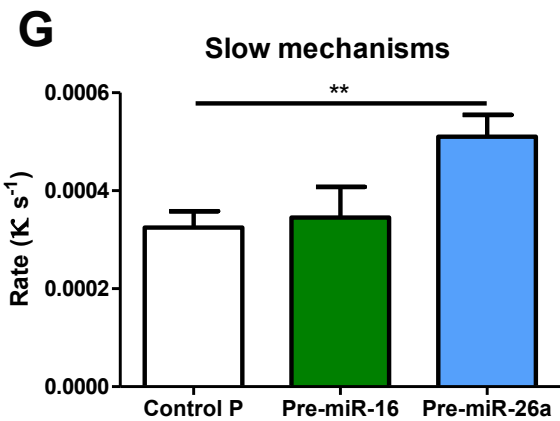
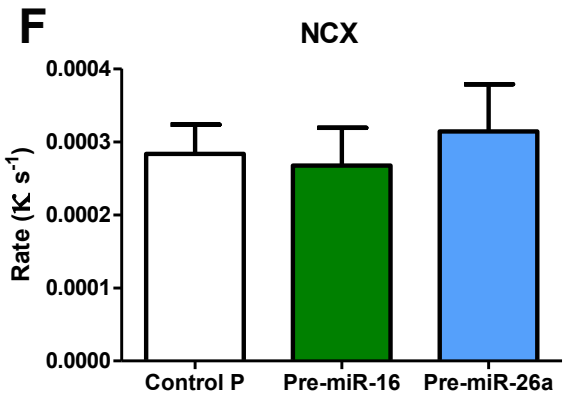
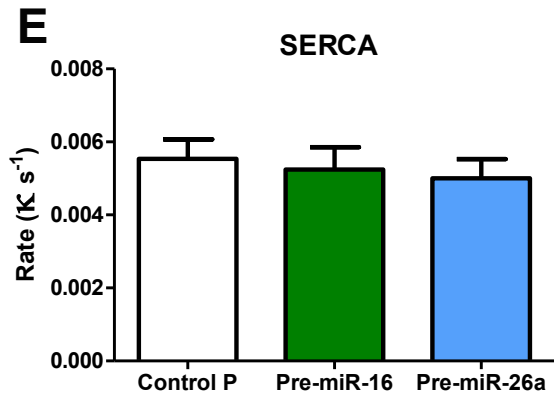
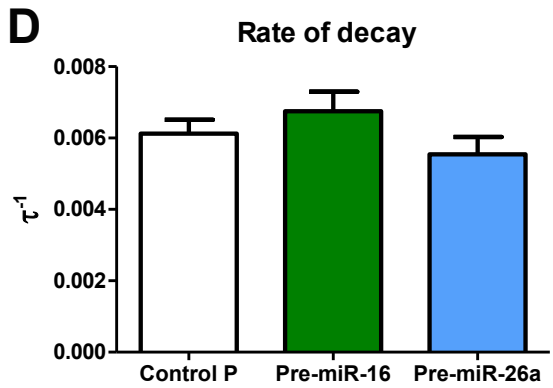
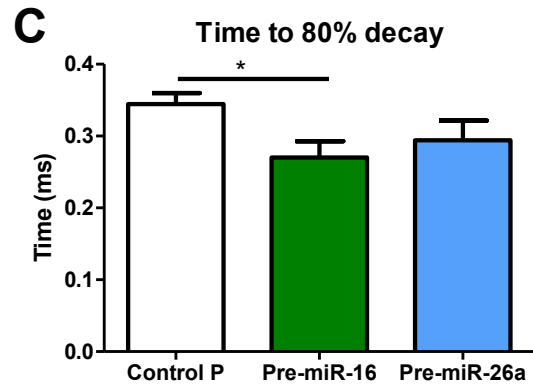
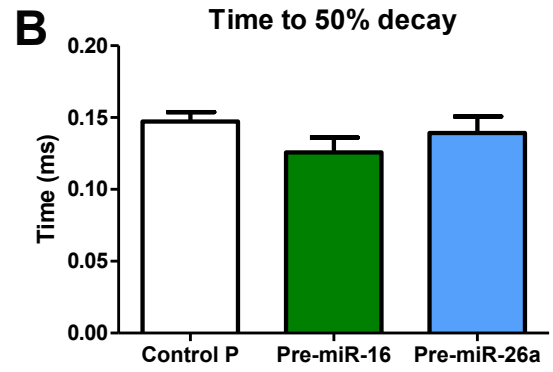
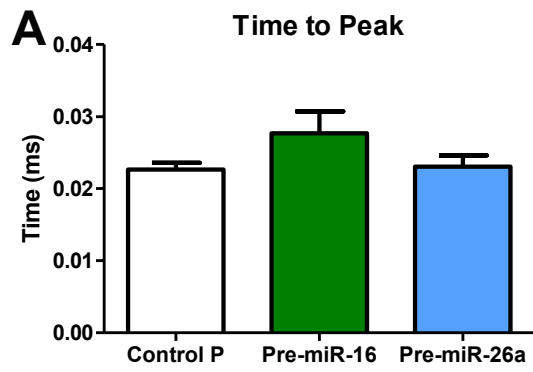


Figure 3.10: miR-16 and miR-26a do not change time to peak or time to 50% decay, and do not alter rate of SERCA or NCX

Epi-fluorescence of miR-16 and miR-26a transfected adult rat apical cardiomyocytes. **A** Time to peak of transfected cardiomyocytes (control $p = 66/4$, pre-miR-26 = $32/4$ and pre-miR-26a = $23/4$). **B** Time to 50% decay of calcium transient from peak (control $p = 66/4$, pre-miR-26 = $31/4$ and pre-miR-26a = $23/4$). **C** Time to 80% decay of calcium transient from peak (control $p = 66/4$, pre-miR-26 = $31/4$ and pre-miR-26a = $23/4$). **D** Rate of calcium transient decay fitted with a mono-exponential function (control $p = 64/4$, pre-miR-26 = $30/4$ and pre-miR-26a = $22/4$). **E** SERCA rate (control $p = 39/4$, pre-miR-26 = $20/4$ and pre-miR-26a = $18/4$). **F** NCX rate (control $p = 24/4$, pre-miR-26 = $13/4$ and pre-miR-26a = $9/4$). **G** Rate of slow mechanisms of calcium transient decay (control $p = 21/4$, pre-miR-26 = $8/4$ and pre-miR-26a = $13/4$). **H** Contribution of SERCA, NCX and slow mechanism rates to overall calcium transient decay. Data is displayed as mean $\pm$ SEM, with significance determined by one-way ANOVA using Tukey's post-hoc test and shown as follows: * = $p < 0.05$, ** = $p < 0.01$.

3.3.7 miR-16 and miR-26a do not change metabolic profiles of adult rat apical cardiomyocytes

To further understand the effect of miR-16 and miR-26a on cardiomyocyte function, we analysed their effect on the metabolic parameters of the cardiomyocyte. This was done using the Seahorse XFp Extracellular Flux Analyzer (Seahorse Biosciences, USA) as described in Section 2.3.5. Figure 3.11A shows a representative image of the protocol used, with each component of mitochondrial respiration highlighted. In short, basal respiration was first measured, before application of oligomycin to inhibit ATP synthase to enable steady state ATP production to be calculated. FCCP was then added to disrupt the mitochondrial membrane and disperse the proton gradient, driving oxidative phosphorylation to maximum capacity. Finally, antimycin A and rotenone were applied to inhibit electron transport chain to completely inhibit oxidative phosphorylation and obtain measures for non-mitochondrial respiration. Values of oxygen consumption rate (OCR) for basal respiration, ATP production, proton leak, maximal respiratory capacity, spare respiratory capacity and non-mitochondrial respiration were obtained from this protocol. miR-16 and miR-26a did not alter the overall metabolic profile of adult rat apical cardiomyocytes (Figure 3.11B and C) or the specific elements previously described that are detailed in Figure 3.11A (Figure 3.11D and E).

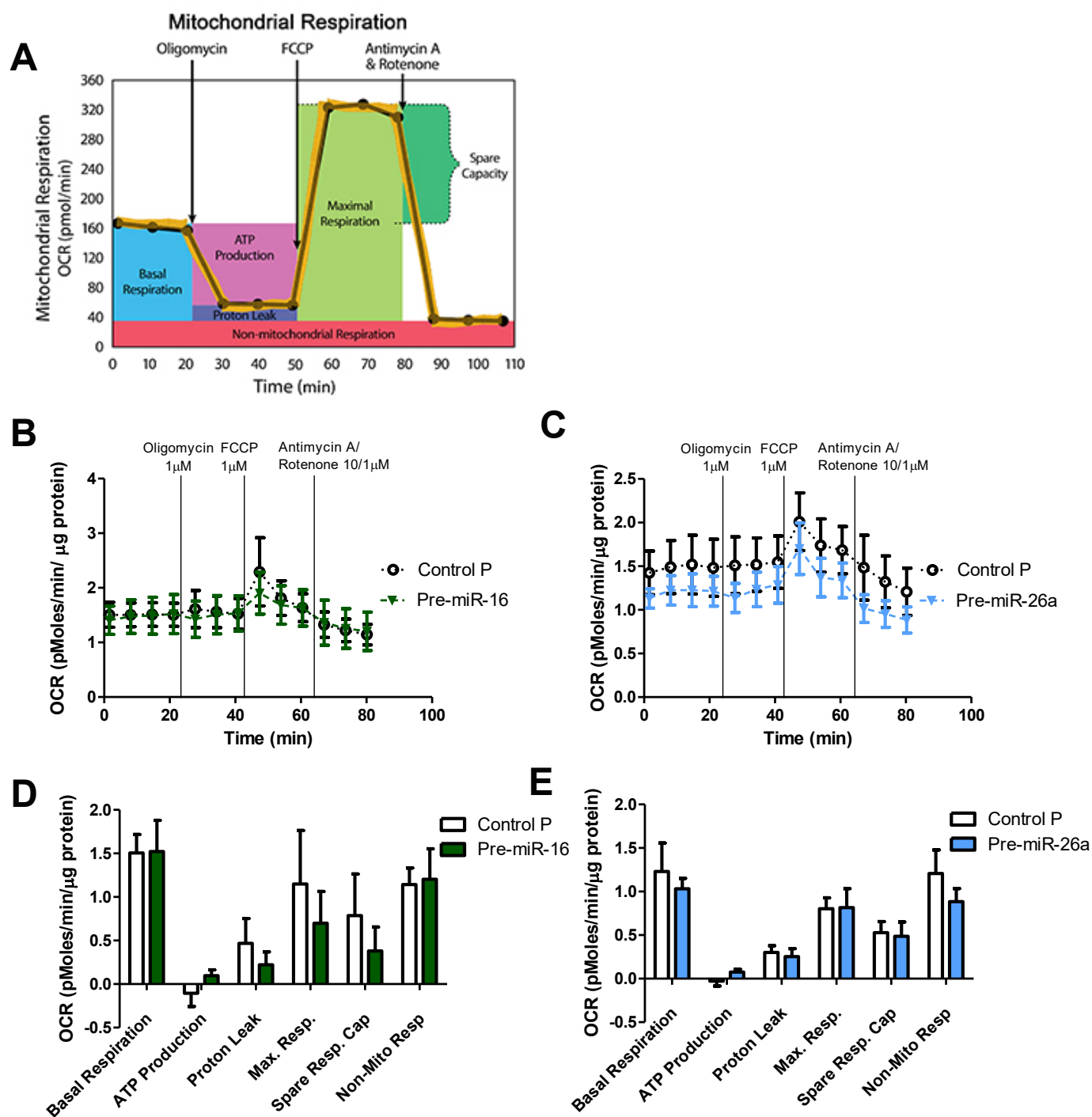


Figure 3.11: miR-16 and miR-26a do not alter metabolic parameters of adult cardiomyocytes

Mitochondrial stress test of miR-16 and miR-26a transfected adult rat apical cardiomyocytes. **A** A representative time course for the mitochondrial oxidative respiration parameters after application of oligomycin to inhibit ATP synthase, FCCP to disrupt the mitochondrial membrane proton gradient, and antimycin A and rotenone to inhibit electron transport chain. **B** Mitochondrial stress test for miR-16 (N=6). **C**

Mitochondrial stress test for miR-26a (N=8). **D** and **E** Calculated values for basal respiration, ATP production, proton leak, maximum respiratory capacity, spare respiratory capacity and non-mitochondrial respiration for miR-16 (N=6) and miR-26a (N=8) respectively. Data is displayed as mean $\pm$ SEM, with differences compared using Student's t-test.

3.4 Discussion

Given that the precise mechanism of TTS is widely debated, and the predisposition of post-menopausal women is unknown (Lyon *et al.*, 2016), novel mechanistic data is required to clarify this issue. miR-16 and miR-26a represent key diagnostic biomarkers that are not only increased in TTS patients (Jaguszewski *et al.*, 2014). We hypothesised that these miRs could be pre-existing factors in this patient population predisposing towards TTS.

The data presented in this chapter demonstrates that TTS-associated miR-16 and miR-26a *in vitro* recapitulate several characteristic elements present within TTS. Here we show that: (1) miR-16 and miR-26a produce a negative inotropic effect in apical rat cardiomyocytes, although do not synergise in this regard. (2) This effect is apical specific, as the miRs do not alter the baseline contractility of basal cardiomyocytes. (3) Reduction in calcium transient amplitude and SR calcium content occur alongside the changes in baseline contractility, although SERCA and NCX function is unaffected. (4) Changes in metabolism do not underlie the changes in contractility observed. (5) Although the peak β_2 AR response is unaltered, miR-16 and miR-26a together increase maximal response to adrenaline in the base and reduce sensitivity in the apex.

3.4.1 TTS-associated miRs recapitulate the apicobasal gradient in hypokinesis seen in TTS

Apical cardiomyocytes were chosen for the initial miR experiments, as this zone is the characteristically affected region in TTS. Additionally, they have previously been observed to exhibit a greater β_2 AR response than basal cardiomyocytes (Paur *et al.*, 2012).

miR-16 and miR-26a were increased by transfection in isolated apical cardiomyocytes to reflect the changes that may occur in TTS. Anti-miR sequences were also used to investigate

the role these miRs play in the tonic regulation of cardiomyocytes. Figure 3.3 demonstrates that upregulation of miR-16 and miR-26a reduced the baseline contractility in apical cardiomyocytes. Whilst it is unknown if TTS patents have impaired baseline contractility prior to developing the syndrome, this is interesting as recapitulates a key disease element. However, downregulation of TTS-associated miRs had no effect. Alongside the low expression levels of miR-16 and miR-26a in heart tissue (Figure 3.1), this suggests that miR-16 and miR-26a do not play a physiological role in the homeostatic control of contractility in isolated cells, but rather have the potential to dysregulate the system when they are raised.

When identified in TTS in human serum, the originating organ of miR-16 and miR-26a was not clarified, but presumed to be of neurological origin (Jaguszewski *et al.*, 2014).

Bioinformatic analysis showed that miR-16 and miR-26a were lowly expressed in mouse heart as seen in Figure 3.1A and B (Meng *et al.*, 2016), but the same in apical and basal cardiomyocytes (Figure 3.1C and D). miR-16 was most highly expressed in spleen, but also elevated in the gastrointestinal system, kidney, liver, lung and brain (Figure 3.1A). miR-26a was highest in lung and brain, and also raised in kidney (Figure 3.1B). These could contribute to the levels found within isolated serum, but the exact source is unknown.

miR-16 and miR-26a are concomitantly increased in TTS *in vivo*, although co-transfection of both miRs did not produce any additional reduction in baseline contractility, as seen in Figure 3.3E. This suggests that either miR-16 and miR-26a act through a unified mechanism, or the concentrations of miRs used is having a ceiling effect on baseline contractility.

The pathognomonic characteristic in TTS that gives rise to the characteristic echocardiographic appearance is the apicobasal gradient in changes in contractility. The initial experiments were carried out in apical cardiomyocytes, so we compared whether the TTS-associated miRs had the same effect in basal cardiomyocytes, since miRs would be

hypothesised to have the same effect on protein expression regardless of cell origin. As such, we isolated apical and basal cardiomyocytes in a paired fashion, transfected them with miR-16 and miR-26a, and measured changes in baseline contractility concurrently. Whilst apical cardiomyocytes observed the expected reduction in baseline contractility with miR-16 and miR-26a, basal cardiomyocytes did not (Figure 3.5). Indeed, there was a significant difference between the contractility of apical and basal miR transfected cardiomyocytes, and no difference between miR-16 and miR-26a basal cardiomyocytes from control transfected cardiomyocytes. This is a particularly pertinent observation and gives strong preliminary evidence that TTS-associated miR-16 and miR-26a could be mechanistically involved in the generation of TTS.

For statistical analysis of the *in vitro* contraction, the n value used for analysis was the number of cardiomyocytes. When treated animals (e.g. heart failure models) are used for studies, data from cardiomyocytes are usually averaged for each animal, and the n value for statistical comparisons is the number of animals. This is done to prevent bias produced by unequal data per animal, for example, large numbers of arrhythmias or calcium sparks from a single animal in a group. For healthy animals, which should be effectively interchangeable, it is acceptable to pool the total cardiomyocyte population, although efforts were made to study approximately equal numbers of cells for each animal. However, to ensure that this was not skewing the analysis, we used an alternative method adapted by our group: hierarchical clustering (Sikkel *et al.*, 2017). This takes account of both between-cardiomyocyte and between-animal variation in a nested analysis. Hierarchical clustering was conducted for the data obtained in Figure 3.3C and D, on the baseline contractility of pre-miR-26a and anti-miR-26a transfected cardiomyocytes. The significance of the difference was increased, rather than reduced, when this was done (Appendix 3).

3.4.2 The reduction in baseline contractility occurs along with a reduction in calcium transient amplitude and SR calcium content

We first investigated whether cardiomyocyte calcium handling was altered using epi-fluorescence to observe calcium transient amplitude and kinetics. This was done alongside caffeine application to potentially open RyR to assess SR calcium content and rate of SERCA, NCX and slow mechanisms of calcium extrusion as described in section 2.5.3.

miR-16 and miR-26a both reduced calcium transient amplitude of adult rat apical cardiomyocytes (Figure 3.9). This change in calcium handling would result in a reduction in cardiomyocyte contractility, and therefore align with the data previously discussed in Section 3.4.1. Although the difference in calcium transient amplitude was modest in comparison to the overall reduction in contractility, it is worth noting that the relationship between calcium transient amplitude and contractility is not linear. There may be other contributing factors to the negative inotropic effect of miR-16 and miR-26a.

A reduction in calcium transient amplitude could result from lower trans-sarcolemmal calcium flux through the LTCC, reduced gain of CICR from lower SR calcium load (Janczewski *et al.*, 1995; Shannon, Ginsburg and Bers, 2000), or reduced RyR open probability (P_o) (Shannon, Ginsburg and Bers, 2000; Bers, 2008). Caffeine-induced calcium transient amplitude was measured to be reduced, and fractional release unchanged (Figure 3.9). This indicates that SR calcium content was reduced, and the ratio between the reduction in calcium transient amplitude and SR calcium content is preserved. Therefore, it is likely that RyR P_o is unchanged, and the reduced calcium transient amplitude is a consequence of the reduced SR calcium load. A reduction in SR calcium content could result from impaired SR calcium reuptake via SERCA or reduced diastolic calcium concentration.

There was no difference in time to peak and time to 50% decay, but there was a significant reduction in time to 80% decay by miR-16 (

Figure 3.10). However, there is no overall change in the rate of decay of the calcium transient, as obtained from the decay constant. Taken together, these findings suggest that changes in calcium transient morphology are minor, although there may be a slight abbreviation of the calcium transient, especially with miR-16. However, this does not align with impairments in SERCA activity that could give rise to the altered SR calcium content.

There are no differences in SERCA or NCX function as calculated from caffeine challenge with miR-16 or miR-26a, but a significant increase in the slow mechanism for calcium extrusion with miR-26a (

Figure 3.10). The slow mechanisms of calcium extrusion are not thought to act on a beat-to-beat basis, but rather buffer calcium over a longer time period (Bers, 2008). It is clear there is no impairment in re-sequestering calcium back into the SR via SERCA. As such, we can deduce the previous reductions in SR calcium content and transient amplitude are not due to impairments in SR calcium reuptake, but instead are more likely to be caused by reduced diastolic calcium, a possible consequence from impaired LTCC function.

A major limitation with the technique used to assess calcium handling lies with the use of Fluo-4. This fluorophore is not ratiometric, and therefore alterations in baseline calcium levels would alter the normalised value for fluorescent amplitude calculated. Resulting from this, absolute conclusions about amplitude or diastolic levels of calcium cannot be drawn. This data requires validation by use of a ratiometric fluorophore such as Fura-2 to obtain absolute values for fluorescence, or patch clamp to directly measure LTCC function.

3.4.3 Metabolic changes of the cardiomyocyte do not explain the changes in contractility observed

The assessment of metabolic function of the cardiomyocytes was carried out by utilisation of the Seahorse XFp Extracellular Flux Analyzer as described in section 3.3.7, which enabled the components of oxidative phosphorylation to be analysed.

miR-16 and miR-26a did not change basal respiratory rate of the transfected cardiomyocytes, or any of the specific components of oxidative phosphorylation previously described in section 3.3.7. Indeed, the maximal respiratory capacity, spare respiratory capacity and non-mitochondrial respiration were unchanged, as were the ATP production and proton leak within the mitochondria (Figure 3.11). From these data, it is possible to conclude that cardiomyocyte energetics are preserved, and therefore do not contribute to the miR induced changes in baseline contractility observed.

It must be noted that even after normalisation, this technique is extremely variable, and consequently a larger N number was required to make robust conclusions. Furthermore, the modest changes in respiratory profile observed following application of oligomycin, FCCP and antimycin A and rotenone reflect data in the literature where adult cardiomyocytes exhibit only small changes when using this system (Yancey *et al.*, 2015). However, comparative changes, albeit minor, could still be reliably measured.

3.4.4 miR-16 and miR-26a alter the β AR response of basal, but not apical cardiomyocytes

Given the differential response to adrenaline observed in the apex and base in TTS, β AR activity was measured. This was done by conducting an adrenaline concentration-response curve, ranging from 3×10^{-10} M to 3×10^{-6} M.

In the base, we observed a raised maximal response with miR-26a alone (Figure 3.6), and miR-16 and miR-26a in combination (Figure 3.7). It was unclear whether the increase in maximal response to adrenaline with miR-26a was a real increase as the entire adrenaline concentration-response curve was displaced vertically. In combination, miR-16 and miR-26a together demonstrated that the increase in response to adrenaline was genuine since maximal response was significantly increased in the absence of any differences in baseline contraction. This mirrors the changes that occur in TTS, with amplification of the hypercontractility observed in the base. This could result from amplified G α s signalling or reduced adrenergic desensitisation.

In the apex, miR-16 and miR-26a alone produced no changes in response to adrenaline (Figure 3.6), although in combination profoundly reduced sensitivity to adrenaline as demonstrated by a significant difference in the logEC₅₀ (Figure 3.7). The reduction in sensitivity in the apex required synergism of the two miRs to occur, suggesting multiple points of action within the same pathway are required. This could also be explained by altered G-protein signalling, with a reduction in sensitivity to adrenaline a possible consequence from increased G α i activity. Taken together, these reproduce key differences in apicobasal response to adrenaline as seen in TTS and provide an important insight into the mechanism of action of TTS-associated miRs.

Paur et al. demonstrated the key involvement of the β_2 AR in the mechanistic generation of TTS (Paur *et al.*, 2012). Indeed, they showed the importance of β_2 AR-G α i stimulus trafficking in inducing the hypokinetic changes in TTS. This resulted in the regional apical reduction in contractility due to the increased density of β AR and magnified β_2 AR response in the zone (Paur *et al.*, 2012). Given this data, the effect of miR-16 or miR-26a on cardiomyocyte β_2 AR response was investigated.

miR-16 and miR-26a did not change the peak contractility observed after induction of β_2 AR response, or the fold or absolute change in contractility from baseline (Figure 3.8). The peak contractility of the β_2 AR response indicates the maximal response following canonical G α s stimulation. This suggests that β_2 AR-G α s activity is unchanged subsequent to miR-16 and miR-26a transfection.

An important limitation to note is the culture induced changes that occur with the β_2 AR function. Chakir et al. identified that RGS2, as a key terminator of β_2 AR-G α i signalling, was increased in the absence of agonist stimulation that occurs with time in culture (Chakir *et al.*, 2011). This results in a larger β_2 AR response as β_2 AR-G α i is inhibited. PTX pre-treatment usually increases the β_2 AR response approximately five fold by inhibiting G α i (Paur *et al.*, 2012), although after culture this was not the case with amplified β_2 AR response and no further increase following PTX pre-treatment (Appendix 2). Therefore, culture significantly altered the β_2 AR response making it hard to draw specific conclusions about β_2 AR-G α i.

3.4.5 Summary of the effect of miR-16 and miR-26a in adult rat cardiomyocytes *in vitro*

In synthesising the experiments conducted in this chapter, it can be noted that miR-16 and miR-26a specifically reduce baseline contractility of apical but not basal cardiomyocytes. This occurs along with a modest reduction in calcium transient amplitude and SR calcium content. Whilst baseline contractility was preserved in basal cardiomyocytes, these exhibited a magnified response to adrenaline *versus* control, and apical cardiomyocytes had reduced sensitivity to adrenaline following miR-16 and miR-26a in combination. Together, these recapitulate key findings present within TTS, and consequently provide strong evidence for the mechanistic involvement of miR-16 and miR-26a in TTS.

4 Comparison of miR effect in an *in vitro* human cardiomyocyte model

4.1 Introduction

The findings in Chapter 3 represent promising mechanistic data for the involvement of miR-16 and miR-26a in TTS, however despite this data being obtained in adult cardiomyocytes, these were isolated from rats. Numerous interspecies differences occur in cardiomyocyte physiology, including in phosphodiesterase activity (Johnson *et al.*, 2012), calcium handling and SR calcium dependency (Bassani, Bassani and Bers, 1994), force-frequency relationships (Janssen and Periasamy, 2007), and post-rest potentiation and myofilament sensitivity (Bers, 1989). As a result, the effects of TTS-associated miR-16 and miR-26 require validation in an *in vitro* human cardiomyocyte model.

Human primary adult cardiomyocytes from cardiomyopathy or heart failure patients are readily available from myectomy samples or explanted whole heart preparations after donor heart transplantation. Cardiomyocytes can be isolated from these tissues, although isolated cardiomyocyte function represents that of the originating diseased myocardium. Indeed, failing human cardiomyocytes lose their positive force-frequency relationship (Davies *et al.*, 1995; Janssen and Periasamy, 2007), have mutated contractile proteins altering calcium sensitivity in hypertrophic cardiomyopathy and uncoupling response to adrenergic stimulation in dilated cardiomyopathy (Marston, 2011), and failing cardiomyocytes have reduced sensitivity to catecholamines (Harding *et al.*, 1992) and have higher Gai activity (Neumann *et al.*, 1988). In addition, cardiomyocyte miR profile is known to be altered in cardiac disease states such as heart failure (Kumarswamy *et al.*, 2012). Consequently, this model was deemed inappropriate for use in this context, and whilst a scarce resource, the

primary cardiomyocytes chosen for use were limited to healthy hearts that could not be used for transplantation.

As an additional resource, iPSC-CM were used, as these have recently become increasingly used for cardiac disease modelling (Burridge *et al.*, 2016). Whilst these do exhibit an immature cardiomyocyte phenotype (Kane, Couch and Terracciano, 2015; Louch, Koivumäki and Tavi, 2015), recently a TTS specific iPSC-CM patient line has been produced that supports the involvement of the β AR in TTS, with increased β_1 AR, β_2 AR and cAMP responses, increased calcium transients and kinetics, increased intracellular lipid accumulation and reduced mitochondrial function. Engineered Heart Tissue (EHT), 3-dimensional, force-generating strips of iPSC-CMs (Schaaf *et al.*, 2011; Mannhardt *et al.*, 2016), made from these TTS iPSC-CMs had reduced baseline force generation compared with controls, higher sensitivity to catecholamines and reduced desensitisation following 24 hours of catecholamines (Borchert *et al.*, 2017). Therefore, it was assessed whether TTS-associated miRs could affect iPSC-CM function similarly to in Chapter 3, and whether the findings from Borchert *et al.* could be explained by alterations in TTS-associated miRs.

4.2 Materials and methods

Specific details for the methods used in this chapter are detailed in Chapter 2.

4.2.1 Culture of primary human cardiomyocytes

Cardiomyocytes were isolated from donor heart tissue as described in Section 2.3.2. They were left to settle to remove the enzyme buffer without centrifugation and changed into defined culture media described in Section 0. Cardiomyocytes were transfected for 48 hours with Lipofectamine 3000 as described in Section 2.6.1 with Pre-miR-negative control #2, Pre-miR-16 and Pre-miR-26a at 100nM.

4.2.2 Culture of iPSC-CM

30,000 of our in house made IMR-90 iPSC-CM (Section 2.4.1) were plated in a glass-bottomed Mattek dish and cultured in RB+ for 48 to 72 hours before transfection over 48 hours with Lipofectamine 3000 as described in Section 2.6.1 with Pre-miR-negative control #2, Pre-miR-16, Pre-miR-26a, Anti-miR-negative control #1, Anti-miR-16 and Anti-miR-26a at 100nM.

Transfection was verified before commencing experiments, showing Lipofectamine 3000 was not toxic, and efficiency of transfection determined by Cy3™ Dye-Labeled Pre-miR Negative Control #1 (ThermoFisher) was found to be $68\pm 7\%$ total cells (Figure 4.1).

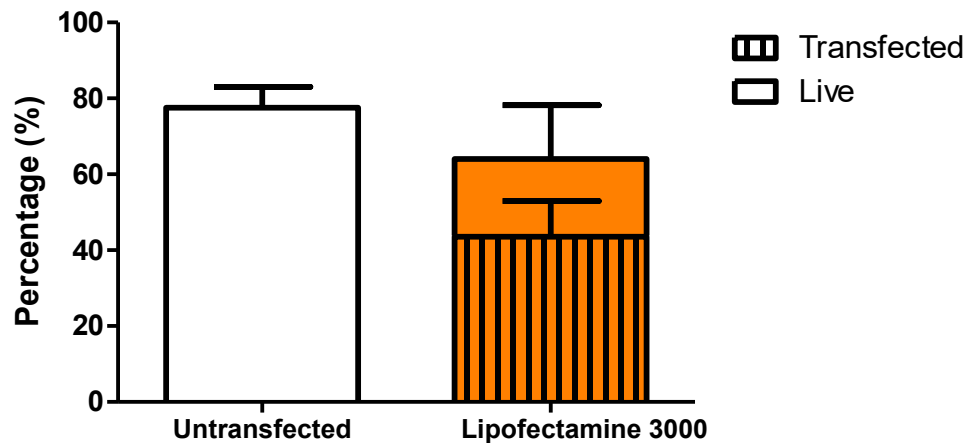


Figure 4.1: Transfection efficiency of Lipofectamine 3000 in iPSC-CM

Tested transfection efficiency of induced pluripotent stem cell-derived cardiomyocytes with the proportion of live transfected cells illustrated.

4.2.3 Ionoptix MCSYS

The Ionoptix MCSYS was used as described in Section 2.3.3 to measure percentage fractional shortening of healthy human adult cardiomyocytes and beating rate of iPSC-CMs. Baseline fractional shortening was measured for human cardiomyocytes, whereas baseline beating rate, as well as β_2 AR response (as described in Section 2.5.1) was measured in iPSC-CM.

4.2.4 Epi-fluorescence

Epi-fluorescence was conducted on iPSC-CM for calcium with Fluo-4 AM (Section 2.4.5), and membrane potential with FluoVolt (Section 2.4.6). Fluo-4 AM was loaded at $4\mu\text{M}$ for 20 minutes (37°C) in DMEM before being unloaded for 20 minutes. Four areas were measured per dish, attempted at 1 and 2 Hz stimulation, and spatially averaged. Calcium transients were analysed using a script for MATLAB R2017a to obtain calcium transient amplitude, time to

calcium transient peak, and time to 50% and 80% decay. FluoVolt was used for action potential measurement, where four areas were similarly measured per dish, attempted at 1 and 2 Hz stimulation, and spatially averaged respectively. These were then manually analysed using PClamp 10 software to obtain values for the rise time, and action potential duration to 50% and 90% repolarisation.

4.2.5 Quantification of miR expression

RNA was kindly isolated and provided by Borchert et al. by using a Qiagen miRNeasy kit. RTqPCR was conducted in my time in Hannover as described in Section 2.6.3. Specifically, the RT was carried out using TaqMan® MicroRNA Reverse Transcription Kit and RTqPCR conducted using Absolute Blue qPCR Mix 2x (ThermoFisher Scientific) master mix and ROX Reference Dye (ThermoFisher Scientific, 1:50). Primers for RT and PCR were Taqman primers for RNU6B, hsa-miR-16 and hsa-miR-26a. Measurements were taken using a ViiA7 384 PCR machine (Applied Biosystems), and analysed using the comparative cycle threshold (CT) method (ΔC_t).

4.2.6 Statistics

Data is displayed as mean $\pm$ SEM, and significance calculated with Student's t-test for the comparison of two groups, or one-way ANOVA with Tukey's post-hoc test for comparison of more than two groups. Significance is shown as appropriate as follows: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

4.3 Results

4.3.1 miR-16 reduces contractility in healthy human adult cardiomyocytes

To validate the findings from Figure 3.3 in a human model, where miR-16 and miR-26a reduced baseline contractility of adult rat apical cardiomyocytes, human donor hearts that could not be transplanted were utilised. Here, in healthy human adult apical cardiomyocytes, miR-16 significantly reduced baseline contractility, and miR-26a verged on a significant reduction (Figure 4.2). This reflects the changes that occur in adult apical rat cardiomyocytes *in vitro* and validates the use of a rat model.

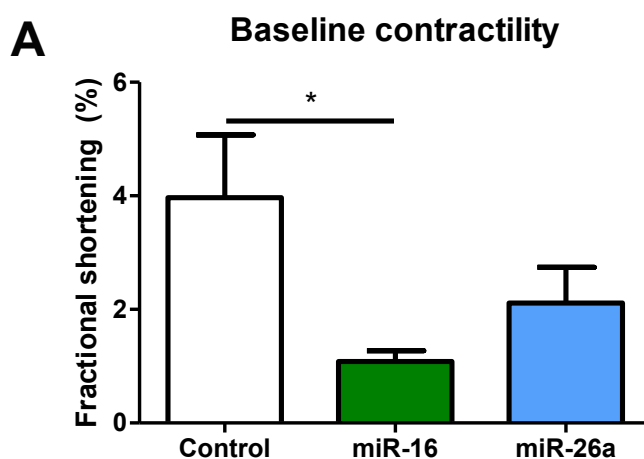


Figure 4.2: Baseline contractility is reduced in healthy human cardiomyocytes by miR-16

A Percentage fractional shortening at baseline of miR-16 and miR-26a transfected healthy human adult apical cardiomyocytes (n/N=5/1). Data is displayed as mean $\pm$ SEM, with significance determined by one-way ANOVA with subsequent Tukey's used for post-hoc analysis and shown as * = $p < 0.05$, using the number of cells for the purposes of statistical comparison.

4.3.2 miR-16 and miR-26a do not change baseline beating rate in induced pluripotent stem cell-derived cardiomyocytes

Due to the lack of availability of primary healthy human adult cardiomyocytes, all subsequent experimentation in this chapter was done on human iPSC-CM. Sherin Saheera conducted the experiments shown in Figure 4.3 and Figure 4.4 alongside myself. Given that contractility of iPSC-CM cannot be readily measured, beating rate was used as an indirect surrogate, as this is heavily influenced by G α i activity in this cell type as discussed in Section 1.1.2 (Fu *et al.*, 2006). Figure 4.3A to D show that beating rate of iPSC-CM was unchanged with transfection of miR-16 and miR-26a precursor sequences, as well as their respective anti-miR oligonucleotides.

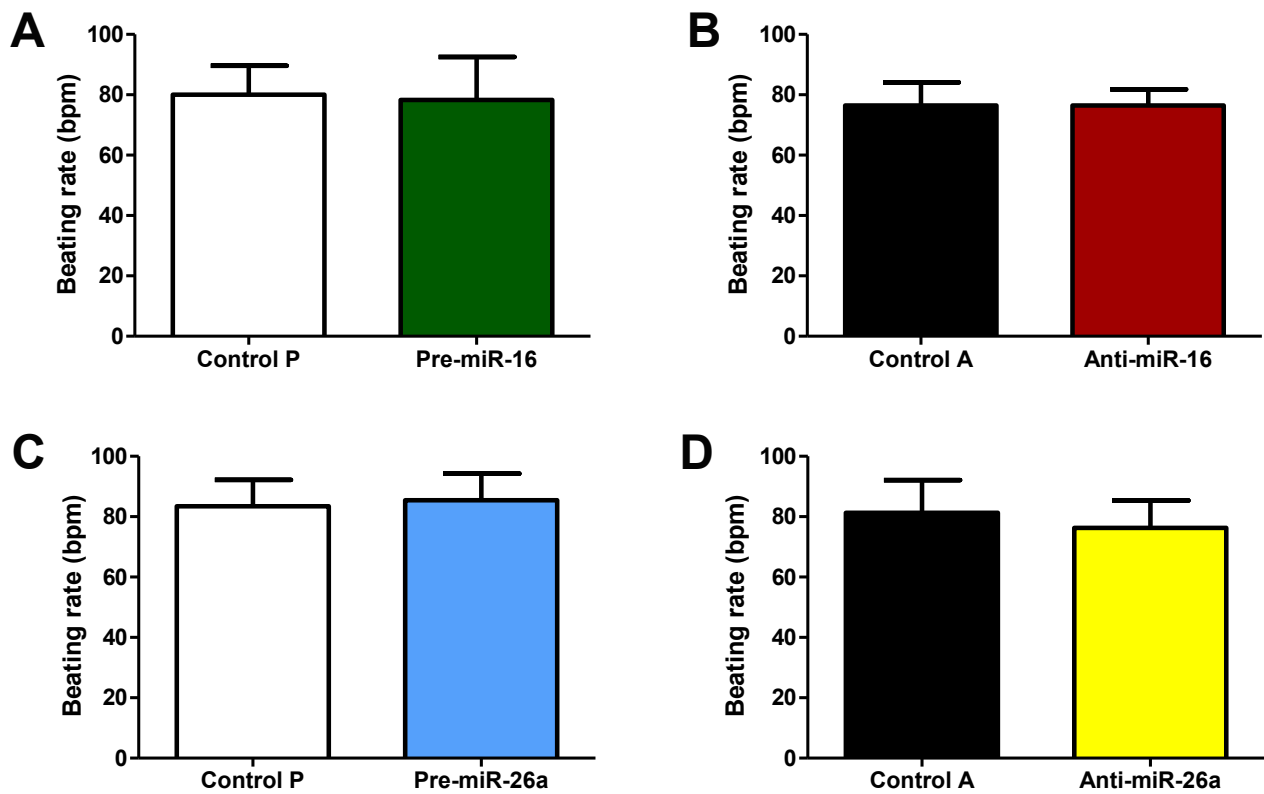


Figure 4.3: Up- or downregulating miR-16 and miR-26a does not affect beating rate of human iPSC-CM

Baseline beating rate of iPSC-CMs with upregulation of miR-16 (A, N=7) and miR-26a (C, N=7), and downregulation of miR-16 (B, N=7) and miR-26a (D, N=7). ‘N’ represents number of iPSC-CM batches measured. Data is displayed as mean $\pm$ SEM, with Student’s t-test carried out for statistical comparisons.

4.3.3 miR-16 and miR-26a do not alter β_2 AR response of human iPSC-CM

Following assessment of baseline beating rate, changes in beating rate were measured following induction of a β_2 AR response as described in Section 2.5.1. As shown in Figure 4.4A to D, miR-16 and miR-26a upregulation and downregulation had no effect on the profile of the β_2 AR response. Indeed, the peak beating rate observed within 5 minutes, which is indicative of maximum $G_{\alpha s}$ activity, is unchanged, as is the reduction in beating rate following this, which represents the desensitisation of the β_2 AR response.

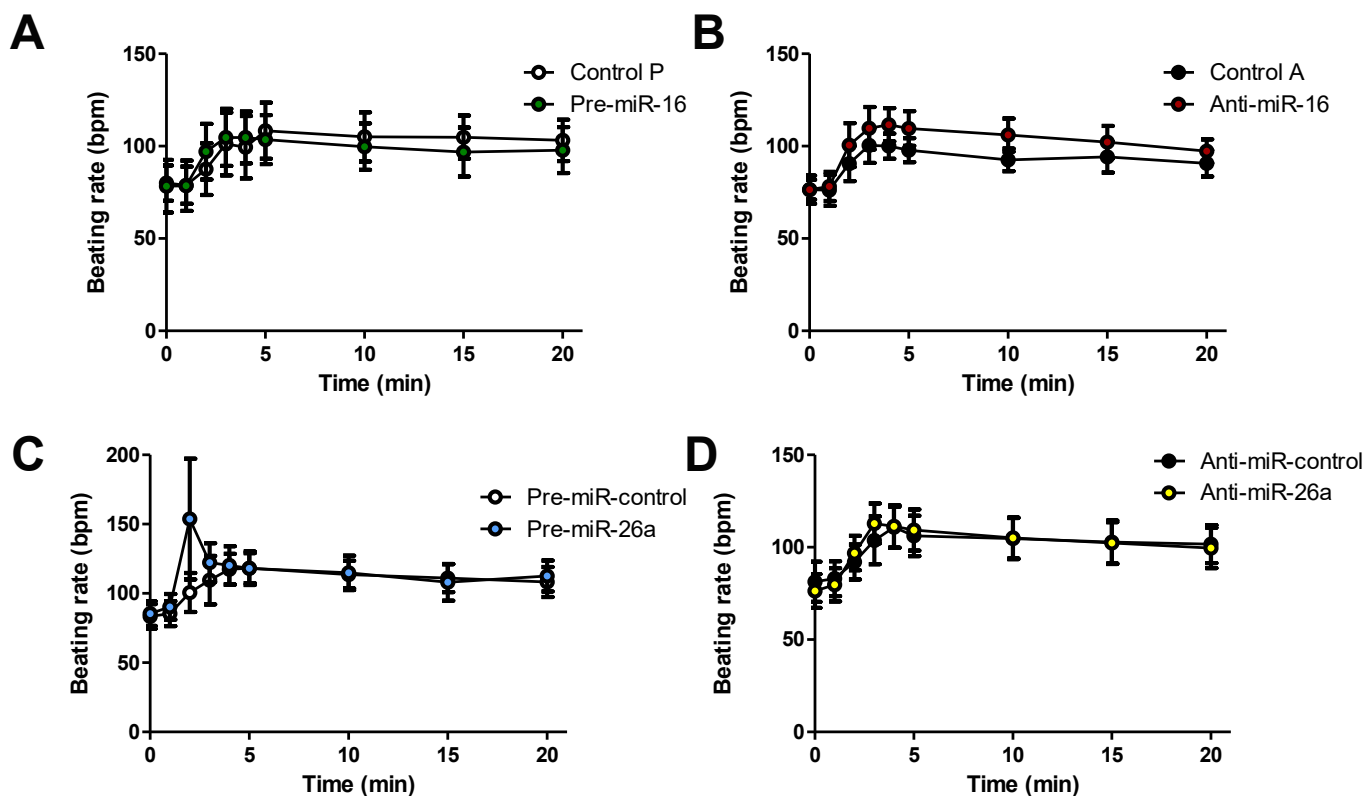


Figure 4.4: miR-16 and miR-26a do not alter β_2 AR response of human iPSC-CM

Beating rate after induction of β_2 AR response obtained by β_1 AR with CGP 20712A and applying the β AR agonist isoprenaline in iPSC-CMs with upregulation of miR-16 (A, N=7) and miR-26a (C, N=7), and downregulation of miR-16 (B, N=7) and miR-26a (D, N=7). 'N' represents number of iPSC-CM batches measured. Data is displayed as mean $\pm$ SEM. Data was compared for significance with RM-ANOVA.

4.3.4 miR-16 and miR-26a do not alter calcium handling of human iPSC-CM

Following assessment of baseline beating rate and β_2 AR response, calcium handling of iPSC-CMs was assessed, since calcium transient amplitude was reduced by miR-16 and miR-26a in adult rat apical cardiomyocytes in Figure 3.9. Laura Wienecke, a medical student from Hannover Medical School, conducted the experiments shown in Figure 4.5 and Figure 4.6. Calcium transient amplitude is unchanged by miR-16 and miR-26a upregulation or downregulation at both 1 and 2 Hz (Figure 4.5A and B). Indeed, this was also the case for time to calcium transient peak (Figure 4.5C and D), time to 50% decay (Figure 4.5E and F) and time to 80% decay (Figure 4.5G and H).

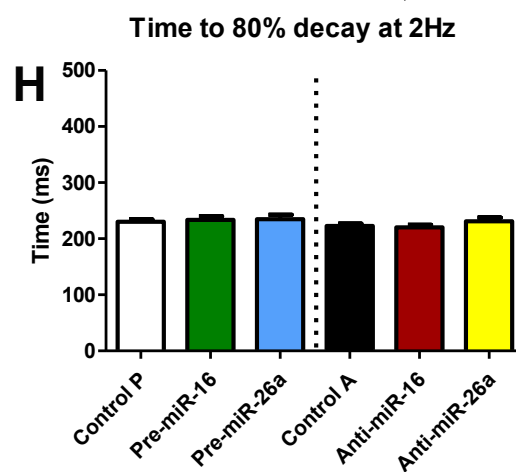
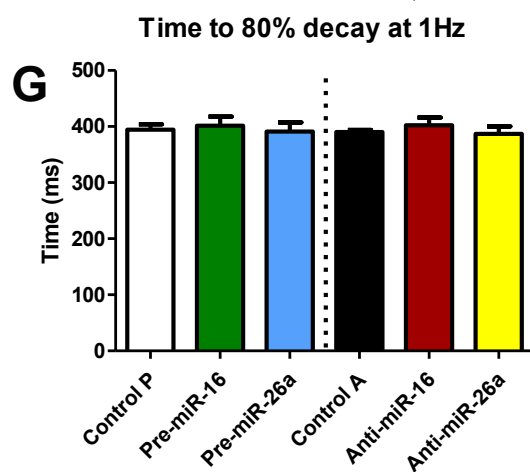
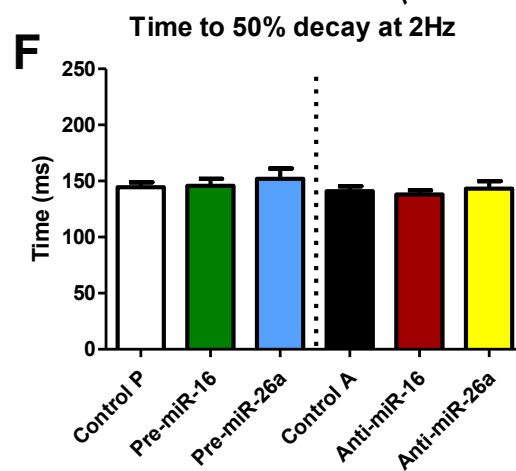
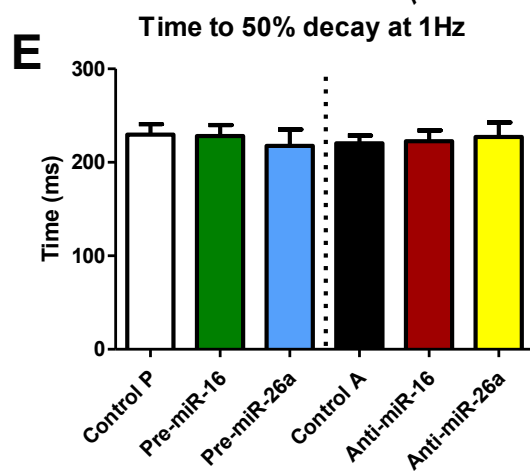
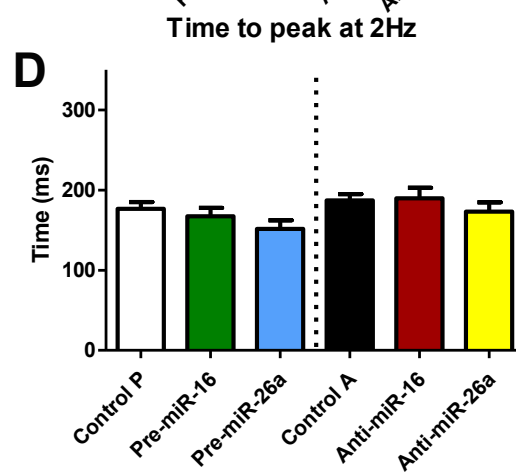
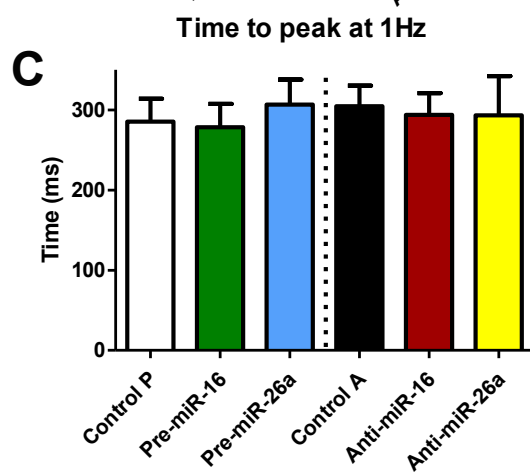
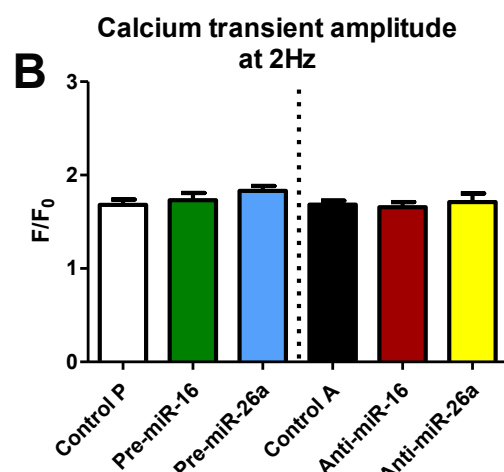
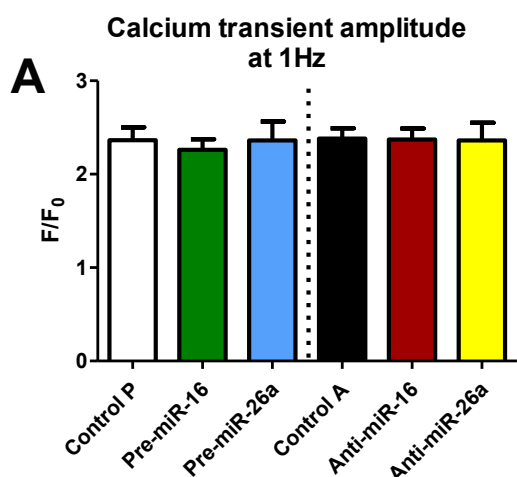


Figure 4.5: miR-16 or miR-26a do not alter iPSC-CM calcium handling

Calcium transient epi-fluorescence with upregulation and downregulation of miR-16 and miR-26a in iPSC-CMs. **A** and **B** Amplitude at 1 and 2 Hz. **C** and **D** Time to calcium transient peak at 1 and 2 Hz. **E** and **F** Time to 50% decay at 1 and 2 Hz. **G** and **H** Time to 80% decay at 1 and 2 Hz. For 1 Hz (**A**, **C**, **E** and **G**) Control P n/N = 10/6, Pre-miR-16 n/N = 6/6, Pre-miR-26a n/N = 5/5, Control A n/N = 11/6, Anti-miR-16 n/N = 6/6, Anti-miR-26a n/N = 4/4. At 2 Hz (**B**, **D**, **F** and **H**) Control P n/N = 13/7, Pre-miR-16 n/N = 7/7, Pre-miR-26a n/N = 7/7, Control A n/N = 13/7, Anti-miR-16 n/N = 7/7, Anti-miR-26a n/N = 5/5. N numbers shown as n/N where 'n' equals the number of dishes and 'N' represents number of iPSC-CM batches measured. Data is displayed as mean $\pm$ SEM and compared with one-way ANOVA using the number of iPSC-CM dishes for statistical comparison.

4.3.5 miR-16 and miR-26a do not alter action potential of human iPSC-CM

Alongside calcium handling, action potential morphology of miR treated iPSC-CMs was also assessed. This cannot be routinely investigated with fluorophores in adult cardiomyocyte, so was of interest to conduct here. Whilst this experiment has only been completed to a low N number, action potential time course was unaltered by increased or reduced miR-16 or miR-26a at 1 or 2 Hz. Indeed, action potential rise time was unchanged (Figure 4.6A and B), as was time to 50% (Figure 4.6C and D) and 90% repolarisation (Figure 4.6E and F).

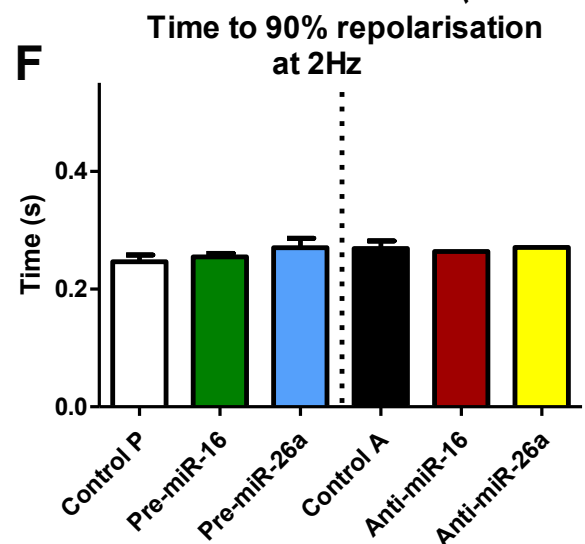
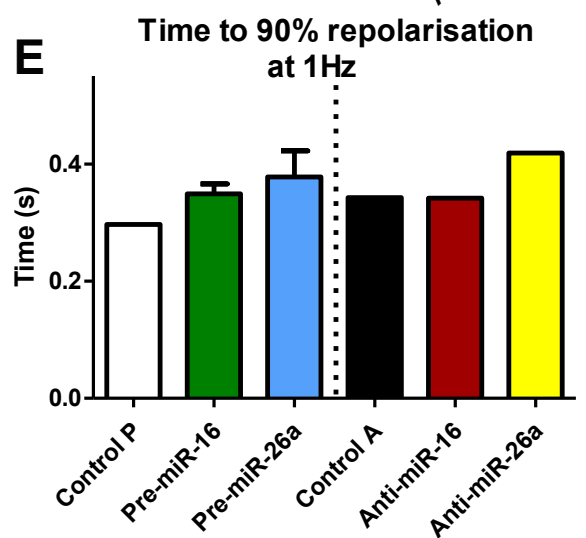
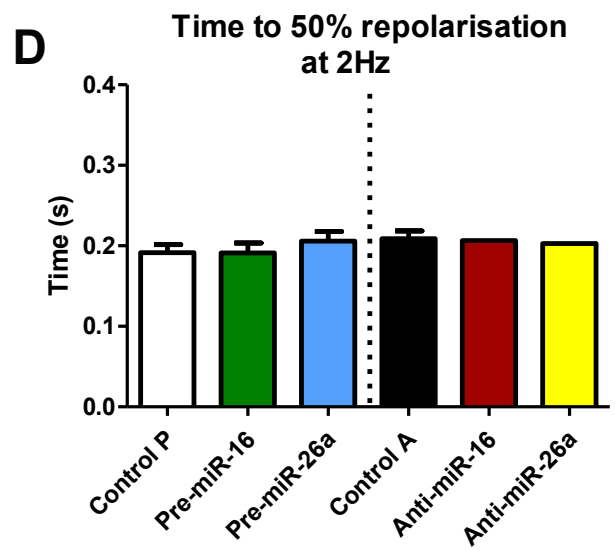
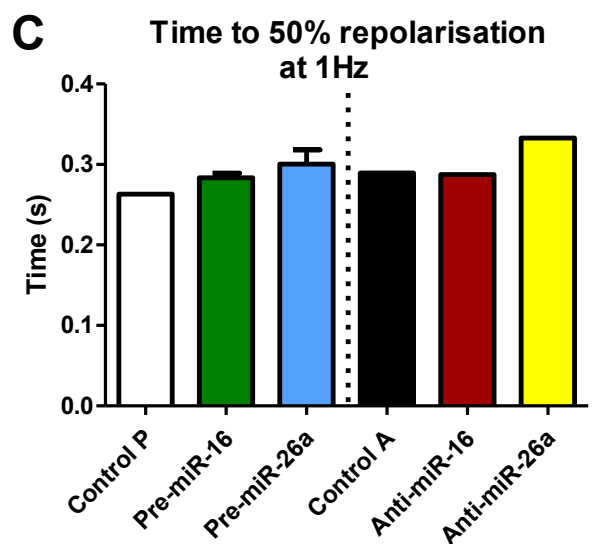
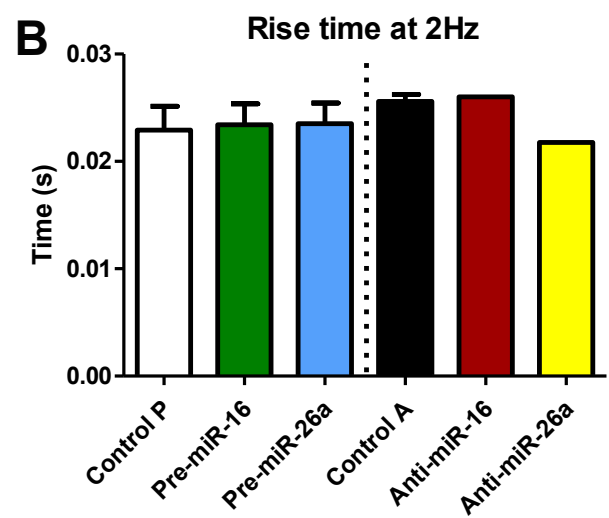
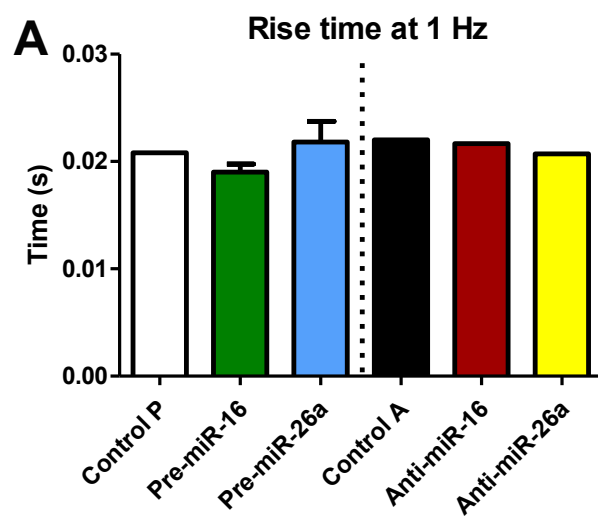


Figure 4.6: miR-16 and miR-26a do not change action potential morphology of iPSC-CM

Membrane potential epi-fluorescence with upregulation and downregulation of miR-16 and miR-26a in iPSC-CMs. **A** and **B** Rise time of action potentials at 1 and 2 Hz. **C** and **D** Time to 50% repolarisation of action potentials at 1 and 2 Hz. **E** and **F** Time to 90% repolarisation of action potentials at 1 and 2 Hz. For 1 Hz (**A**, **C**, **E** and **G**) Control P n/N = 2/2, Pre-miR-16 n/N = 3/3, Pre-miR-26a n/N = 3/3, Control A n/N = 2/2, Anti-miR-16 n/N = 2/2, Anti-miR-26a n/N = 2/2. At 2 Hz (**B**, **D**, **F** and **H**) Control P n/N = 3/3, Pre-miR-16 n/N = 3/3, Pre-miR-26a n/N = 3/3, Control A n/N = 3/3, Anti-miR-16 n/N = 2/2, Anti-miR-26a n/N = 2/2. N numbers shown as n/N where 'n' equals the number of dishes and 'N' represents number of iPSC-CM batches measured. Data is displayed as mean $\pm$ SEM and compared with one-way ANOVA using the number of iPSC-CM dishes for statistical comparison.

4.3.6 Expression of miR-16 and miR-26a are unchanged in an iPSC-CM TTS patient line

Finally, as the findings by Borchert et al. from the iPSC-CM TTS patient lines failed to delineate a mechanism for their findings, we investigated whether miR-16 or miR-26a were altered in this cell line. They kindly provided RNA from these cells to enable this comparison to be made. However, both miR-16 and miR-26a were unchanged in the TTS iPSC-CM line *versus* the control line used by Borchert et al. (Figure 4.7A and B).

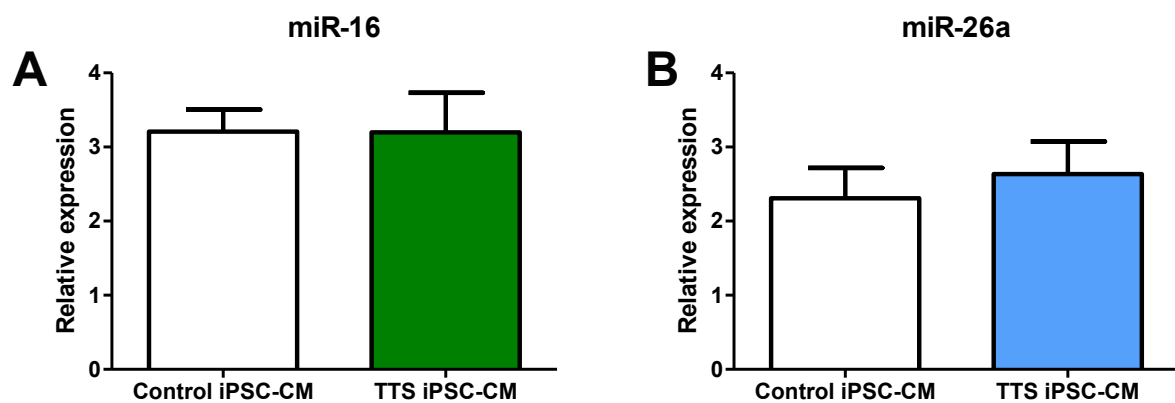


Figure 4.7: Expression levels of miR-16 and miR-26a are unchanged in a TTS iPSC-CM patient line

miR-16 (A, N=4) and miR-26a (B, N=4) expression in TTS iPSC-CM. Data is displayed as mean $\pm$ SEM, with Student's t-test carried out for statistical comparison.

4.4 Discussion

Whilst the findings from Chapter 3 demonstrate that in adult rat cardiomyocytes, miR-16 and miR-26a recapitulate several key features present in TTS, the numerous inter-species differences previously discussed (Bers, 1989; Bassani, Bassani and Bers, 1994; Janssen and Periasamy, 2007; Johnson *et al.*, 2012) demonstrate the importance of validating these findings in a human cardiomyocyte model.

The data presented in this Chapter show that (1) healthy human adult cardiomyocytes experience a reduction in baseline contractility with miR-16 and possibly miR-26a, (2) baseline beating rate, β_2 AR response, calcium handling and action potential of iPSC-CM are unchanged following modulation of miR-16 or miR-26a, and (3) miR-16 and miR-26a are unchanged in the TTS iPSC-CM patient line produced by Borchert *et al.*

4.4.1 Effect of TTS-associated miRs in rat are validated in healthy human adult cardiomyocytes

Given that miR-16 significantly reduced baseline contractility in healthy human adult apical cardiomyocytes, and miR-26a trended towards a reduction (Figure 4.2), showing that TTS-associated miRs produce similar findings to those found in Chapter 3 in a human model. This provides validation that rat is an appropriate model for the investigation into the effects of miR-16 and miR-26a, despite the inter-species differences previously mentioned.

Whilst healthy human adult cardiomyocytes are the ideal model, the rarity of this tissue makes experimentation extremely difficult. Indeed, in this experiment, five cardiomyocytes were measured per condition from one donor heart. To date, we have been fortunate enough to receive three healthy hearts, however, as the culture of these cardiomyocytes required optimisation and the quality of the tissue following cardiopulmonary resuscitation was

compromised, this system was only successfully implemented once. This will be continued whenever possible, however recapitulation of the main finding from Chapter 3 provides supporting evidence for the remaining data despite the low N number used.

The use of failing human cardiomyocytes was carefully considered, as this is a more available, albeit still rare and precious, resource. As previously discussed, as these are from patients with end-stage heart failure, they exist in a pathological state wherein many physiological parameters are altered. Indeed, failing human cardiomyocytes lose their positive force-frequency relationship (Davies *et al.*, 1995; Janssen and Periasamy, 2007), undergo many electrophysiological changes with action potential prolongation (Nass *et al.*, 2008), have reduced sensitivity to catecholamines (Harding *et al.*, 1992) and have higher Gai activity (Neumann *et al.*, 1988). In addition, many sarcomeric proteins such as myosin binding protein C and titin may be mutated in primary cardiomyopathies altering calcium sensitivity in hypertrophic cardiomyopathy and uncoupling response to adrenergic stimulation in dilated cardiomyopathy (Marston, 2011). miR profile also is perturbed in heart failure, with reduced levels of miR-1 in heart failure (Kumarswamy *et al.*, 2012). As a result, cardiomyocytes from failing hearts were not used, as the findings would not be translatable to a normal physiological state and may confound further miR modulation.

4.4.2 iPSC-CM as a model for the investigation of miR-16 and miR-26a

Due to the difficulties obtaining human cardiomyocytes for experimentation previously discussed, iPSC-CM were used as a readily available human cardiomyocyte model. However, miR-16 or miR-26a did not produce any differences in baseline beating rate (Figure 4.3), β_2 AR response (Figure 4.4), calcium handling (Figure 4.5) or action potential (

Figure 4.6) in iPSC-CM. Given that calcium transient amplitude was altered in rat adult apical cardiomyocytes (Figure 3.9), it can be concluded that TTS-associated miRs were not reproducing their effects in iPSC-CMs.

It should be noted that beating rate is not the same as contractility, although this parameter cannot be easily measured in iPSC-CM. Real force generation requires EHTs to be made, and these require a large cell number and cannot be transfected with liposomal transfection methods used elsewhere. However, as no alterations in beating rate or electrophysiological properties were seen with miR-16 or miR-26a, EHTs were not utilised.

Whilst it was important to carry out these experiments on iPSC-CM, they were likely an inappropriate model for the investigation of TTS-associated miRs in a human model. iPSC-CM are an extremely immature form of cardiomyocyte, being compared to the level of development of a mid-gestational cardiomyocyte (Kane, Couch and Terracciano, 2015; Louch, Koivumäki and Tavi, 2015). iPSC-CMs spontaneously beat, have disorganised sarcomeres, lack key electrophysiological currents and exhibit very limited polarisation towards chamber sub-type (Kane and Terracciano, 2017). The effects of miR-16 and miR-26a were seen to be extremely specific in Chapter 3, differentially affecting apical and basal cardiomyocytes. In this regard, iPSC-CM are unlikely to be an appropriate model, as lack key properties of ventricular cardiomyocytes, being more generic, unlikely the highly functionally adapted apical and basal adult cardiomyocytes (Wright *et al.*, 2018).

4.4.3 TTS-associated miRs are not changed in aTTS patient iPSC-CM line

A TTS specific iPSC-CM patient line has recently been produced by Borchert et al. that provides mechanistic insight into how cardiomyocytes from TTS patients are differently regulated to healthy control. TTS iPSC-CMs exhibited increased β_1 AR, β_2 AR and cAMP responses, a finding which supports the mechanistic involvement of the β AR in the

generation of TTS. Additionally, calcium transient amplitude and kinetics were increased, and increased intracellular lipid accumulation and reduced mitochondrial area was seen on confocal microscopy. This suggests there is metabolic dysfunction in TTS iPSC-CMs, an interesting finding considering the increased lipid droplet accumulation observed in endomyocardial biopsy of TTS patients. EHTs made from TTS iPSC-CMs had reduced baseline force generation compared with controls, higher sensitivity to catecholamines with reduced desensitisation following 24 hours of treatment (Borchert *et al.*, 2017). However, the investigators failed to identify any specific mutations to explain these changes, despite whole exome sequencing. Therefore, epigenetic or non-coding RNA post-transcriptional differences could exist in these patients, not least because the originating patient cells were exposed to extremely high catecholamine concentrations in TTS.

Particularly when considering the reduction in baseline force production of EHTs and increased β AR response, as we observe similar findings in apical and basal cardiomyocytes respectively with miR-16 and miR-26a, we wanted to assess whether these could be differentially expressed in TTS iPSC-CMs. However, once isolating RNA and conducting RTqPCR, miR-16 and miR-26a were not changed in these cells suggesting that whilst miR-16 and miR-26a cause similar changes, they are not the only factors necessary for predisposing the adrenergic system towards TTS generation.

4.4.4 Summary of the effect of TTS-associated miRs in a human model

Whilst there was no effect of miR-16 or miR-26a on iPSC-CM, this chapter has validated the action of TTS-associated miRs in rat cardiomyocytes, since healthy human adult cardiomyocytes also experience a reduction in baseline contractility. This supports the data gained in Chapter 3, strengthening the evidence for the mechanistic involvement of miR-16 and miR-26a in the generation of TTS.

5 Translation into a preclinical *in vivo* rat model of takotsubo syndrome with miR upregulation utilising adeno-associated virus

5.1 Introduction

In vivo models of TTS have been used to understand disease mechanisms that underpin TTS, and subsequently to assess the efficacy of possible treatment options available. Two key models involve systemic administration of catecholamines, specifically adrenaline administered via the external jugular vein (Paur *et al.*, 2012) and intraperitoneal injection of isoprenaline (Shao, Redfors, *et al.*, 2013). These have been particularly insightful due to the lack of understanding of the pathogenesis of TTS, and the paucity of patient tissue samples.

Specifically, these preclinical models of TTS have elucidated the importance of catecholamines in the induction of TTS, the mechanistic role of β_2 AR-Gi signalling, and consequently the cardioprotective effect that this is associated with (Paur *et al.*, 2012; Shao, Redfors, Scharin Täng, *et al.*, 2013). Indeed, they have also provided the appreciation that TTS is a multifactorial disease involving whole-body responses to stress, and multiple disease mechanisms that include direct myocardial catecholamine stunning, altered metabolism and subsequent inflammatory changes (Paur *et al.*, 2012; Shao, Redfors, *et al.*, 2013; Shao, Redfors, *et al.*, 2013; Surikow *et al.*, 2018).

Consequently, the application of miRs in an *in vivo* model of TTS constitutes an important step to translate the *in vitro* data collected within Chapter 3 in a clinically applicable *in vivo* model of TTS. This is of particular importance given the need to contextualise the *in vitro* miR experiments conducted thus far, as although they recapitulate several key TTS disease elements, this lacks the complexity of whole-organism physiology, and the TTS disease

setting. Therefore, upregulation of miR-16 and miR-26a was required *in vivo*, with subsequent induction of TTS to specifically investigate whether they have a mechanistic role in the predisposition to, or exacerbation of TTS.

5.2 Methods

The methods detailed here are described in full in Chapter 2.

5.2.1 Echocardiographic imaging

Adult male Sprague-Dawley rats were anaesthetised with isoflurane (Section 2.7.1) before having their thorax shaved and depilated (Section 2.7.2). Ultrasound gel was applied to the chest, and the echocardiographic probe positioned to reveal an on-axis parasternal long-axis view of the LV and fixed in place. A B-mode image, along with M-mode images at base, mid-LV and apex were taken at baseline and every 5 minutes during the TTS protocol (Section 2.7.3). These areas were defined as the lower third, midpoint and upper third respectively.

During this procedure, heart rate was sampled using the heated ultrasound table described in Section 2.7.2.

5.2.2 Intravenous administration of catecholamines to induce TTS

Following the establishment of a satisfactory echocardiographic view of the LV at baseline, the echocardiography probe was displaced vertically, and a superficial vertical incision was made superior to the mid-point of the left clavicle using surgical scissors (Section 2.7.3), and surrounding subcutaneous tissue blunt dissected away to reveal the external jugular vein. This was cannulated using a 26G cannula. Once venous access had been established, the cannula was flushed with sterile PBS and sealed.

Baseline echocardiographic images were taken, before injection of the adrenaline bolus over approximately 10 seconds. For induction of TTS in Section 5.3.1, 55µg/kg adrenaline was injected, whereas this was titrated down to 18µg/kg when TTS was induced in the AAV

treated animals (Section 5.3.10), since the former dose resulted in increased mortality as rats were older and heavier, with increased fat content.

5.2.3 Sampling of blood

When the external jugular vein was cannulated, blood could be sampled. For experimentation within this Chapter, blood was sampled at baseline, as well as at 20 minutes and 60 minutes post-adrenaline administration (Section 2.7.5). The blood was allowed to remain at room temperature for up to an hour or stored on ice for longer time periods in a Vacutainer SST Advance blood tube, before serum was isolated as per the protocol outlined by Qiagen in the miRNeasy Serum/Plasma isolation kit in Section 2.6.2 (Qiagen, 2012).

5.2.4 RTqPCR

RNA was isolated using the Qiagen miRNeasy Serum/Plasma isolation kit (Qiagen, 2012) using cel-miR-39 as a spike-in control prior to RNA isolation, since no reliable endogenous control exists for miRs in blood. RNA concentration was quantified using NanoDrop 2000 and 10ng RNA used in a RT reaction using the TaqMan® MicroRNA Reverse Transcription Kit with specific primers for cel-miR-39, hsa-miR-16 and hsa-miR-26a.

RTqPCR was then carried out the protocol in TaqMan® Small RNA Assays (Applied Biosystems, 2011) using TaqMan® Universal PCR Master Mix II, with UNG and primers for cel-miR-39, hsa-miR-16 and hsa-miR-26a. Expression was quantified using the Δ CT method. This process is described in full in Section 2.6.3.

5.2.5 AAV infection

miR-16 and miR-26a upregulation *in vivo* was carried out using AAV9. This contained a generalised EF1 α promoter which drove an mCherry reporter. Primary sequences for miR-16

and miR-26a were located in the intronic regions of the EF1 α promoter, since this is common in miR biogenesis (Ha and Kim, 2014). This yielded a control virus that was AAV9-EF1 α -mCherry and a miR virus which was AAV9- EF1 α -pri-miR-16+pri-miR-26a-mCherry (Figure 5.1). These are referred to AAV-control and AAV-miR throughout this Chapter.

These were administered to rats via tail vein injection (Section 2.7.4). Male Sprague-dawley rats weighing between 75g and 150g were placed into a heated chamber for 10 minutes to vasodilate peripheral vessels. They were lightly and briefly anaesthetised using the induction protocol described in section 2.7.1. Tails were washed in warm water to clean the tail and rats placed on a heated mat maintain vasodilation. They were wiped with ethanol wipes for sterilisation prior to injection. 300 μ L AAV-control or AAV-miR at specified doses were injected using a 30G needle. An initial dose response was carried out using a maximum of 5×10^{12} gene copies (gc). Following this, a final 50% dose of 2.5×10^{12} gc was selected, and 10 rats infected with each virus.

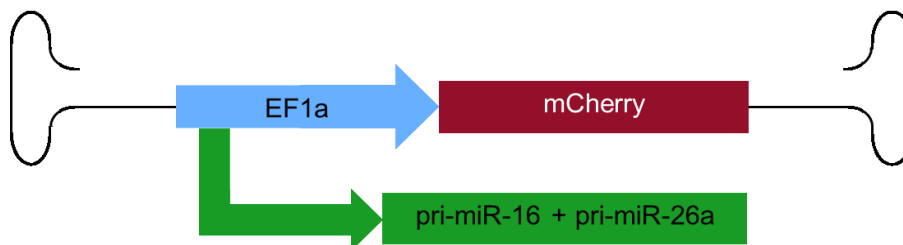


Figure 5.1: AAV9 design

Schematic representation of AAV9- EF1 α -pri-miR-16+pri-miR-26a-mCherry. EF1 α promoter is shown in blue, the mCherry reporter in red, and primary-miR-16 and primary-miR-26a in green within the intronic regions of the promoter.

5.2.6 Removal of organs

After the echocardiographic procedure was completed, the rats were terminated by non-Schedule 1 method under anaesthesia by removal of organs. Specifically, the heart was excised under deep anaesthesia (Section 2.7.2). The heart was promptly washed in ice-cold PBS, before being divided into apical and basal samples which were split for freezing in liquid nitrogen for RNA or protein isolation or fixed in PFA for fluorescent imaging of mCherry.

Brains were also removed following excision of the hearts. This was done by decapitation of the cadaver and removal of skin from the dorsal and posterior aspects of the skull. Scissors were carefully fed up through the foramen magnum and the skull cut on either side dorsally and laterally. This was continued until just above the junction of the zygomatic arch to yield a flap of bone that could be removed near the junction of sutures that form the bregma point. The brain was carefully removed using the flat edge of the scissors. The brain was divided and frozen in liquid nitrogen for subsequent RNA and protein isolation.

5.2.7 Widefield fluorescent microscopy

Once fixed in PFA as described in Section 2.6.5, samples were cryosectioned in OCT, mounted in Hard-set Vectashield and imaged on a Zeiss AxioObserver widefield microscope. Images were analysed using Image J.

5.2.8 Monitoring of AAV treated rats

After consulting a veterinarian from Imperial CBS, a behavioural monitoring plan was devised. The rat grimace scale (Sotocina *et al.*, 2011) was used to monitor whether the AAV treated rats experienced any pain daily during the 6-week monitoring period. The activity level of the rats was evaluated with the help of Hannah Jones, an animal technician in CBS,

to grade the average activity level of a cage of animals treated with either AAV-control or AAV-miR. Low, medium and high levels of activity were assigned values of 0, 1 and 2.

AAV treated rats were weighed at baseline and then weekly using a bench-top set of digital scales within the CBS animal facility. This enabled the growth of AAV treated rats to be monitored and compared.

5.2.9 Statistics

Data is displayed throughout as mean $\pm$ SEM. To compare the relative changes between two data sets, Student's t-tests was used. Where there were more than two statistical comparisons to be made, one-way analysis of variance (ANOVA) was used with a post-hoc Tukey's test.

When comparing two groups with time, repeated measures ANOVA was conducted with subsequent Bonferroni's post-hoc test. Individual time-points were compared to baseline using a hypothetical value of zero. Level of significance is presented throughout as follows:

*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$.

5.3 Results

5.3.1 Takotsubo syndrome was successfully recapitulated *in vivo*

Based on our acute catecholamine-induced TTS model developed by Paur et al. (Paur *et al.*, 2012), we re-established this to reliably induce TTS for downstream testing of the effect of TTS-associated miRs. Figure 5.2A, B and C show the response to adrenaline at apex, mid-left ventricle (mid-LV) and base respectively, injected via the external jugular vein. This was measured from baseline, 'B', and every 5 minutes after adrenaline injection for an hour. In all three zones, there was a significant increase in contractility at the 5-minute timepoint, indicative of the expected positive inotropic effect of adrenaline. There was a profound reduction in contractility in the apex from 15 minutes onwards, that is statistically significant between 15 and 45 minutes, and recovered between 50 to 60 minutes (Figure 5.2A). In the mid-LV, the negative inotropic phase was brief, being significantly reduced at 15, 20 and 30 minutes, and recovered back to level of baseline from 40 minutes onwards (Figure 5.2B). At the base, the hypocontractility was only significant at 15 minutes, and recovered from 20 minutes onwards. There was a trend towards hypercontractility in the base from 45 minutes onwards (Figure 5.2C). A representative M-mode trace is shown in Figure 5.2D, where baseline contractility and contractility after adrenaline administration are shown.

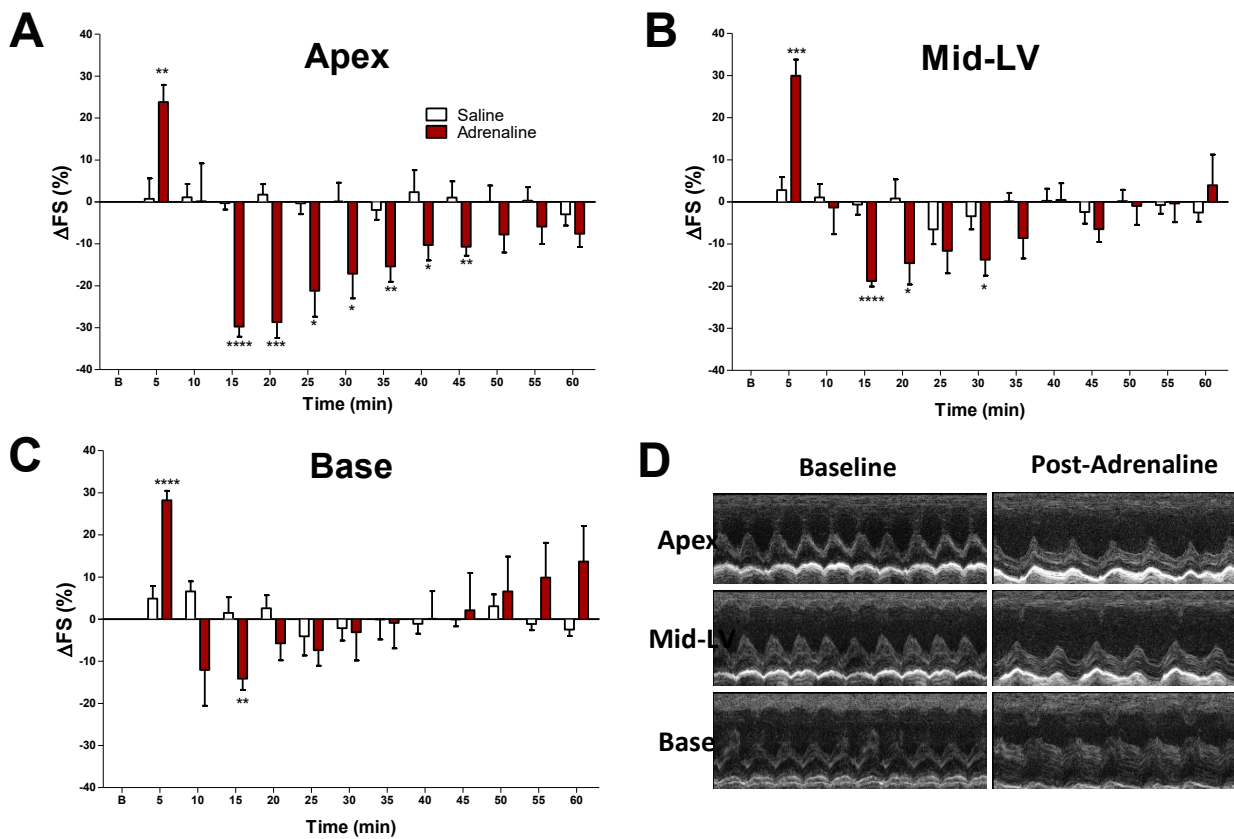


Figure 5.2: TTS was recapitulated *in vivo* by bolus injection of adrenaline

Fractional shortening percentage at baseline and every 5 minutes following for 60 minutes using the intravenous adrenaline model to induce TTS. Fractional shortening was measured at apex (A), mid-LV (B) and base (C) comparing rats treated with adrenaline to saline (N=6). D Representative M-mode traces for apex, mid-LV and base at baseline and following adrenaline administration. Data is displayed as mean $\pm$ SEM, with significance determined by comparing each timepoint to baseline and shown as follows: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

5.3.2 Serum levels of miR-16 and miR-26a were unchanged in this *in vivo* model of TTS

From the catecholamine-induced TTS *in vivo* model established in Figure 5.2, blood was obtained from the external jugular at baseline, and at 20 minutes and 60 minutes after adrenaline administration. miRs were isolated from the obtained serum, and levels of miR-16 and miR-26a quantified by RTqPCR. Figure 5.3A and B show that miR-16 and miR-26a respectively were not changed relative to baseline within an hour following adrenaline administration. Indeed, there is no difference in miRs between saline and adrenaline treated animals, indicating that TTS-associated miRs are not increased acutely by adrenaline, and they are not the direct effectors of the changes in contractility observed. Thus, if they are mechanistically involved in TTS, it would be in the predisposition or exacerbation of the syndrome.

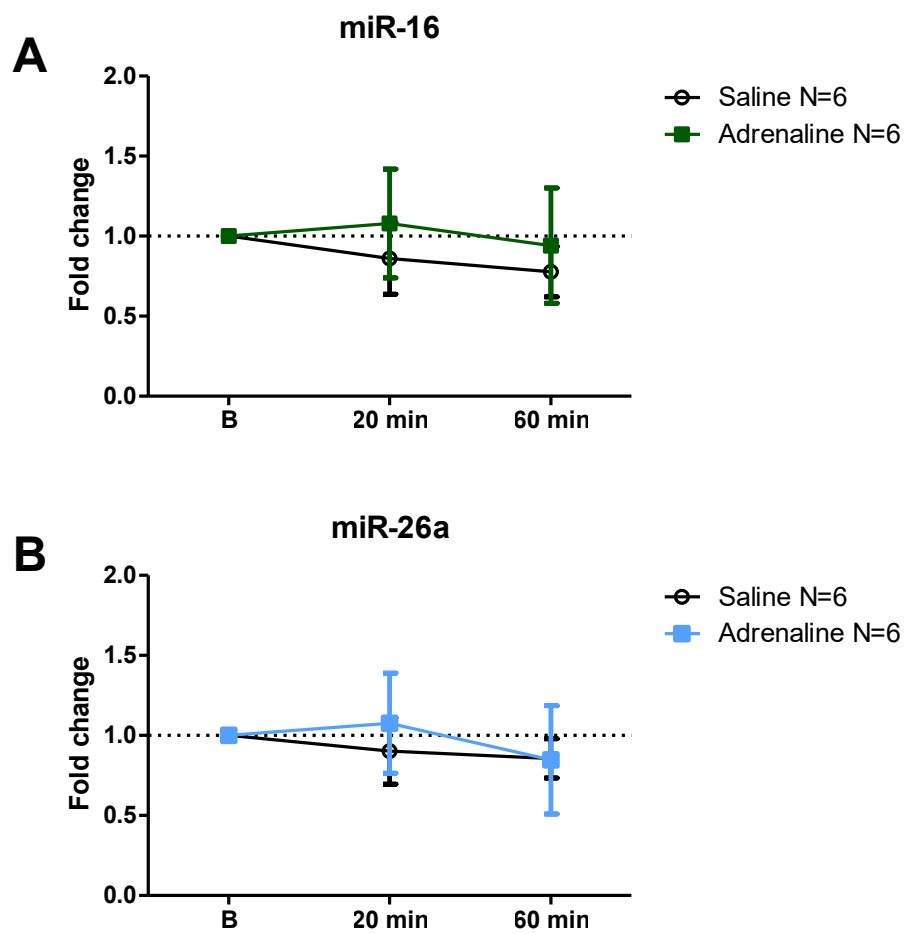


Figure 5.3: Serum levels of miR-16 and miR-26a do not change acutely after intra-venous adrenaline injection

miR-16 (**A**) and miR-26a (**B**) expression levels in rat serum after administration of adrenaline or saline with respect to baseline (N=6). Data is displayed as mean $\pm$ SEM.

5.3.3 Adenovirus associated virus 9 dose optimisation

To test the efficacy of this virus, a dose-response range was established, with 0 gene copies (gc), 6.25×10^{11} gc, 1.25×10^{12} gc, 2.5×10^{12} gc and 5×10^{12} gc being selected, and displayed as percentages of the maximum dose. These doses were made up in 300 μ L sterile PBS, and each dose was administered via tail vein injection (Section 2.7.4) to one animal for AAV-control and AAV-miR six weeks prior to removal of organs. The hearts were divided into apex and base as described in Section 2.7.5, before being fixed in PFA and cryosectioned as described in Section 2.6.5, mounted in Hard-set Vectashield and imaged on a Zeiss AxioObserver widefield microscope. Representative areas for AAV-control and AAV-miR are displayed in Figure 5.4A and B respectively. When averaging the whole heart section, 50% viral dose corresponding to 2.5×10^{12} gc yielded the highest levels of mCherry fluorescence (Figure 5.4C and D). The representative images in Figure 5.4A illustrate that at 12.5% and 25%, relatively few myocytes show an increase in mCherry immunofluorescence, whereas at 50% for AAV-control at apex and base and apex for AAV-miR, there is an almost ubiquitous increase in fluorescence such that the whole myocardium has increased mCherry expression. At 100%, mCherry fluorescence appears to decrease once again, due to a presumed toxic effect of the viral dose.

From the isolated hearts, apical and basal tissue sections were also frozen in liquid nitrogen for miR analysis. RNA was isolated from this tissue and miRs quantified using RTqPCR as described in Section 2.6.2 and Section 2.6.3 respectively. It can be seen in Figure 5.5A and B that there was a trend towards increased miR-16 and miR-26a respectively in heart tissue at the 50% AAV-miR dose only. miRs were also isolated from serum (Section 2.6.2) obtained via the external jugular vein as described in Section 2.7.3. Figure 5.6A and B shows the miR-16 and miR-26a serum levels respectively, with a trend towards increased expression

between 12.5% and 50% for AAV-control, and 100% for AAV-miR. This data was extremely variable, and likely reflected the low N number.

The fluorescent imaging, along with the tissue miR expression was used to select the 50% dose to yield a final dose of 2.5×10^{12} gc to be used for subsequent experimentation. It must be noted that this dose did appear to not increase fluorescence in the basal myocardium for AAV-miR. Due to the significant expense of the AAV, this dose-response range was only completed to N=1 per condition, and thus data obtained was preliminary and only used for dose ranging.

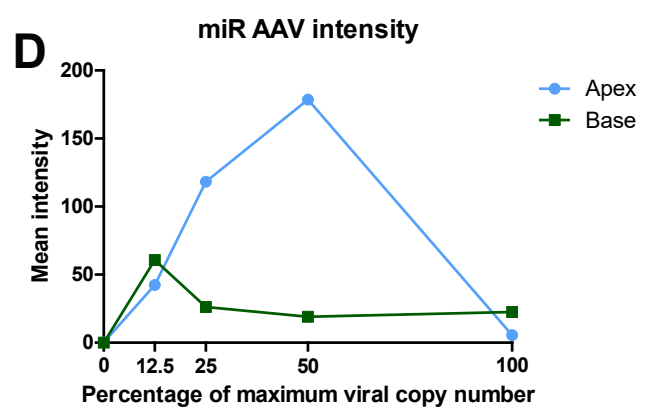
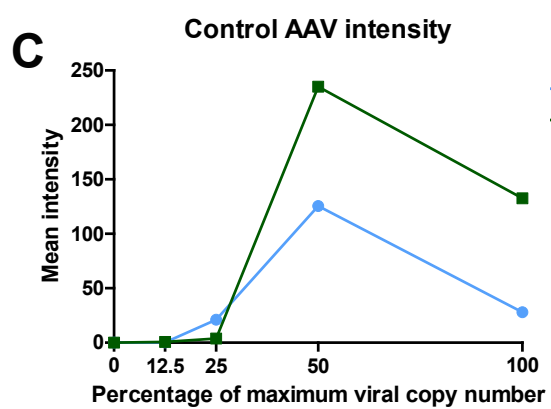
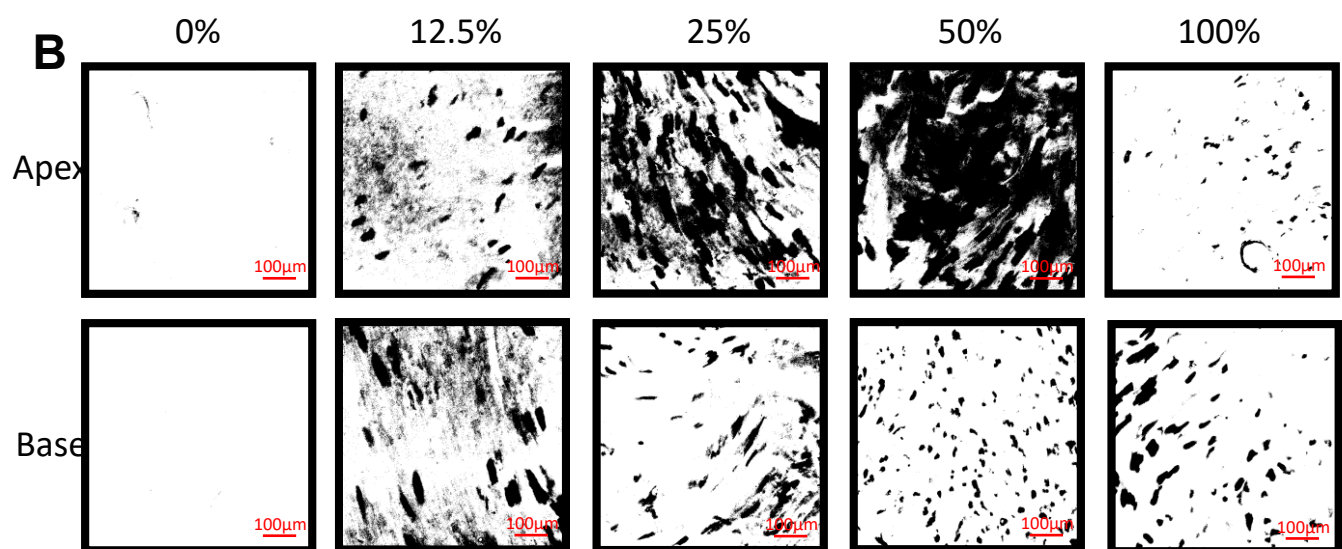
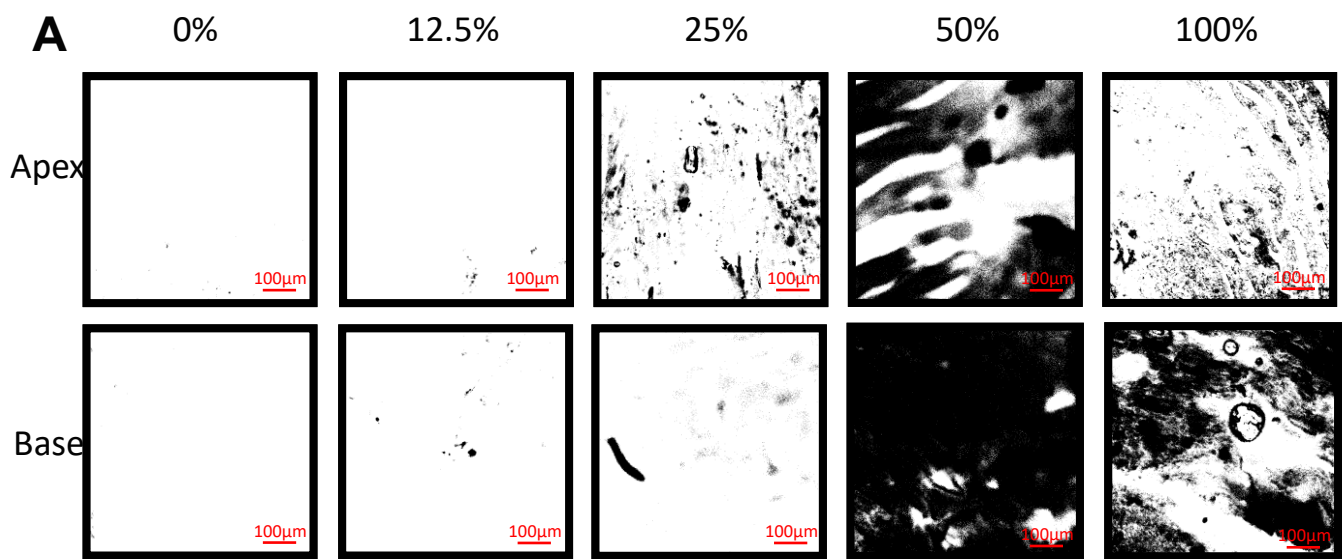


Figure 5.4: mCherry expression via AAV9 infection is expressed 6 weeks after tail vein injection

Fluorescent widefield assessment of the myocardium showing representative images for AAV-control (**A**) and AAV-miR (**B**), where black represents an increase in mCherry fluorescence. The quantified mean fluorescence was calculated from the average fluorescence of 3 randomly selected areas (**C** and **D** respectively). N=1 throughout, data shown as individual data points.

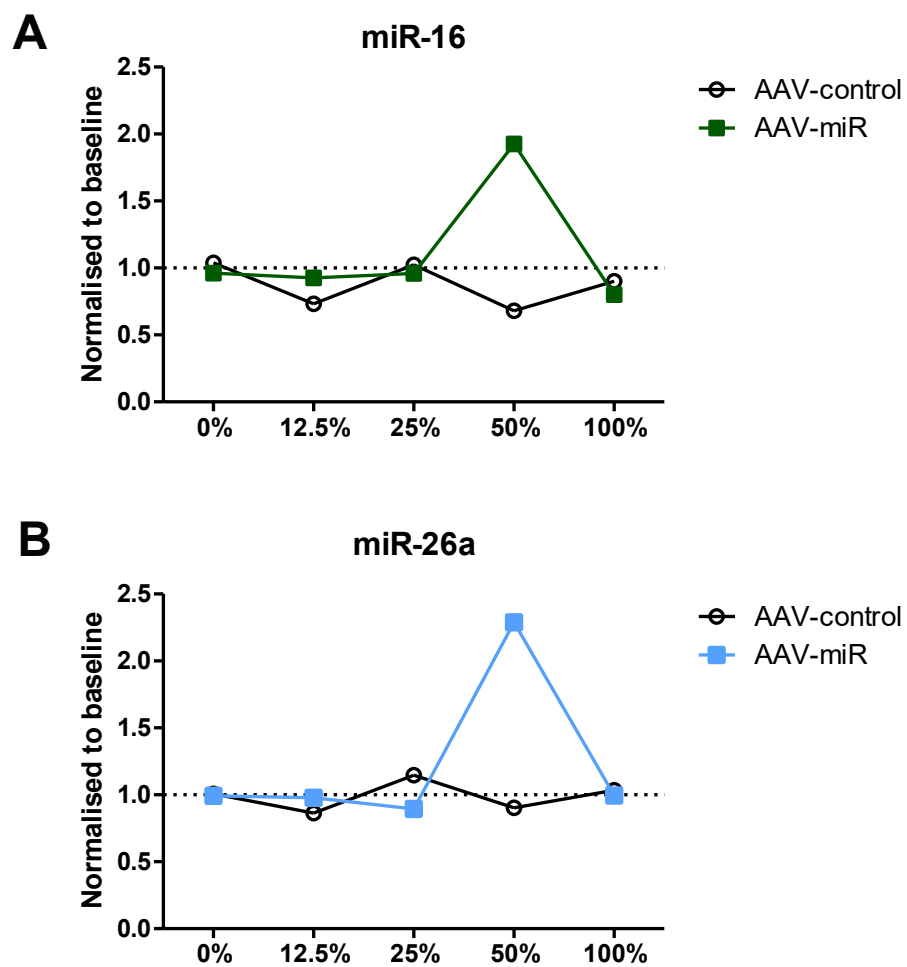


Figure 5.5: AAV dose response miR expression in heart tissue

miR-16 (A) and miR-26a (B) expression levels in isolated hearts (N=1). Data shown as individual data points.

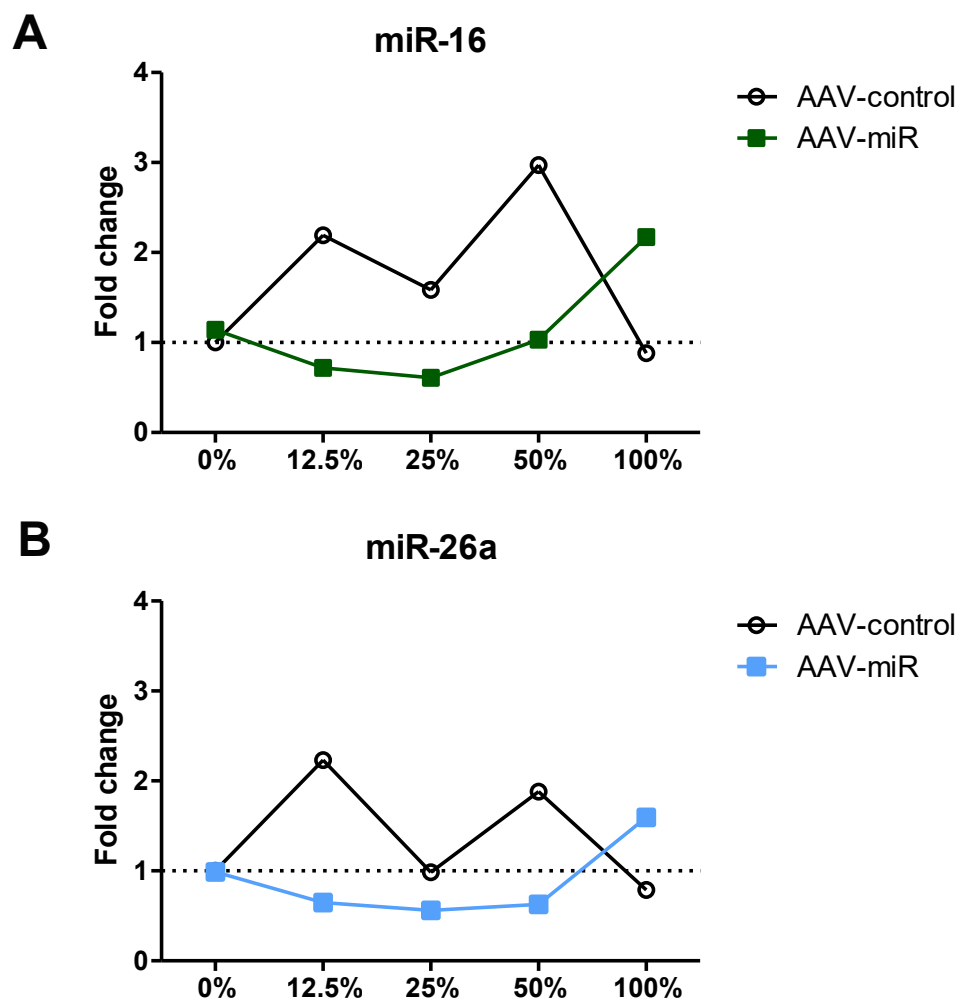


Figure 5.6: AAV dose response miR expression in peripheral blood

miR-16 (**A**) and miR-26a (**B**) expression levels in serum obtained from AAV treated rats (N=1). Data shown as individual data points.

5.3.4 mCherry fluorescence is increased 6 week after AAV treatment

After selection of the AAV dose, AAV-control and AAV-miR was injected into a cohort of rats as described in Section 2.7.4. Tissue from AAV-control and AAV-miR treated animals was isolated after induction of TTS to validate rat infection. This was split into apex and base and fixed in PFA for cryosectioning. mCherry fluorescence was assessed by widefield microscopy in Figure 5.7, where it can be seen that at both apex and base, AAV-control and AAV-miR have significantly higher fluorescence than control untreated rat tissue (Figure 5.7A). Indeed, there is no differences between the fluorescence in AAV-control and AAV-miR by two-way ANOVA. The degree of infection can be seen for each animal in Figure 5.8, where black represents an increase in mCherry expression above that of control transfected tissue. The majority of the rats yielded a large increase in fluorescence, seen by the ubiquitous increase in black expression, whereas a minority had only a small increase in fluorescence, observable by the discrete or absent pattern of expression. This was likely due to the challenging nature of administering viral infection via a tail vein injection.

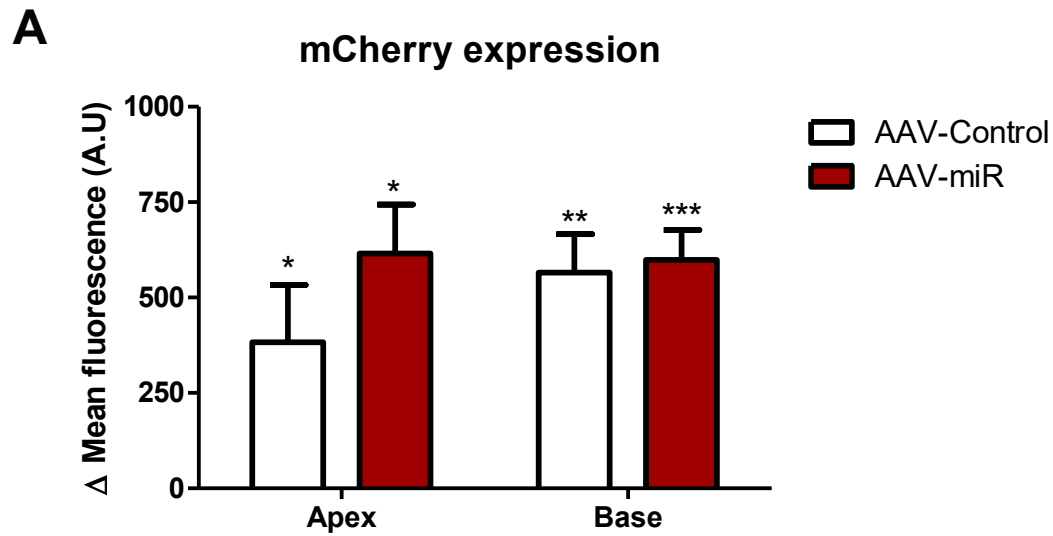


Figure 5.7: mCherry is increased after AAV infection

Mean fluorescent intensity of heart sections from AAV-control and AAV-miR treated rats as measured via widefield fluorescent microscopy. **A** Quantification of mean fluorescent intensity of AAV-control and AAV-miR treated rat tissue relative to an averaged value for untreated rat tissue (N=7). Data is displayed as mean $\pm$ SEM, with significance determined by comparing each condition with change from the averaged value for untreated rat tissue by t-test, and comparison between AAV-miR and AAV-control carried out by two-way ANOVA. Significance is shown as follows: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

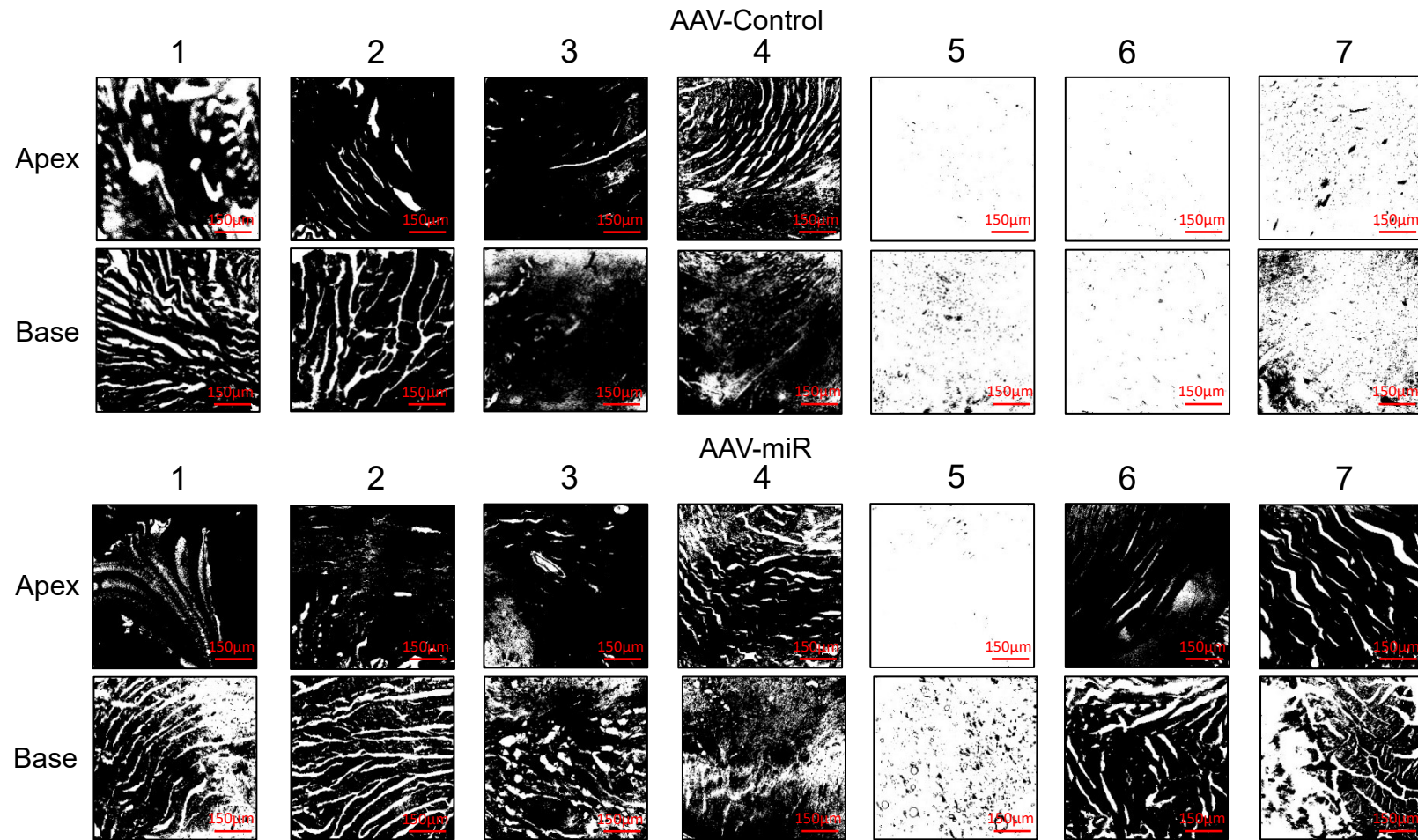


Figure 5.8: Representative images of mCherry fluorescent intensity

Representative images of mCherry fluorescent intensity of AAV-control and AAV-miR heart sections from treated animals at apex and base where black represents an increase in mCherry fluorescence.

5.3.5 Six weeks following AAV treatment, serum levels of miR-16 and miR-26a are unchanged

Blood was sampled from the external jugular vein at baseline before induction of TTS, and at 20 minutes and 60 minutes post-adrenaline administration. miRs were isolated from the serum, and miR-16 and miR-26a expression levels were measured using RTqPCR. Baseline expression in AAV-control and AAV-miR treated animals as shown in Figure 5.9A and B respectively, where although the data trended towards increased expression levels, there were no statistically significant differences. Similarly, in Figure 5.10A and B, miR-16 and miR-26a expression levels are unchanged between AAV-control and AAV-miR treated animals at 20 minutes or 60 minutes following adrenaline administration.

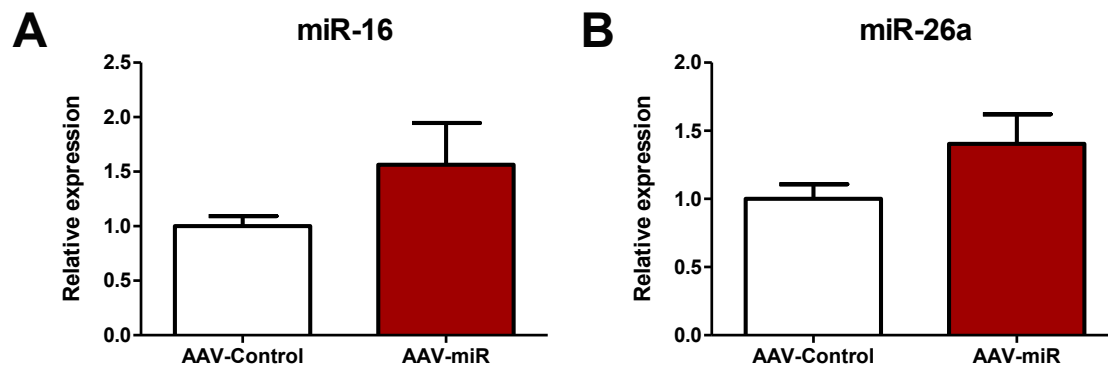


Figure 5.9: miR-16 and miR-26a baseline expression is unchanged

miR-16 (A) and miR-26a (B) expression levels in serum obtained from AAV-control and AAV-miR treated rats at baseline (N=10). Data shown mean $\pm$ SEM.

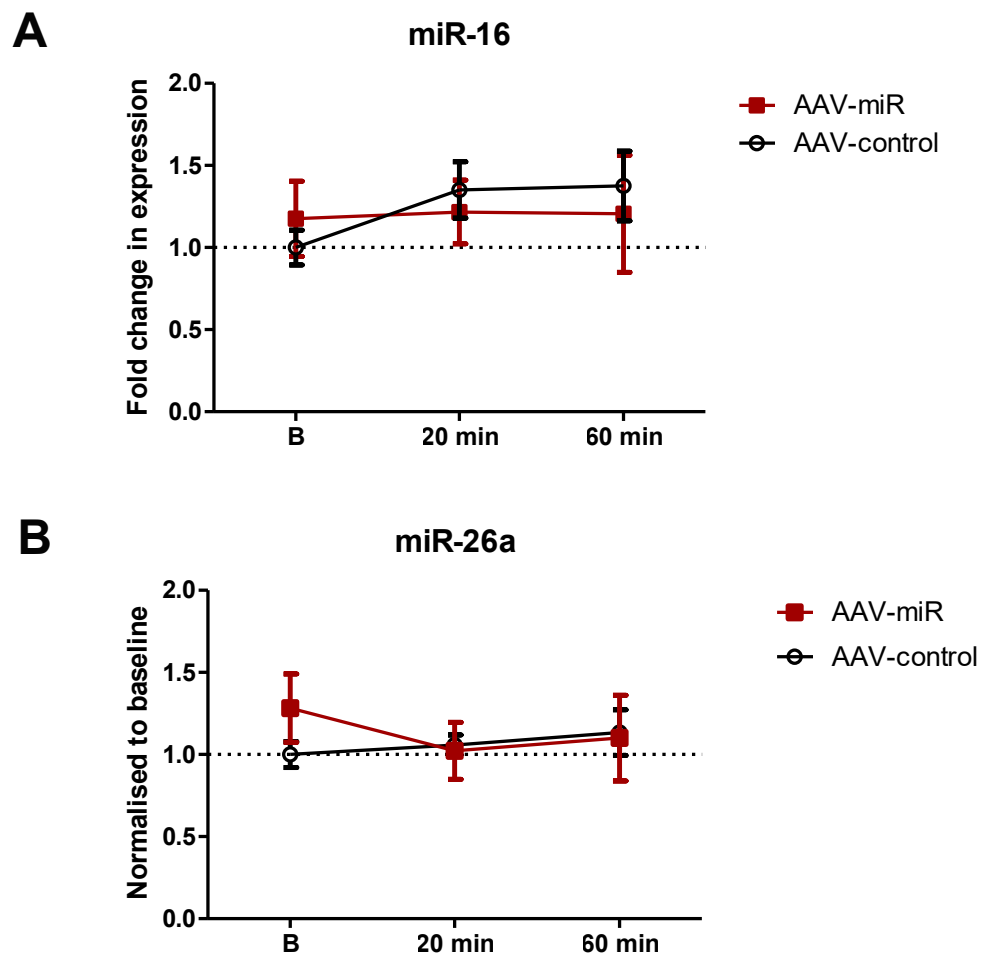


Figure 5.10: Serum miR-16 and miR-26a expression are not altered after adrenaline bolus 6 weeks after AAV treatment

miR-16 (A) and miR-26a (B) expression levels in serum obtained from AAV-control and AAV-miR treated rats at baseline, 20 minutes and 60 minutes relative to the baseline value of AAV-control (N=7). Data shown mean $\pm$ SEM.

5.3.6 At the 6 week timepoint, miR-16 and miR-26a expression is unchanged with AAV-miR in the heart

miR expression was also analysed in apical and basal tissue isolated from AAV-control and AAV-miR treated animals in Figure 5.11. miR-16 was unchanged at the apex or base with AAV-miR in comparison to AAV-control (Figure 5.11A and B), as was the case for miR-26a (Figure 5.11C and D). Whilst this was not expected, following discussion with other experts within the field, this is a common finding that will be discussed in Section 5.4.

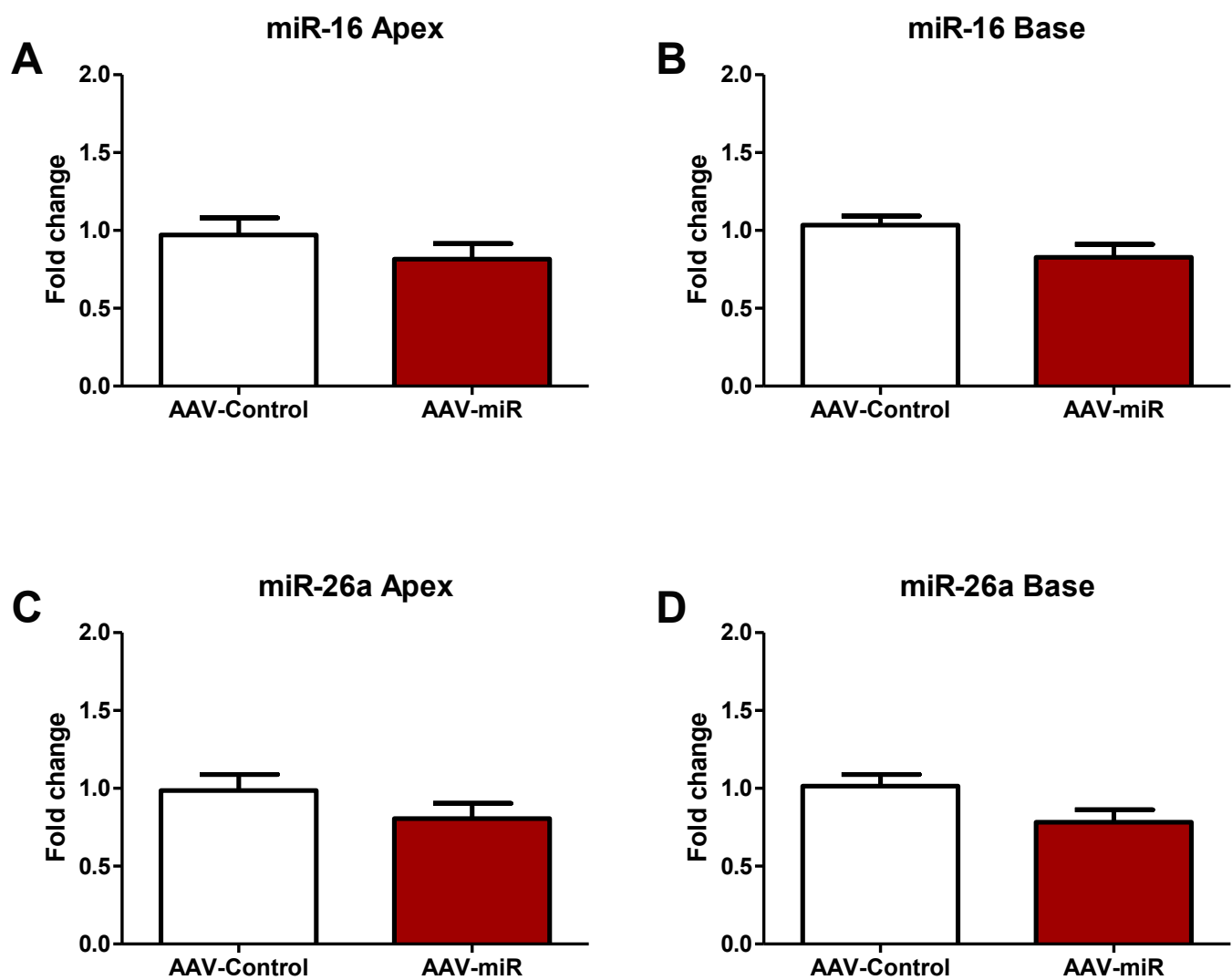


Figure 5.11: After 6 weeks, miR-16 and miR-26a expression are unchanged in heart tissue with AAV-miR treatment

miR-16 (A) and miR-26a (B) expression levels in heart tissue obtained from AAV-control and AAV-miR treated rats (N=7). Data shown mean $\pm$ SEM.

5.3.7 Upregulation of miR-16 and miR-26a *in vivo* does not alter baseline contractility

After 6 weeks, animals were anaesthetised for echocardiography and subsequent TTS induction. The baseline percentage fractional shortening was first assessed in the apex, mid-LV and base in Figure 5.12. There were no significant differences throughout, although the mean fractional shortening trended towards a slight reduction in AAV-miR treated animals in the apex and mid-LV (Figure 5.12A and B). Resting heart rate under isoflurane was also unchanged by AAV-miR (Figure 5.12D).

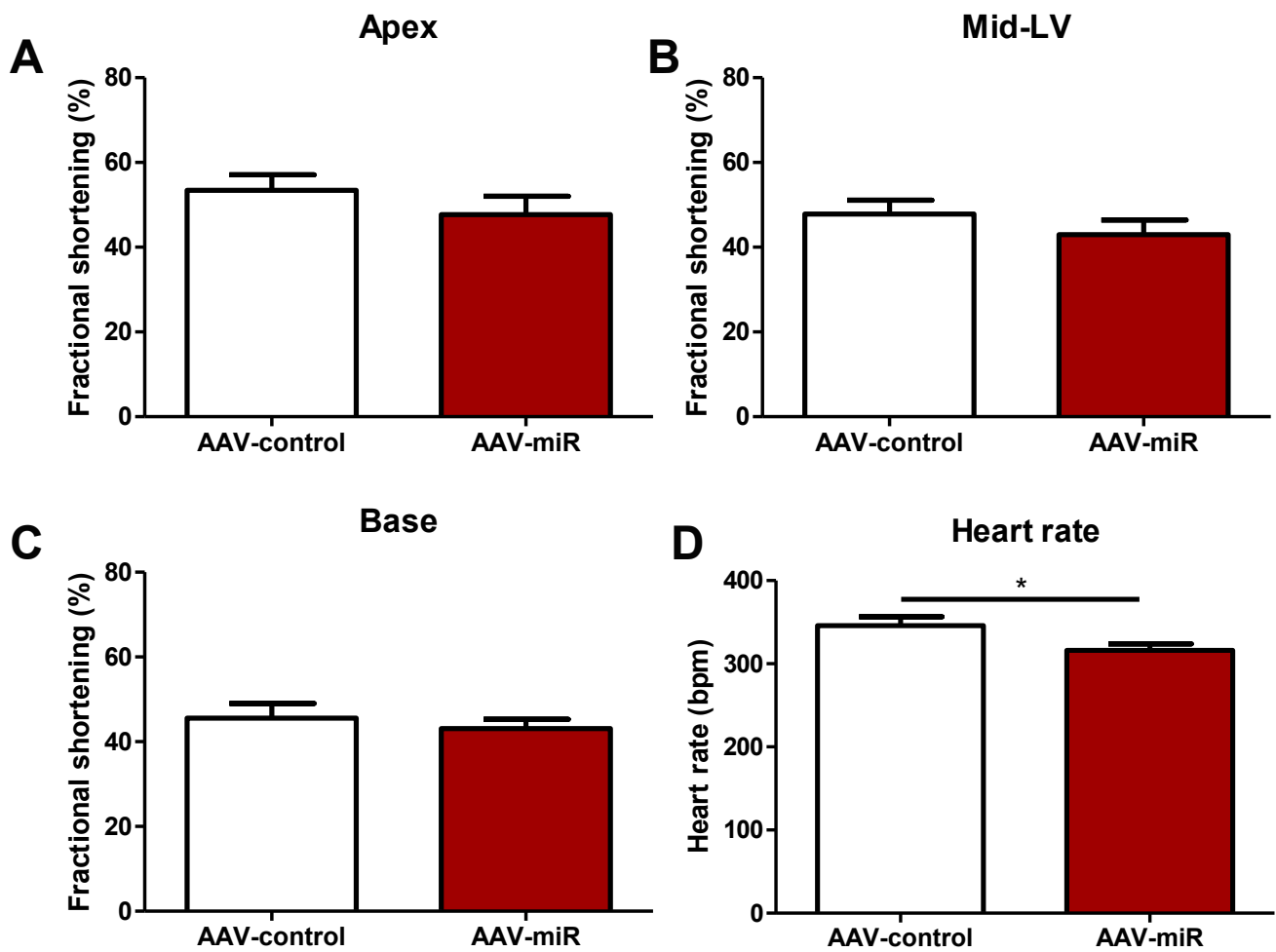


Figure 5.12: Baseline contractility is unaffected by miR-16 and miR-26a *in vivo*

Fractional shortening percentage measured at baseline following AAV-miR and AAV-control infection, shown at apex (A), mid-LV (B) and base (C), N=10. D Resting heart rate under isoflurane (N=7 for AAV-control and N=10 for AAV-miR). Data is displayed as mean $\pm$ SEM, with significance determined by Student's t-test.

5.3.8 Peak contractile response to adrenaline at 5 minutes is increased in the base *in vivo*

Following administration of 18µg/kg adrenaline as described in Section 2.7.3, peak contractile response was measured in apex, mid-LV and base at 5 minutes shown in Figure 5.13A, B and C respectively. There was no difference between the contractility of AAV-control and AAV-miR treated animals at this timepoint in the apex or the mid-LV, however the AAV-miR treated animals were observed to have significantly greater contractility in the base. This suggests that the basal myocardium had an amplified contractile response to adrenaline, as in TTS.

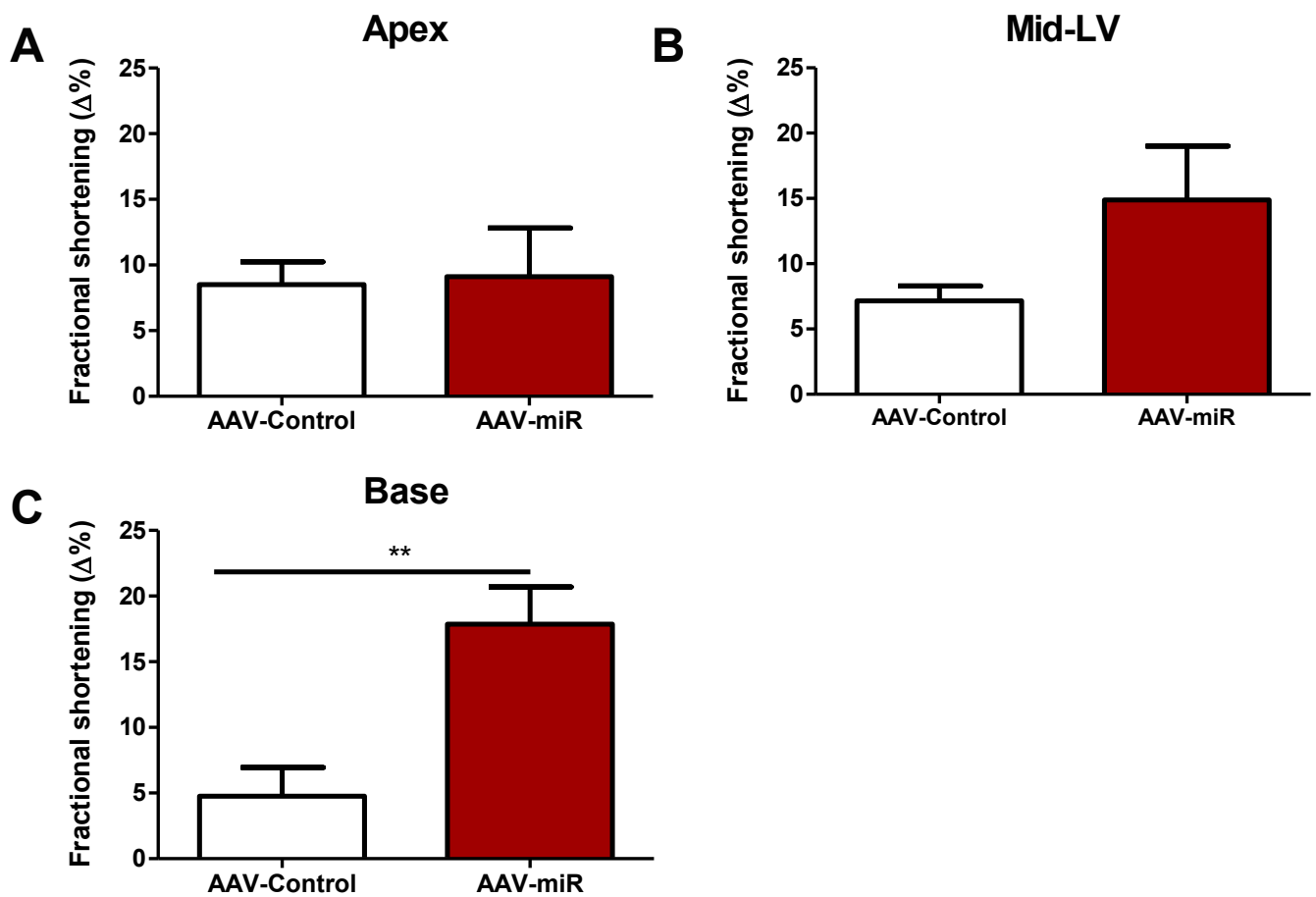


Figure 5.13: Response to adrenaline is increased in the base following treatment with AAV9-pri-miR-16+pri-miR-26a

Fractional shortening percentage measured at 5 minutes following administration of adrenaline shown at apex (A), mid-LV (B) and base (C) comparing AAV-control with AAV-miR (N=7). Data is displayed as mean $\pm$ SEM, with significance determined by Student's t-test and shown as follows: ** = $p < 0.01$.

5.3.9 AAV-miR causes significantly greater hypocontractility at 10 minutes post-adrenaline in the apex

After a further five minutes, 10 minutes post-adrenaline administration, AAV-miR treated animals exhibited significantly greater reduction in contractility at the apex than AAV-control treated animals (Figure 5.14A), with AAV-control itself modestly reduced in comparison to Figure 5.2. This indicates that AAV-miR exacerbates the hypokinetic changes in the apex seen in TTS. There was no difference between AAV-control and AAV-miR treated animals in the mid-LV or base at 10 minutes (Figure 5.14B and C respectively), although the AAV-miR treated animals appear to no longer undergo the negative inotropic changes in the base.

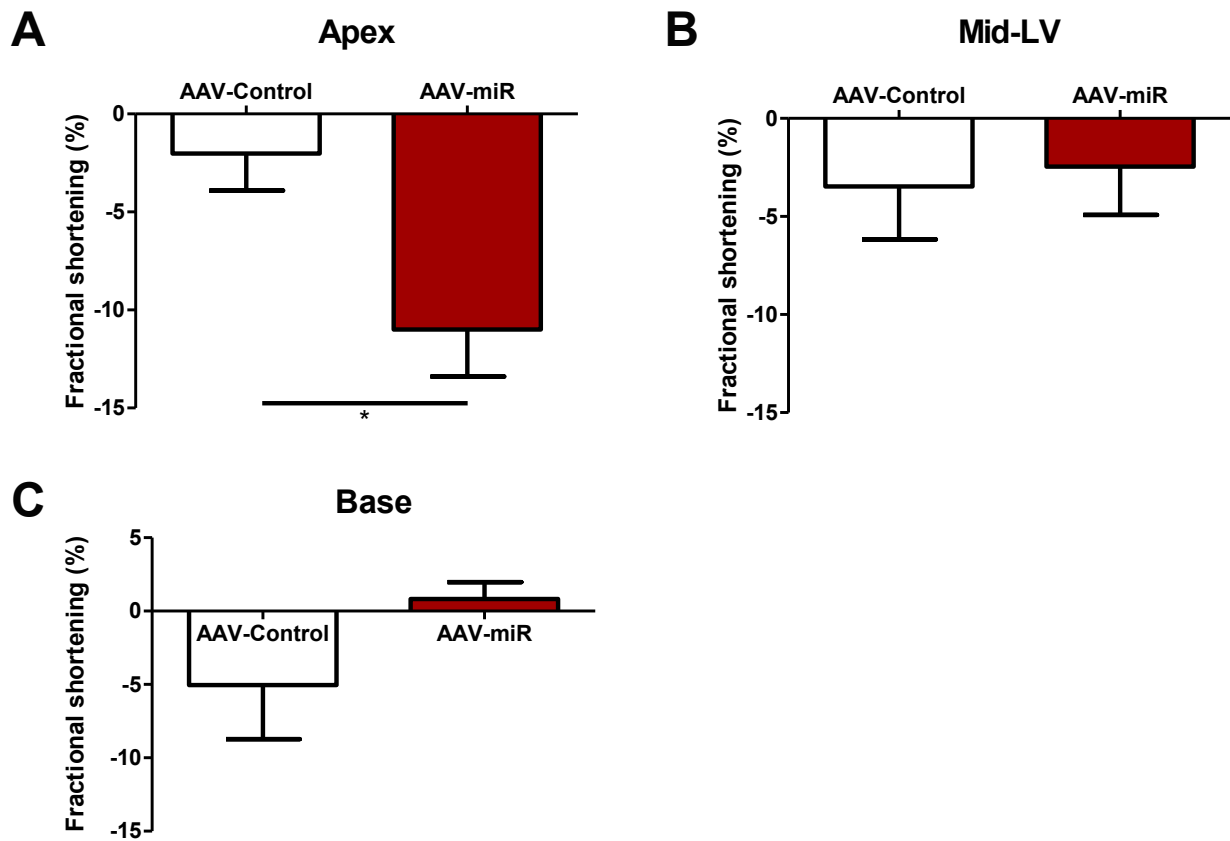


Figure 5.14: AAV-miR significantly reduces contractility in the apex

Fractional shortening percentage measured at 10 minutes following administration of adrenaline shown at apex

(A), mid-LV (B) and base (C) comparing AAV-control with AAV-miR (N=7). Data is displayed as mean $\pm$

SEM, with significance determined by Student's t-test and shown as follows: * = $p < 0.05$.

5.3.10 AAV-miR treatment alters time course of TTS induced, with greater hypokinesia in the apex and hypercontractility in the base

When observing the overall time course of the induced TTS seen in Figure 5.15, significance shown on the graphs indicate statistically significant differences from baseline. Figure 5.15A shows the data obtained from the apex and shows that AAV-control treated animals had a statistically significant increase in contractility at 5 minutes, whereas AAV-miR treated animals do not. Indeed at 10 minutes, AAV-miR treated animals had a significant reduction in contractility that did not occur in AAV-control. This suggests that TTS was not induced in AAV-control but was in AAV-miR. In the mid-LV (Figure 5.15B), both AAV-control and AAV-miR had a significant increase in contractility after 5 minutes. Whilst there were no other differences from baseline, overall, with a repeated measures ANOVA, the time course of the contractility changes observed in AAV-miR treated animals was significantly different from control, as indicated by the significance shown between the groups in the figure legend. In the base (Figure 5.15C) AAV-control exhibited no significant differences from baseline, whereas AAV-miR was significantly more contractile at 5 minutes, 30 minutes, 40 minutes, 55 minutes and 60 minutes. Indeed, on repeated measures ANOVA, there was a significant difference between AAV-control and AAV-miR overall and at 5 minutes and 20 minutes, suggesting that AAV-miR largely increased the hypercontractility in the base in comparison to AAV-control.

Taken together, the contractility changes observed in apex and base with AAV-miR strongly provide evidence for the mechanistic involvement of miR-16 and miR-26a in TTS, as TTS appeared to only be induced in AAV-miR and not AAV-control, and consequently these exacerbations recapitulate the pathognomonic changes that occur in TTS.

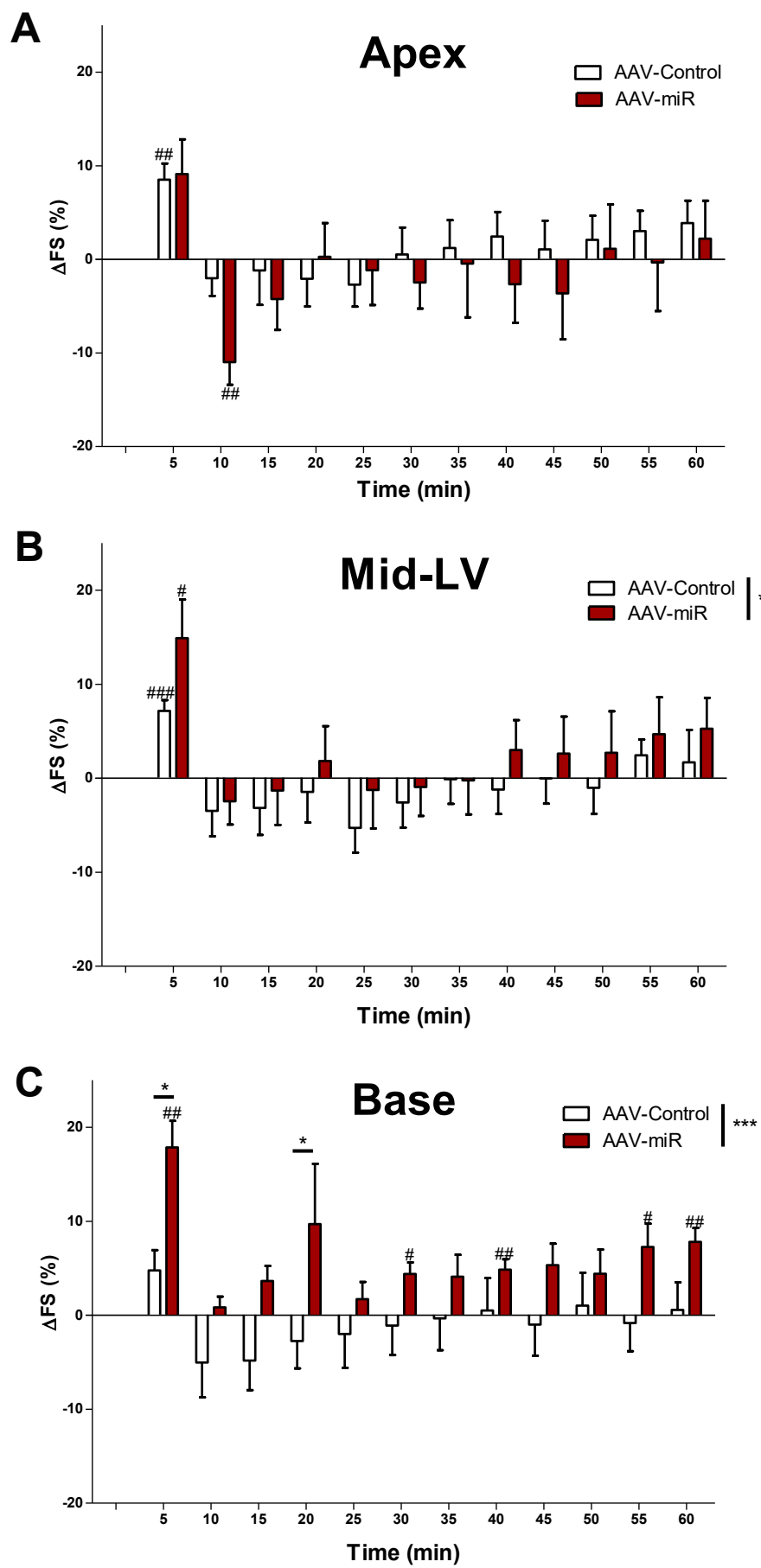


Figure 5.15: AAV-miR causes greater hypokinesia in the apex, and hypercontractility the base

Fractional shortening percentage measured at baseline and every 5 minutes following for 60 minutes using the intravenous adrenaline model to induce TTS. Fractional shortening was measured at apex (**A**), mid-LV (**B**) and base (**C**) comparing rats treated with AAV-control (N=7) and AAV-miR (N=6). Data is displayed as mean $\pm$ SEM, with significance for the overall dataset using a repeated measures ANOVA, displayed as the significance between the groups in the figure key, and a t-test comparing each timepoint to baseline.

Significance is shown as follows: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ for comparison with repeated measures ANOVA, and with # = $p < 0.05$, ## = $p < 0.01$, ### = $p < 0.001$ with t-test compared to the paired baseline.

5.3.11 AAV-miR reduces animal weight and increases animal activity

Owing to the literature previously discussed, that miR-16 was shown to be increased in stress and depression (Bai *et al.*, 2012; Katsuura *et al.*, 2012), and miR-26a to be reduced following treatment of depression (Radu Enatescu *et al.*, 2016), we wanted to evaluate the activity levels, psychological status, and growth rates of our AAV-miR treated rats.

Activity levels were scored as follows by Hannah Jones, an animal technician, or myself, where 0 equalled low, 1 equalled medium, and 2 equalled high levels of activity. Figure 5.16A shows that there was an increased activity in the AAV-miR treated animals relative to AAV-control. Indeed, there was also a significant effect of AAV-miR on rat weight, with a significant reduction by repeated measures ANOVA over the course of the 6 weeks (Figure 5.16B). There were no other perceived signs of stress or pain observed as determined by the Rat Grimace Score (Sotocina *et al.*, 2011).

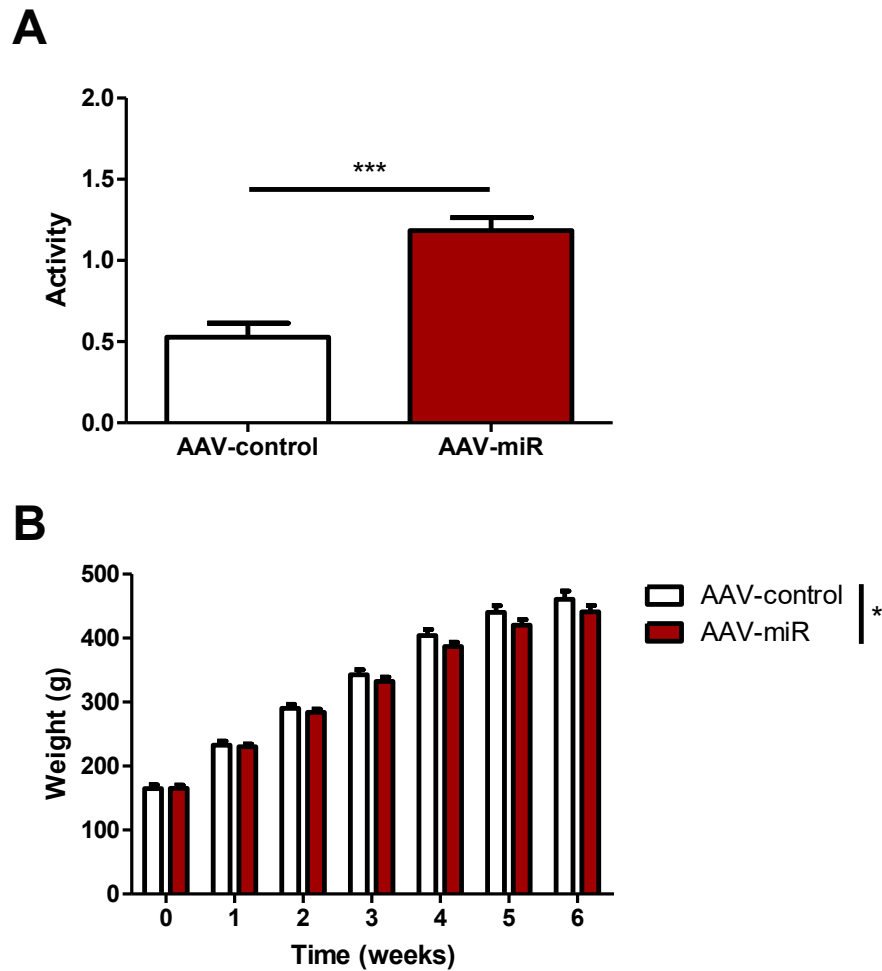


Figure 5.16: AAV-miR treated animals had increased activity and reduced weight

A Activity levels of AAV-control and AAV-miR treated animals. $n/N=37/3$ 'n' corresponds to the number of observed days and 'N' equals the number of cages observed that contained between 2 and 5 rats. **B** Weight of AAV-control and AAV-miR treated rats measured weekly ($N=10$). Data shown as mean $\pm$ SEM. Significance was determined for **A** using a Student's t-test, and was significant whether based on 'n', the number of observed days, or 'N' the number of cages. Statistical differences from **B** were compared using repeated measures ANOVA comparing AAV-control and AAV-miR, as indicated in the figure legend. Significance is shown as follows: * = $p<0.05$, *** = $p<0.001$.

5.4 Discussion

Whilst the data presented in Chapter 3 demonstrates that miR-16 and miR-26a recapitulate several key elements within TTS, this lacks the complexity of whole-organism physiology, and the TTS disease setting. Within this Chapter, our intravenous catecholamine model of TTS was re-established, and this enabled several observations to be made. Notably (1) miR-16 and miR-26a do not change within the hour following adrenaline administration, (2) AAV-miR treatment exacerbates the hypokinesia at the apex and hypercontractility at the base, and (3) AAV-miR treatment alters rat behaviour and growth rates.

5.4.1 Takotsubo syndrome was successfully recapitulated *in vivo*

The first step for experimentation within this Chapter was ensuring the *in vivo* TTS model was ready for downstream miR manipulation. Given that two well established catecholamine-induced models exist, careful consideration was required in order to determine which was more suitable for our requirements. A key difference between the intravenous adrenaline model established by Paur et al. and the intraperitoneal isoprenaline model established by Shao et al. is the time over which they occur. The former observed negative inotropic changes at the apex and mid-LV between 10 and 15 minutes, which recovered within an hour in both zones. In contrast, the hypokinetic changes occur between 50 and 60 minutes in the isoprenaline model and persists for several days. Whilst the choice of catecholamine is different, the mode of administration provides a major reason for the difference in time course. The bolus of adrenaline given intravenously provides an abrupt surge in catecholamines within the blood that much more closely reflects the changes within humans, although the prolonged absorption with intraperitoneal administration provides the delayed recovery seen in TTS patients.

The isoprenaline model exhibits a greater degree of akinesia than the adrenaline model, and this could be due to the choice of anaesthetic. Indeed, the group that established the isoprenaline model have demonstrated that isoflurane, the anaesthetic used by Paur et al., has cardioprotective effects when inducing TTS (Oras, Redfors, Ali, Lundgren, *et al.*, 2017). Indeed, they have even assessed the applicability of isoflurane as a treatment option (Oras, Redfors, Ali, Alkhoury, *et al.*, 2017).

Considering these factors, we chose to use our own model established by Paur et al. due to the extensive validation that we had previously carried out (Paur *et al.*, 2012). The model was successfully recapitulated in Figure 5.2 with minor differences. At apex, mid-LV and base, there was an expected positive inotropic effect of adrenaline at 5 minutes, causing a significant increase in contractility with respect to baseline.

We observed a profound reduction in contractility in the apex from 15 minutes onwards, that is statistically significant between 15 and 45 minutes, and recovered between 50 to 60 minutes (Figure 5.2A). In the mid-LV, this negative inotropic phase was brief, being significant at 15, 20 and 30 minutes, which recovered back to level of baseline from 40 minutes (Figure 5.2B). The negative inotropic phases that we observe occur in a similar profile to that published by Paur et al., although we observed a slightly more prolonged negative inotropic phase in the apex, and less in the mid-LV.

We observed a hypocontractile phase at the base whereas Paur et al. only observed lesser hypercontractility during the same timepoints. This was significant at 15 minutes, recovered from 20 minutes onwards, and trended towards hypercontractility from 45 minutes onwards (Figure 5.2C). Paur et al. did not see reductions in contractility in the base, but instead noted significant increases in contraction at 50 and 55 minutes.

Whilst there are minor differences in the contractile profile observed between the data presented in Figure 5.2 and Paur et al., TTS was robustly induced, and these small changes were likely due to the changes in operator, campus and surgical theatre, and the small difference in adrenaline administered. Importantly, the data presented here shows that we have recapitulated a reliable model to use as a platform for downstream investigations into the mechanisms of TTS, specifically the involvement of miR-16 and miR-26a.

5.4.2 Serum levels of miR-16 and miR-26a were unchanged in this *in vivo* model of TTS

Once the *in vivo* model of TTS had been established, this enabled blood to be sampled for analysis of circulating miRs. The data presented in Figure 5.3 shows that miR-16 and miR-26a do not change within the hour following adrenaline administration. Whilst the original findings by Jaguszewski et al. sample circulating blood miR content within a larger window of up to 24 hours after TTS onset (Jaguszewski *et al.*, 2014), this nevertheless provides important information into the role that miR-16 and miR-26a are having in TTS.

These data show that changes in miR-16 and miR-26a from baseline are not directly causing the contractile changes seen in TTS, or indeed are required for TTS induction. This was expected given that miRs post-transcriptionally regulate protein expression by silencing or degrading mRNA (Ha and Kim, 2014), therefore requiring hours to days as a mechanism of action.

Whilst there is a difference in the time period between the blood sampled in our experiment, and that obtained by Jaguszewski et al., this result does suggest that the miRs may not be increasing as a direct result of adrenaline itself, or at least within the short time period sampled here. This makes it more likely that miR-16 and miR-26a are pre-existing factors which are raised in patients with TTS.

5.4.3 AAV-miR treatment alters time course of TTS induced, with greater hypokinesia in the apex and hypercontractility in the base

Ten male Sprague-Dawley rats were infected with either AAV-control or AAV-miR via tail vein injection as per the schematic in Figure 2.7, before induction of TTS after 6 weeks. The rationale behind this choice in AAV9 protocol is discussed in detail in Section 2.7.4. TTS was induced as described in Section 2.7.3 and shown in Figure 5.2, however the adrenaline concentration used in the data presented in this Chapter required the adrenaline to be reduced from 55 μ g/kg to 18 μ g/kg due to the first four AAV treated animals dying from sudden cardiac death when using 55 μ g/kg. The dose of adrenaline given was likely to be proportionally higher in these rats, since they were older and heavier, with an increased body fat percentage. Given that adrenaline is not fat-soluble, this would result in a proportionally higher intravascular dose given, as the dosage was scaled according to body weight only. As such, the dose administered was reduced by half a log dose in molarity.

The baseline percentage fractional shortening was not changed in the apex, mid-LV or base after 6 weeks when comparing AAV-miR and AAV-control (Figure 5.12). It does appear as the fractional shortening trends towards a reduction in the apex and mid-LV, although this is not significant. The significant reduction in heart rate in AAV-miR treated animals is unlikely to be a compensatory change, since rats exhibit a positive force-frequency relationship at this physiological range (Janssen and Periasamy, 2007). Therefore, this is likely to be a direct effect of AAV-miR which could result from changes such as increased G α i activity, although will be discussed later (Chapter 7).

At the 5-minute time point following administration of adrenaline, the AAV-miR treated rats exhibited a significantly higher absolute contractility in the base of the LV than AAV-control treated rats, whereas there was no difference at the apex or mid-LV (Figure 5.13). When

comparing the change in contractility to baseline, this is significantly higher with AAV-miR, but not AAV-control in the base, with both AAV-miR and AAV-control in the mid-LV, and only AAV-control, and not AAV-miR in the apex (Figure 5.15). Taken together, this demonstrates that AAV-miR amplifies the initial positive inotropic response to adrenaline in the base only, and this positive inotropic phase is abrogated to a degree with AAV-miR in the apex.

After a further five minutes, 10 minutes following the injection of the adrenaline bolus, the positive inotropic effect of adrenaline was lost (Figure 5.15). Indeed, at the apex, AAV-miR treated animals observed a reduction in contractility that was significantly different from baseline contractility (Figure 5.15), and significantly lower than in the AAV-control treated rats (Figure 5.14). There were no differences in the mid-LV or base between AAV-control and AAV-miR treated animals, but the AAV-miR treated animals appear to no longer undergo the negative inotropic changes in the base. The changes that occurred at 10 minutes post-adrenaline administration demonstrate that AAV-miR exacerbated the severity of TTS induced, with a greater degree of hypokinesia at the apex of the LV.

In addition to the differences from baseline already discussed at 5 and 10 minutes, the AAV-miR treated rats also exhibited significantly greater contractility than baseline in the base at 30 minutes, 40 minutes, 55 minutes and 60 minutes, whereas AAV-control did not (Figure 5.15). When using repeated measures ANOVA to compare the overall time course of TTS induced in the AAV-miR and AAV-control treated rats, this is significantly different in the base and mid-LV over the course of the whole hour as well as at 5 and 20 minutes in the base.

Interestingly, when looking over the course of the hour, it does not appear that TTS was induced in AAV-control treated rats, since there were no significant differences from baseline

after the initial positive inotropic phase at 5 minutes. This is in contrast to AAV-miR, where TTS was clearly induced.

Synthesising the changes discussed above, TTS induction in AAV-miR treated animals resulted in a larger response to adrenaline and a greater degree of hyperkinesia in the base, and significantly more hypokinesia in the apex. This demonstrates that AAV-miR caused a worse form of TTS, where the same dose of adrenaline in AAV-control treated animals exhibited modest or even absent TTS-associated changes in contractility. This proves strong evidence for the mechanistic involvement of miR-16 and miR-26a in the predisposition to, and exacerbation of TTS.

5.4.4 Use of AAV9 for cardiac infection in rat *in vivo*

AAV9 was selected as this has been shown to be the most efficient AAV serotype for use in the heart, and selectively infects cardiomyocytes within the heart (Prasad *et al.*, 2011). Indeed, AAV9 can effectively cross endothelial barriers to reach the heart (Inagaki *et al.*, 2006; Pacak *et al.*, 2006; Bostick *et al.*, 2007). This is also the case for crossing the blood-brain barrier (DiMattia *et al.*, 2012), and this was desired as although the origin of the miRs in circulating blood found by Jaguszewski *et al.* is unknown, it was presumed to be neuronal (Jaguszewski *et al.*, 2014), and miR-16 has been shown to be increased in stress and depression (Bai *et al.*, 2012; Katsuura *et al.*, 2012), and miR-26a to be reduced following treatment of depression (Radu Enatescu *et al.*, 2016). Given the strong correlation that TTS has with psychiatric disorders (Templin *et al.*, 2015), we combined AAV9 with a generalised promoter to obtain upregulation of miRs in other organs, including the central nervous system.

Whilst older AAV serotypes, such as AAV2, require up to 6 week transduction time to observe upregulation and functional effects (Aikawa, Huggins and Snyder, 2002; Prasad *et*

al., 2007), AAV9 has been shown to be efficiently upregulated in liver after 1 week, and sustained until week 6 (Inagaki *et al.*, 2006). This most efficient when delivered via the portal vein for the liver, followed by tail vein and intraperitoneally. However, as this is for the liver, portal vein and intraperitoneal administration is unlikely to be as efficient for use in the heart. It could be surmised that the most effective route of administration would be intracoronary, although this could not be done due to practical limitations. We chose to inject AAV9 via the tail vein, and wait 6 weeks for further experimentation based on previous AAV9 work published in our lab (Lyon *et al.*, 2011; Kumarswamy *et al.*, 2012).

The initial dose response conducted suggested that 2.5×10^{12} gc of AAV-control or AAV-miR yielded the highest degree of mCherry fluorescence (Figure 5.4), and miR-16 and miR-26a expression levels in heart tissue with AAV-miR (Figure 5.5), although changes in serum appeared non-specific (Figure 5.6). It was presumed that the higher dose was not more efficient due to toxicity. Despite being a higher dose than our laboratory has previously used (Lyon *et al.*, 2011; Kumarswamy *et al.*, 2012), we chose 2.5×10^{12} gc for further experimentation. It must be noted that the initial AAV dose-response was only completed to N=1, owing to the substantial expense of AAV experimentation, and this was especially problematic due to the challenging nature of tail vein injections. Despite this, increase in tissue fluorescence can clearly be seen, and thus we chose to proceed.

Following the infection protocol and TTS induction (Figure 2.7), hearts were excised and fixed or frozen in liquid nitrogen. Indeed, both AAV-control and AAV-miR produced significant increases in mCherry fluorescence at apex and base when compared to an average compiled from control untreated tissue (Figure 5.7). This demonstrates that infection with both AAV-control and AAV-miR was successful. There is variability between the

fluorescence of some of the individual samples (Figure 5.8), perhaps representing the technical nature of AAV administration.

Whilst not significant, miR-16 and miR-26a trended towards increased expression levels in the serum of AAV-miR treated rats (Figure 5.9). However, tissue expression of miR-16 and miR-26a was not changed in the heart (Figure 5.11). This could be due to the time point sampled, as Inagaki *et al.* demonstrated that the initial peak in gene expression with AAV9 infection occurs after 1 week (Inagaki *et al.*, 2006). It is possible that the miRs exhibit temporal changes in expression that exert functional effects that persist after when we can no longer observe direct differences in expression. This is likely, as differences were observed despite the viruses being identical apart from the intronic regions containing pri-miR-16 and pri-miR-26a. The target genes for miR-16 and miR-26a will also be examined in Chapter 6, where this will be discussed further.

5.4.5 AAV-miR reduces animal weight and increases animal activity

As miR-16 and miR-26a have previously been associated with changes in mental state as previously discussed in Section 5.4.4, we monitored animal behaviour to investigate whether TTS-associated miRs had these anticipated psychogenic effects.

Activity levels of AAV-miR treated animals was significantly higher than in AAV-control, indicating that miR-16 and miR-26a altered animal behaviour. Whilst it is not possible to directly attribute this to changes in stress, this is an important preliminary observation to show miR-16 and miR-26a are altering the behaviour of AAV-miR treated rats.

We also observed a reduction in weight in the AAV-miR treated rats with respect to AAV-control. This change correlates with the increase in activity levels but could also be due to changes in mental state which could subsequently alter appetite. Robust behavioural analysis

is required in order to comment further about the specific effect TTS-associated miRs are having on activity and animal growth.

5.4.6 Summary

This Chapter has shown that miR-16 and miR-26a do not change within the hour following adrenaline administration, AAV-miR treatment exacerbates the hypokinesia at the apex and hypercontractility at the base, and AAV-miR treatment alters rat behaviour and growth rates. Taken together, this provides strong evidence *in vivo* for the involvement of miR-16 and miR-26a in the predisposition to and exacerbation of TTS.

6 Identification of the mechanism of action of miR-16 and miR-26a on cardiomyocyte contractility

6.1 Introduction

miRs are short non-coding sequences of nucleotides that post-transcriptionally regulate protein expression by silencing or degrading mRNA (Ha and Kim, 2014). This results in changes in protein expression that subsequently enables for regulation of whole systems of proteins by a single miR. They are subject to intense research as they are dysregulated in many forms of cardiac disease (Thum and Condorelli, 2015). Indeed, miR-1 has been shown to be reduced in heart failure (Kumarswamy *et al.*, 2012), miR-21 increased and miR-29 decreased in cardiac fibrosis (van Rooij *et al.*, 2008; Patrick *et al.*, 2010), and miR-208 and miR-133 dysregulated in cardiac hypertrophy (Carè *et al.*, 2007; Van Rooij *et al.*, 2007). Interestingly, miR-133 has also been shown to modulate the adrenergic system, counteracting the deleterious apoptotic effects caused by chronic β_1 AR stimulation (Castaldi *et al.*, 2014).

Given that miR-16 and miR-26a have been identified as biomarkers for TTS (Jaguszewski *et al.*, 2014), it is feasible that miRs could also be mechanistically involved in the generation of TTS. The data presented in Chapters 3 and 5 provide strong evidence for this, in particular, their impact in the predisposition to TTS. The purpose of the experiments in this Chapter is to identify the mechanism of action by which this occurs. miR targets will be predicted using *in silico* methods, before investigation of mRNA and protein expression of putative miR targets *in vivo* using AAV-miR upregulation as described in Chapter 5. Identified targets will be used to guide final confirmatory mechanistic *in vitro* experiments.

6.2 Methods

6.2.1 *In silico* target prediction

miRWalk 2.0 was used to predict target gene binding by analysing 3'UTR (untranslated region) sequence homology and cross-comparing 12 different miR databases as detailed in Section 2.8.1 (Dweep and Gretz, 2015). This list was stratified using Panther DB to obtain a list of genes associated with contractility (Mi *et al.*, 2013, 2017).

6.2.2 Luciferase assays

Primers for luciferase assays were designed using Primer3 to contain 5'-*SpeI* and 3'-*HindIII* restriction sites for incorporation into the pmiRReport vector. Target sequences were then checked in TargetScan and Ensembl to confirm the putative miR binding sites (Section 2.6.7). Target genes were constructed from rat cDNA using PCR and incorporated into the vector. These were expanded in *E. coli* and sequenced to check the desired gene had been cloned (Section 2.6.8). Luciferase assays were performed in HEK293 cells with miR upregulation and normalised to β -galactosidase plasmid activity (Section 0).

6.2.3 Quantitative real-time PCR (RTqPCR)

RNA was isolated as described in Section 2.6.2, and quantified using specific Taqman primers for CACNA1C, CACNB1, RGS4, GNB1 and GNG12, with GAPDH, TBP and 18S used as endogenous controls (Section 2.6.3). Data was analysed using the comparative cycle threshold (CT) method (Δ Ct) as previously described (Schmittgen and Livak, 2008).

6.2.4 Western blot

Protein was isolated (Section 0) and samples prepared for western blot as described in Section 2.6.10. Primary antibodies were 1:200 rabbit anti-CACNA1C (Abcam, ab58552),

1:10,000 rabbit anti-vinculin (Abcam, ab129002), 1:500 rabbit anti-RGS4 (Abcam, ab9964), 1:500 rabbit anti-CACNB1 (Sigma, AV34953), 1:333 rabbit anti-GNB1 (ThermoFisher Scientific, PA1-725) and 1: 500 rabbit anti-GNG12 (ThermoFisher Scientific, PA5-75620), with 1: 3000 anti-rabbit horse radish peroxidase (HRP)-linked secondary antibody (Cell Signalling Technologies, 7074S). Membranes were developed using Clarity™ Western ECL Blotting Substrates and imaged using a ChemiDoc imaging system. Images were analysed using Image J, comparing the intensity of each band to a paired value for vinculin, used as the loading control.

6.2.5 PTX pre-treatment

PTX was used to inhibit Gαi. This was done by pre-treating the cells overnight with 1.5µg/mL PTX for 12 hours at 37°C.

6.2.6 Calcium current patch clamp

Calcium current patch clamp is described in full in Section 0.

6.2.7 Statistics

Data is represented as mean± SEM. Student's t-tests was used to compare the relative changes between two data sets. Where there were more than two statistical comparisons to be made, one-way analysis of variance (ANOVA) was used with Tukey's post-hoc test. If two variables were present within the dataset, two-way ANOVA was conducted with subsequent Bonferroni's post-hoc test. Level of significance is presented throughout as follows:

*=p<0.05, **=p<0.01, ***=p<0.001.

6.3 Results

6.3.1 *In silico* prediction of microRNA targets

In order to find the mechanism of action of miR-16 and miR-26a, an *in silico* analysis of their predicted targets was carried out as described in Section 2.8.1. After excluding all genes that are not involved in the regulation of contractility, 33 targets remained for miR-16, and 29 for miR-26a (Table 6.1 and Table 6.2 respectively). This included genes involved in the regulation of the adrenergic system, calcium handling and protein kinases. miRs classically act by silencing or degrading mRNA, so the list was further narrowed down by considering a reduction in which genes could produce results seen in Chapter 3 and 5. For miR-16, this was considered to be CACNB1 (LTCC β 1 subunit), GNB1 (G β 1), GNG12 (G γ 12) and RGS3, and for miR-26a, this was CACNA1C (LTCC α 1c subunit), CACNB2 (LTCC β 2 subunit) and RGS4. ADRBK1 (GRK2) is required for stimulus trafficking to occur, and was included for this reason. Throughout this chapter they are referred to by their gene names, although their protein names are included in the brackets above.

Table 6.1: Predicted targets for miR-16

Targets of miR-16 proposed to influence cardiomyocyte contractility identified using miRWalk 2.0 and Panther DB.

Hsa-miR-16	Protein
ADCY1	Adenylate cyclase type 1
ADRA2A	Alpha-2A adrenergic receptor
ADRB2	Beta-2 adrenergic receptor
ADRBK1	G-protein coupled receptor kinase 2 (GRK2)
CACNA1B	Voltage-dependent N-type calcium channel subunit alpha-1B
CACNA1E	Voltage-dependent R-type calcium channel subunit alpha-1E
CACNB1	Voltage-dependent L-type calcium channel subunit beta-1
CACNB4	Voltage-dependent L-type calcium channel subunit beta-4
CDC42	Cell division control protein 42 homolog
CREB5	Cyclic AMP-responsive element-binding protein 5
GNAI3	Guanine nucleotide-binding protein G(k) subunit alpha
GNAL	Guanine nucleotide-binding protein G(olf) subunit alpha
GNB1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1
GNG12	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12
GRM4	Metabotropic glutamate receptor 4
GYS1	Glycogen [starch] synthase, muscle
HTR4	5-hydroxytryptamine receptor 4
ITPR1	Inositol 1,4,5-trisphosphate receptor type 1
MAP2K3	Dual specificity mitogen-activated protein kinase kinase 3
MAP3K4	Mitogen-activated protein kinase kinase kinase 4
MAPK3	MAP kinase-activated protein kinase 3
MKNK1	MAP kinase-interacting serine/threonine-protein kinase 1
PHKA1	Phosphorylase b kinase regulatory subunit alpha, skeletal muscle isoform
PHKG2	Phosphorylase b kinase gamma catalytic chain, liver/testis isoform
PRKAR2A	cAMP-dependent protein kinase type II-alpha regulatory subunit
PRKCA	Protein kinase C alpha type
PRKCZ	Protein kinase C zeta type
RGS3	Regulator of G-protein signalling 3
SNAP25	Synaptosomal-associated protein 25
SSR1	Somatostatin receptor type 1
SSTR3	Somatostatin receptor type 3
VAMP1	Vesicle-associated membrane protein 1
VAMP8	Vesicle-associated membrane protein 8

Table 6.2: Predicted targets for miR-26a

Targets of miR-26a proposed to influence cardiomyocyte contractility identified using miRWalk 2.0 and Panther DB.

Hsa-miR-26a	Protein
ADCY2	Adenylate cyclase type 2
ADCY6	Adenylate cyclase type 6
ADRBK1	G-protein coupled receptor kinase 2 (GRK2)
CACNA1C	Voltage-dependent L-type calcium channel subunit alpha-1C
CACNB2	Voltage-dependent L-type calcium channel subunit beta-2
CACNB4	Voltage-dependent L-type calcium channel subunit beta-4
CLTC	Clathrin heavy chain 1
CREBBP	CREB-binding protein
GPSM1	G-protein-signaling modulator 1
GPSM2	G-protein-signaling modulator 2
GRM1	Metabotropic glutamate receptor 1
GSK3B	Glycogen synthase kinase-3 beta
ITPR1	Inositol 1,4,5-trisphosphate receptor type 1
MEF2C	Myocyte-specific enhancer factor 2C
MEF2D	Myocyte-specific enhancer factor 2D
MKNK2	MAP kinase-interacting serine/threonine-protein kinase 2
PLCB1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-1
PRKCB	Protein kinase C beta type
PRKCD	Protein kinase C delta type
PRKCQ	Protein kinase C theta type
PRKX	cAMP-dependent protein kinase catalytic subunit PRKX
PYGL	Glycogen phosphorylase, liver form
RAP1A	Ras-related protein Rap-1A
RGS4	Regulator of G-protein signaling 4
SNAP29	Synaptosomal-associated protein 29
SSR1	Somatostatin receptor type 1
STX3	Syntaxin-3
TAB2	TGF-beta-activated kinase 1 and MAP3K7-binding protein 2
VAMP1	Vesicle-associated membrane protein 1

6.3.2 Luciferase assay validates CACNB1 and GNB1 as miR-16 targets

The targets identified in Section 6.3.1 were validated by performing luciferase assays. The luciferase plasmids for this experiment, and the experiment conducted for miR-26a in Section 6.3.3, were designed and produced by myself with Jan Fiedler and Annette Just whilst at Hannover Medical School. The luciferase plasmids for ADRBK1, CACNB1, GNB1, GNG12 and RGS3 were transfected into HEK-293 cells, along with a β -galactidose plasmid for normalisation and miR-16 upregulation, and luciferase assays were carried out. Luciferase activity was significantly reduced with miR-16 transfection for CACNB1 and GNB1 (Figure 6.1B and C respectively), and on the verge of significance for GNG12 ($p = 0.08$, Figure 6.1D), whereas luciferase activity was unchanged with ADRBK1 and RGS3 (Figure 6.1A and E respectively). This demonstrates CACNB1 and GNB1 are targets of miR-16.

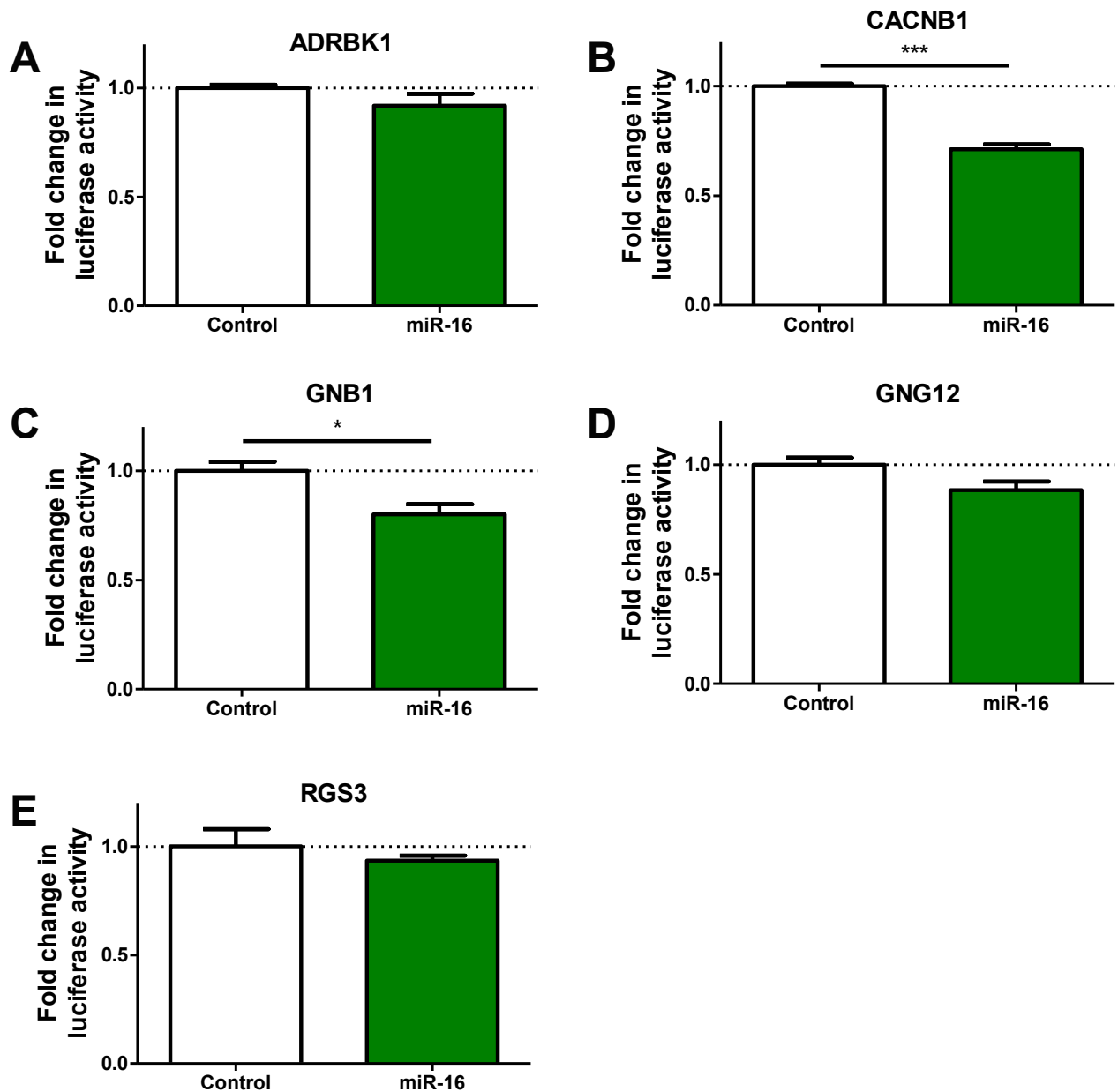


Figure 6.1: miR-16 reduces activity of CACNB1 and GNB1 luciferase plasmids

Luciferase activity of HEK 293 cells after miR-16 transfection for ADRBK1 (A), CACNB1 (B), GNB1 (C), GNG12 (D) and RGS3 (E) luciferase plasmids. Data is displayed as mean $\pm$ SEM. $n/N = 9/3$ where n = number of transfections and N = number of biological repeats, with N being used for statistical analysis, which was conducted by Student's unpaired t-test and represented as follows: * = $p < 0.05$, *** = $p < 0.001$.

6.3.3 Luciferase assay validates CACNA1C and RGS4 as miR-26a targets

Luciferase assays were carried out as in Section 6.3.2, with respective miR-26a targets. With miR-26a transfection, CACNA1C and RGS4 luciferase activity was significantly reduced (Figure 6.2B and D respectively), whereas ADRBK1 and CACNB2 were not (Figure 6.2A and C respectively). This validates that CACNA1C and RGS4 are targets of miR-26a.

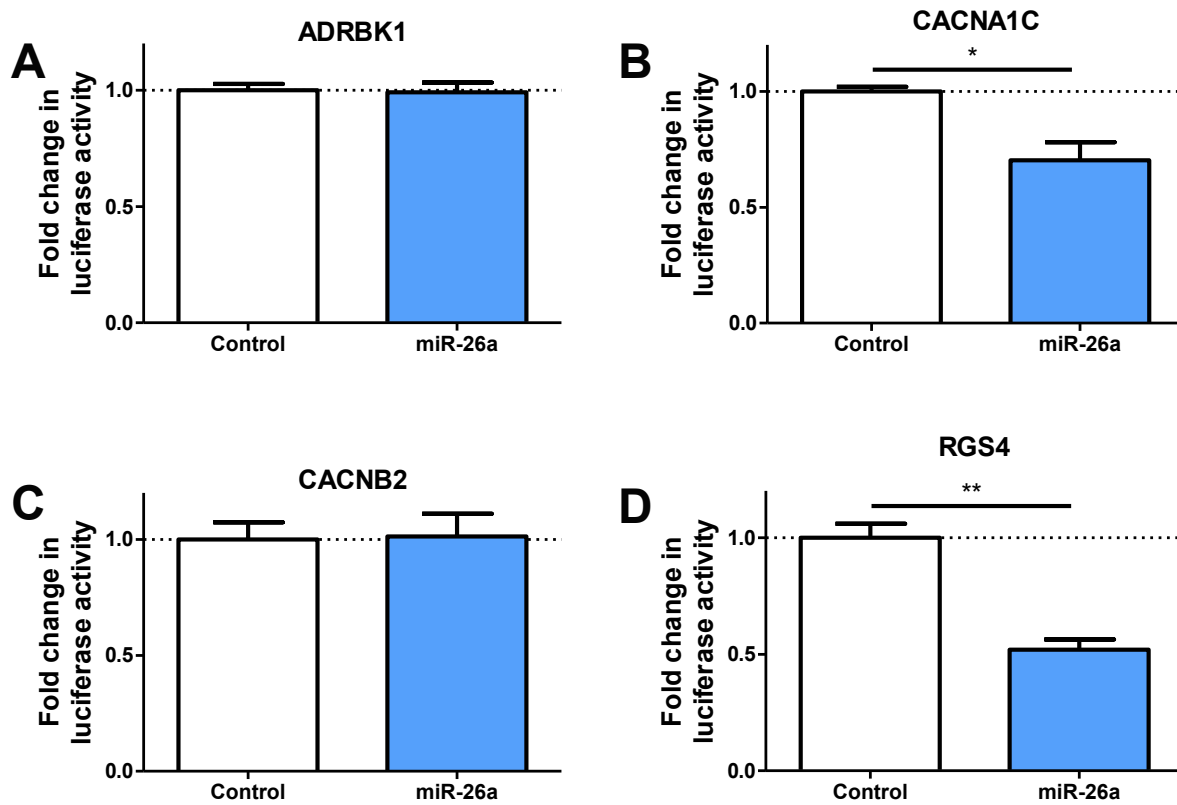


Figure 6.2: miR-26a reduces activity of CACNA1C and RGS4 luciferase plasmids

Luciferase activity of HEK 293 cells after miR-26a transfection for ADRBK1 (A), CACNA1C (B), CACNB2 (C) and RGS4 (D) luciferase plasmids. Data is displayed as mean $\pm$ SEM. $n/N = 9/3$ where n = number of transfections and N = number of biological repeats, with N being used for statistical analysis, which was conducted by Student's unpaired t-test and represented as follows: * = $p < 0.05$, ** = $p < 0.01$.

6.3.4 miR-16 and miR-26a do not synergise to target ADRBK1

Since ADRBK1 was a potential target of by both miRs, miR-16 and miR-26a were transfected together to see if combination could influence ADRBK1 expression. Cotransfection also had no effect on luciferase activity (Figure 6.3), confirming that ADRBK1 is not a target for miR-16 or miR-26a independently, or both together.

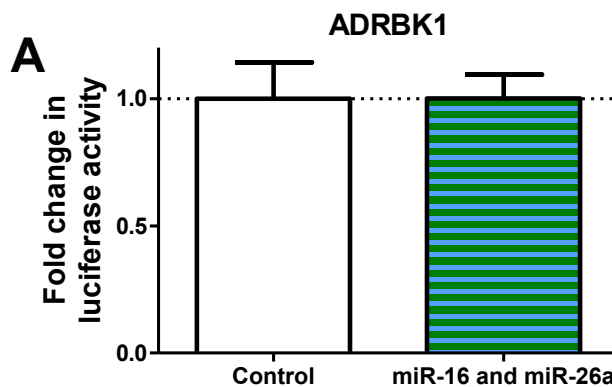


Figure 6.3: miR-16 and miR-26a do not synergise to target ADRBK1

Luciferase activity of HEK 293 cells after miR-16 and miR-26a transfection for ADRBK1 (A) luciferase plasmid. Data is displayed as mean $\pm$ SEM. $n/N = 9/3$ where n = number of transfections and N = number of biological repeats, with N being used for statistical analysis, which was conducted by Student's unpaired t-test.

6.3.5 CACNA1C, CACNB1 and GNB1 mRNA are reduced *in vivo* after AAV-miR

After identification of miR targets by *in silico* prediction and subsequent luciferase assay, RTqPCR was conducted to evaluate the expression levels in apical and basal heart tissue samples from AAV-miR treated animals. CACNA1C, CACNB1 and GNB1 expression levels were all significantly reduced by AAV-miR in comparison to AAV-control treated animals (Figure 6.4A, B and D respectively), whereas RGS4 was not (Figure 6.4C). GNG12 was also investigated, as was on the verge of significance on the luciferase assay, although was unchanged (Figure 6.4E). GNG12 investigation was discontinued, as was not significant on luciferase assay or RTqPCR.

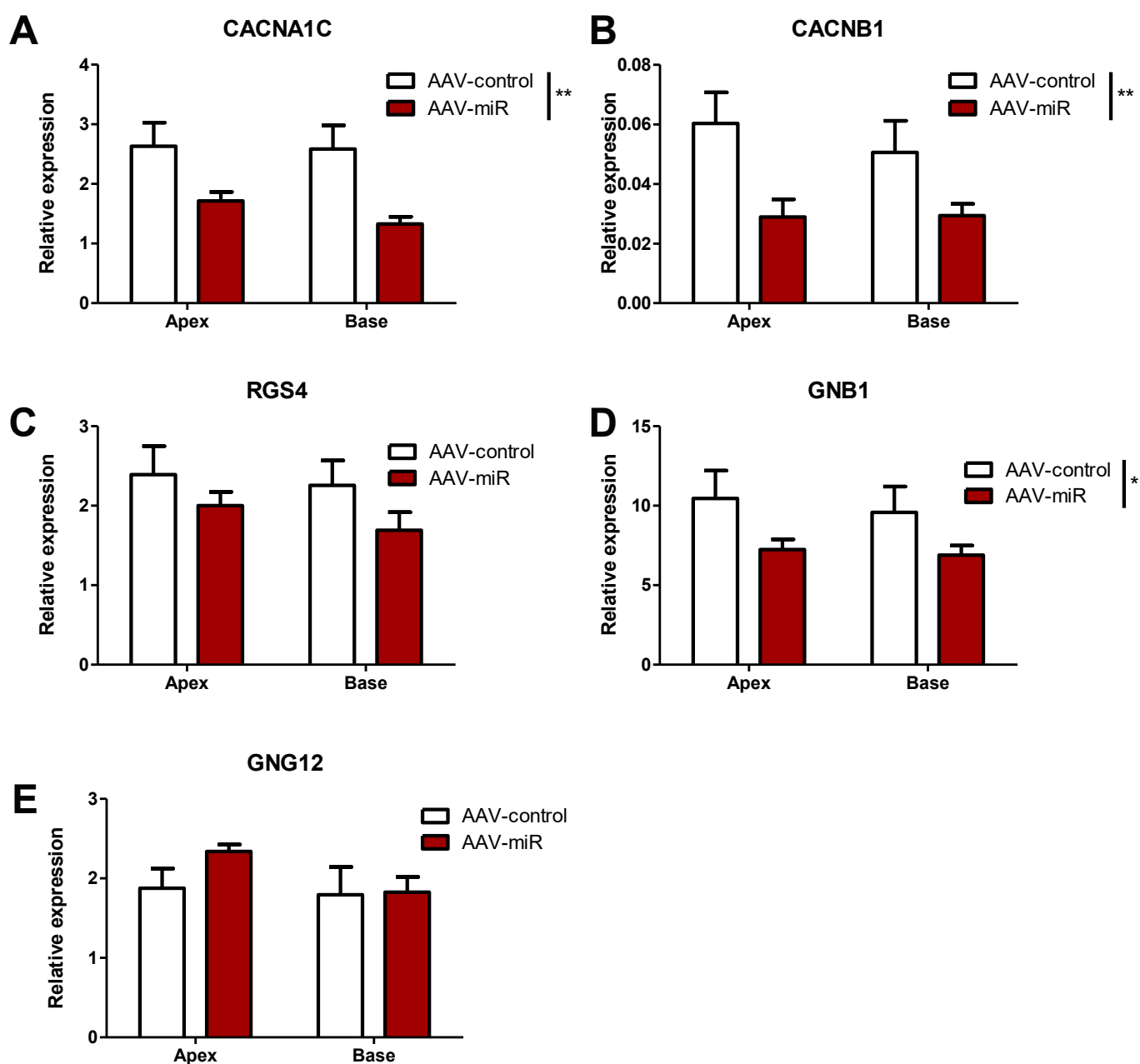


Figure 6.4: CACNA1C, CACNB1 and GNB1 mRNA expression are reduced *in vivo* with AAV-miR

Relative expression of CACNA1C (A), CACNB1 (B), RGS4 (C), GNB1 (D) and GNG12 (E) quantified by RTqPCR from AAV treated whole rat hearts, split into apex and base. Data is displayed as mean $\pm$ SEM. N = 7 where N = number of rats. Statistical analysis was carried out using two-way ANOVA, with subsequent Bonferroni's post hoc. Significance is represented as follows: * = $p < 0.05$, ** = $p < 0.01$.

6.3.6 CACNB1, RGS4 and GNB1 protein are reduced *in vivo* after AA9 miR-16 and miR-26a upregulation

Since RTqPCR is not proof of *in vivo* miR targeting, as miRs can degrade or silence mRNA, western blots were carried out to analyse protein expression. RGS4 was included for this reason. Giles Chick (Cardiovascular BSc student), Jerome Fourre and Brian Wang (PhD students) conducted these western blot experiments with myself. Of the remaining targets, CACNB1, RGS4 and GNB1 protein levels were all reduced *in vivo* by AAV-miR in comparison to AAV-control, with no difference between apex and base (Figure 6.5A, B and C respectively). This indicates that these are regulated *in vivo* by miR-16 and miR-26a. CACNA1C could not be blotted for technical reasons.

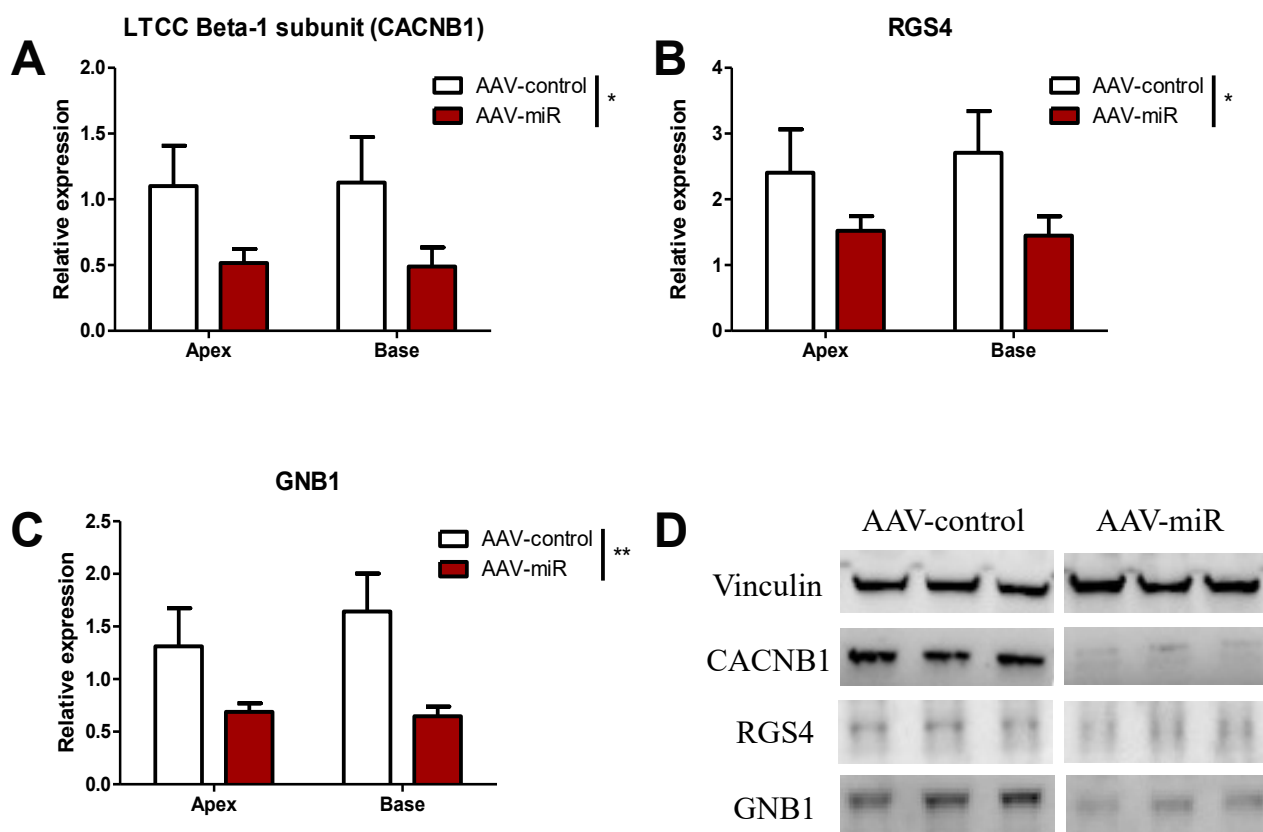


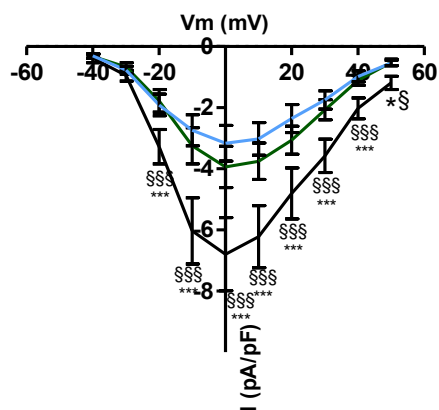
Figure 6.5: LTCC β 1 subunit (CACNB1), RGS4 and G-Protein Subunit Beta-1 (GNB1) protein expression are reduced by AAV-miR

Relative expression of CACNB1 (A), RGS4 (B) and GNB1 (C) quantified by western blot from AAV-treated whole rat myocardium, split into apex and base. Data is displayed as mean $\pm$ SEM. N = 7 where N = number of rats. Statistical analysis was carried out using two-way ANOVA, with subsequent Bonferroni's post hoc. Significance is represented as follows: * = $p < 0.05$, ** = $p < 0.01$.

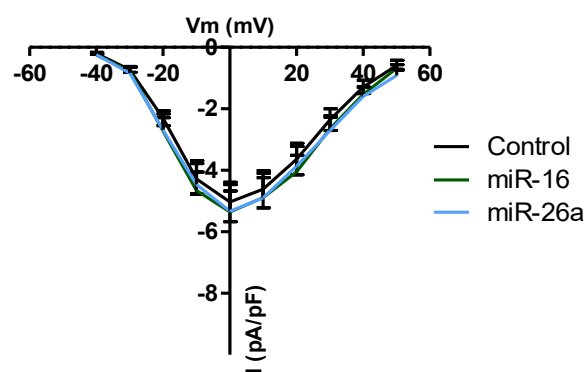
6.3.7 Peak calcium current is reduced with miR-16 and miR-26a in the apex only

As CACNB1 was reduced and encodes a protein involved in LTCC function, we investigated this using patch clamp. This experiment was conducted by Eef Dries, a post-doc with extensive patch clamping experience. In apical cardiomyocytes, peak calcium current was significantly reduced with miR-16 and miR-26a (Figure 6.6A), although unchanged in the base (Figure 6.6B). In contrast, inactivation and recovery curves for apex and base were unchanged with miR-16 or miR-26a (Figure 6.6C to F).

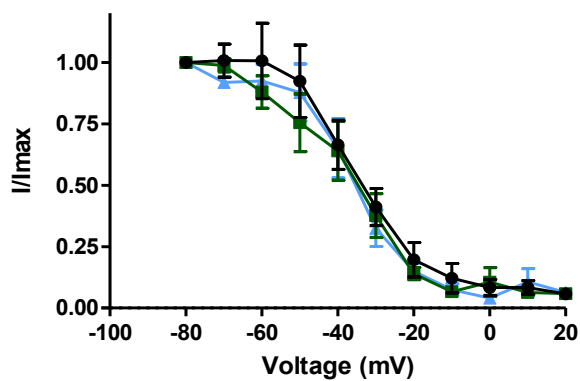
A Peak current in apex



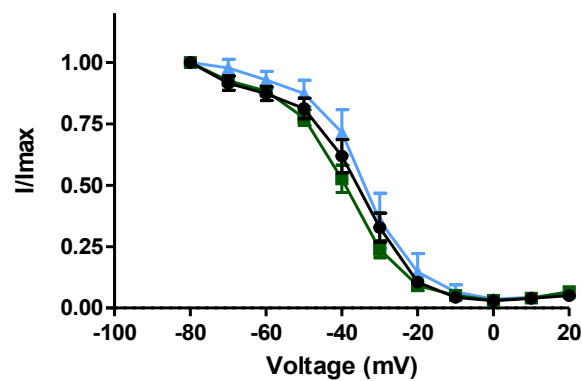
B Peak current in base



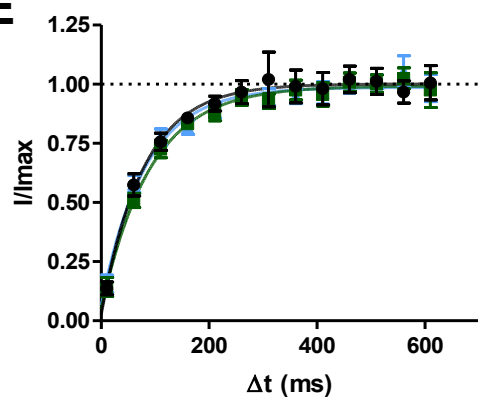
C Inactivation in apex



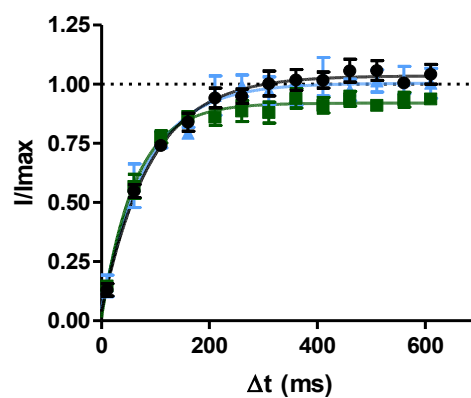
D Inactivation in base



E Recovery from inactivation in base



F Recovery from inactivation in apex



G

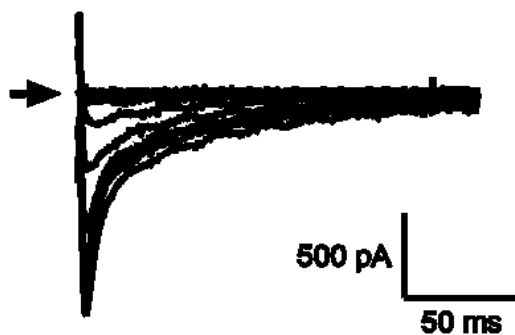


Figure 6.6: Calcium current patch clamp of miR-16 and miR-26a transfected cardiomyocytes

Patch clamp of miR-16 and miR-26a transfected adult rat cardiomyocytes. **A** Peak calcium current amplitude in apex (Control n/N=11/7, miR-16 n/N=11/7 and miR-26a n/N=11/6). **B** Peak calcium current amplitude in base (Control n/N=16/8, miR-16 n/N=14/7 and miR-26a n/N=10/6). **C** LTCC current inactivation in apex (Control n/N=11/6, miR-16 n/N=9/6 and miR-26a n/N=7/4). **D** LTCC current inactivation in base (Control n/N=9/6, miR-16 n/N=9/5 and miR-26a n/N=8/5). **E** Recovery from inactivation in apex (Control n/N=8/6, miR-16 n/N=8/5 and miR-26a n/N=7/5). **F** Recovery from inactivation in base (Control n/N=6/5, miR-16 n/N=7/5 and miR-26a n/N=7/4). **G** A representative patch clamp protocol. Statistical comparisons were made with RM-ANOVA. Data is displayed as mean $\pm$ SEM, with significance shown as follows: * = $p < 0.05$ for miR-16 *versus* control, § = $p < 0.05$ for miR-26a *versus* control.

6.3.8 PTX pre-treatment abolishes the negative inotropic effect of both miR-16 and miR-26a

Since GNB1 and RGS4 are both involved in G-protein signalling, the role of *Gai* in the baseline negative inotropy induced by miR-16 and miR-26a was investigated. This was done by PTX pre-treatment to inhibit *Gai* activity (Mangmool and Kurose, 2011). This resulted in a restoration in the baseline contractility of miR-16 (Figure 6.7A, experiment conducted by Rory Clayton) and miR-26a (Figure 6.7B) transfected cells where no change was observed in control transfected cells.

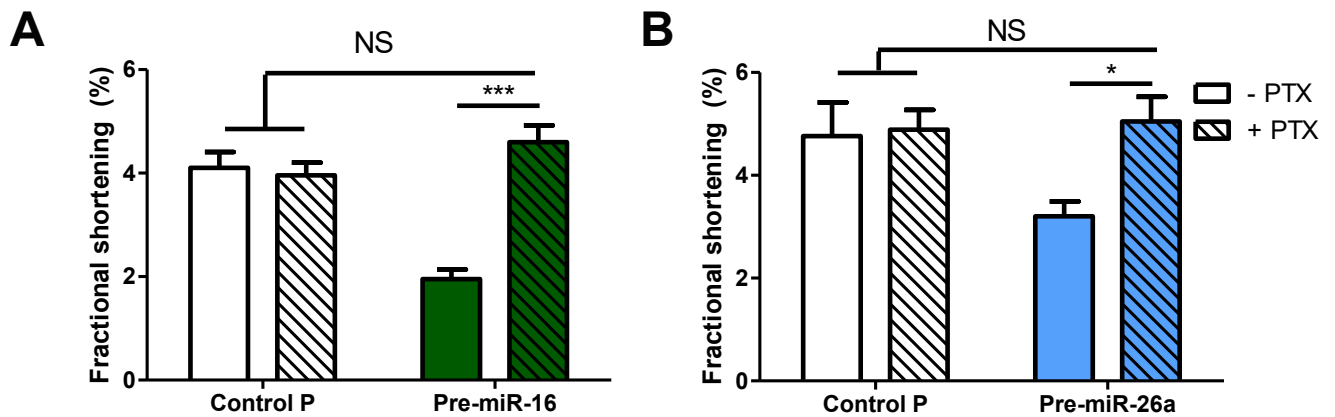


Figure 6.7: PTX pre-treatment restores contractility of miR-16 and miR-26a transfected cardiomyocytes

Baseline fractional shortening of miR-16 (A) and miR-26a (B) transfected adult rat apical cardiomyocytes (as in Chapter 3) pre-treated with PTX. $n/N = 30/6$ throughout, where 'n' is equal to the number of cardiomyocytes and 'N' represents number of rats. Data is displayed as mean $\pm$ SEM, with significance shown as follows: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

6.4 Discussion

Given the key elements of TTS recapitulated with miR-16 and miR-26a *in vitro*, and the clear predisposition and exacerbation of TTS demonstrated with AAV-miR treatment *in vivo*, we investigated the mechanisms by which this may occur.

6.4.1 Utilisation of bioinformatics for miR target identification

Identification of putative miR targets can be carried out by *in silico* methods, or by using sequencing and proteomic approaches. Since RNAseq would not capture all miR targets, as miRs can silence or degrade mRNA, it would require combination with proteomics to evaluate changes in actual protein levels. This would carry considerable expense and be subject to time course differences present between various *in vitro* and *in vivo* models used. As such, we chose to adapt readily available bioinformatic techniques to identify specific genes of interest.

To predict which genes miR-16 and miR-26a are able to target, a bioinformatic process was designed to robustly locate genes associated with cardiomyocyte contractility, as shown in Figure 2.8. miRWalk 2.0 was used to predict target gene binding by analysing 3'UTR (untranslated region) sequence homology by cross-comparing 12 different miR databases (Dweep and Gretz, 2015). These databases included miRWalk, MicroT4, miRanda, miRBridge, miRDB, miRMap, PICTAR2, PITA, RNA22, RNAhybrid and Targetscan. This produced a list of thousands of genes, and a stringent threshold was set, such that only genes located in 7 or more databases were included. This list was then stratified using the Panther DB protein classification system to obtain a list of genes associated with contractility (Mi *et al.*, 2013, 2017).

This generated the list of 33 targets for miR-16, and 29 for miR-26a (Table 6.1 and Table 6.2 respectively). This included genes involved in the regulation of the adrenergic system, calcium handling and protein kinases. We used bioinformatic analysis that identifies targets based upon sequence homology of miR with 3'UTR region of target genes. Since this is predicted, it yields targets that require validation, however enabled for rapid identification of potential targets.

6.4.2 Validation of predicted targets

miRs classically act by silencing or degrading mRNA, so the list of targets to be investigated was further narrowed down by selecting genes that could explain results seen in Chapter 3 and 5 by a reduction in expression. For miR-16, this was considered to be CACNB1, GNB1, GNG12 and RGS3. For miR-26a, this was CACNA1C, CACNB2 and RGS4. ADRBK1 was also included since it encodes GRK2 which is required for stimulus trafficking to occur. Plasmids containing a luciferase enzyme gene and each respective gene of interest was synthesised in-house, and luciferase assays conducted using HEK293 cells as reporter systems. This enabled for proof of miR targetting in a reporter cell type, as validation of biological miR-target interaction. Luciferase assays performed validated CACNB1 and GNB1 as miR-16 targets, and CACNA1C and RGS4 as miR-26a targets (Figure 6.1 and Figure 6.2 respectively). In combination with the *in silico* analysis, this technique robustly identified confirmed miR targets for specific investigation in a cardiac model.

6.4.3 AAV-miR regulation of proteins *in vivo*

First, RNA and protein were isolated from AAV-control and AAV-miR treated animals, 6 weeks after infection. RTqPCR was conducted for the targets identified by luciferase assay to test for preliminary involvement in our model. CACNA1C, CACNB1 and GNB1 mRNA were reduced *in vivo* after AAV-miR (Figure 6.4A, B and D), although RGS4 was not

(Figure 6.4C). Since miRs degrade or silence mRNA, data obtained from RTqPCR was not definitive and mRNA levels could be unchanged if miR target mRNA is silenced, therefore subsequent western blot was required. CACNB1, RGS4 and GNB1 protein levels were all reduced *in vivo* by AAV-miR in comparison to AAV-control, with no difference between apex and base (Figure 6.5A, B and C respectively). This indicates that these are regulated *in vivo* by miR-16 and miR-26a. CACNA1C could not be blotted for technical reasons, but since CACNB1 is targeted by miR-16, whereas CACNA1C is targeted by miR-26a, changes in LTCC function was specifically assessed for each miR by calcium current in Chapter 3.

The lack of difference between apex and base was expected, given that the biological effect of miRs on protein expression would be expected to be similar regardless of location (Ha and Kim, 2014). As such, it is extremely likely that the difference in functional outcome observed in TTS from these miRs results from the inherent differences in adrenergic system and compartmentation demonstrated between apical and basal cardiomyocytes (Wright *et al.*, 2018).

CACNB1 codes for the L-type calcium channel beta-1 subunit ($\text{Ca}_v\beta$), which forms a heterotetrameric polypeptide complex along with the $\alpha 1$, $\alpha 2/\delta$, β , and, in some tissues, γ subunits. β and $\alpha 2/\delta$ form accessory subunits that modulate the biophysical properties and trafficking of the $\alpha 1$ subunit to the membrane (Bodi *et al.*, 2005). $\text{Ca}_v\beta$ is responsible for the regulation of channel surface expression, degradation, and gating (Buraei and Yang, 2013). Indeed, LTCC current is markedly reduced if accessory subunits are absent, reintroduction of $\text{Ca}_v\beta$ enhances surface expression of LTCC and increases channel open probability (Dolphin, 2003). This is facilitated by shifting the voltage dependence of activation to more hyperpolarised voltages (Weissgerber *et al.*, 2006). As such, a reduction in $\text{Ca}_v\beta$ would result in a hypothesised reduction in cardiomyocyte LTCC current.

GNB1 codes for G-Protein Subunit Beta-1 ($G\beta$), which forms a dimeric protein with $G\gamma$ to bind $G\alpha$ when inactive, constituting the heterotrimeric G-protein. Upon GPCR activation, $G\alpha$ and $G\beta\gamma$ dissociate to exert their downstream effects. Liberation of $G\alpha$ results in inotropy and lusitropy secondary to $G\alpha_s$, which is opposed by activity from $G\alpha_i$. $G\beta\gamma$ can independently activate GRKs, which act to terminate adrenergic signalling by resulting in receptor internalisation. Consequently, a reduction in $G\beta$ would result in enhanced $G\alpha$ signalling, to increase activity from both $G\alpha_s$ and $G\alpha_i$, as well as reducing desensitisation to adrenergic stimulation by reducing GRK activity.

RGS4 belongs to a family of proteins that accelerate $G\alpha$ GTPase activity markedly (up to 2000-fold) to result in termination of signalling from $G\alpha$ and $G\beta\gamma$, as well as directly antagonise $G\alpha$ -mediated signal generation (Anger, Zhang and Mende, 2004). The vast weight of experimental evidence supports the action of RGS proteins on $G\alpha_i$ and $G\alpha_q$ subunits, with a lack of data suggesting interaction with $G\alpha_s$ (Zhang and Mende, 2011; Magalhaes, Dunn and Ferguson, 2012). A reduction in RGS4 would consequently potentiate signalling from $G\alpha_i$.

6.4.4 miR-16 and miR-26a alter LTCC function

Given that calcium handling proteins were demonstrated to be regulated by AAV-miR, and miR-26a was shown to directly target CACNB1, the direct effect of these miRs on LTCC current was investigated. Both miRs significantly reduced peak calcium current in the apex, but not base (Figure 6.6A and B). There were no differences in channel inactivation or recovery (Figure 6.6C to F), suggesting existing channel function is maintained, although shuttling of new channels may be affected. This shows that LTCC is impaired specifically in the apex by TTS-associated miRs, and in combination with increased $G\alpha_i$ activity, identifies the mechanism by which they are exerting their functional effects. Interestingly, LTCC can

be organised into caveolae coupled to β_2 AR (Balijepalli *et al.*, 2006), which may explain the differential effect observed between apex and base at rest and during stress.

6.4.5 Negative inotropic mechanism of miR-16 and miR-26a in apical cardiomyocytes is G α i dependent

Since GNB1 and RGS4 both regulate G-protein function and would potentiate G α i as aforementioned, we inhibited G α i with PTX to investigate the role in the reduction in baseline contractility of apical cardiomyocytes *in vitro*. This restored the baseline contractility with both miR-16 and miR-26a to levels of control transfection cardiomyocytes (Figure 6.7A and B), demonstrating that the negative inotropic effect of miR-16 and miR-26a is G α i dependent. It can be concluded that both RGS4 and GNB1 are independently increasing G α i activity, since are targeted by different miRs (Section 6.3.1).

This is particularly interesting, as Paur *et al.* previously demonstrated that the mechanism of TTS was likely to be β_2 AR-G α i. Given that G α i activity is increased by both miR-16 and miR-26a, it is likely that this would exacerbate the severity of TTS induced *in vivo*.

6.4.6 Summary

We have validated CACNB1, RGS4 and GNB1 as targets of TTS-associated miR *in vivo* by luciferase assay, RTqPCR and western blot. These systems have been demonstrated to be functionally altered, since the mechanism of the negative inotropic changes with miR-16 and miR-26a has been shown to be G α i dependent and occurred alongside reductions in peak calcium current.

7 Discussion

7.1 Overview

TTS represents a poorly understood disease, with significant mortality and long-term morbidity (Templin *et al.*, 2015; Schwarz *et al.*, 2017). Understanding the pathogenic mechanism of TTS is required to improve ongoing patient care. We hypothesised that TTS-associated miR-16 and miR-26a could represent novel factors predisposing to or exacerbating the changes in contractility observed in TTS.

Within this thesis, we have shown *in vitro* that miR-16 and miR-26a specifically reduce baseline contractility of apical cardiomyocytes. This includes alterations in calcium handling, with reduced calcium transient amplitude and SR calcium content. Together, they reduce sensitivity to adrenaline in the apex, and amplify maximal response to adrenaline in the base, but do not change β_2 AR response. *In vivo*, in our established model of TTS, serum levels of miR-16 and miR-26a are not changed acutely by adrenaline. AAV-miR treatment produces greater hypokinesia in the apex and hypercontractility in the base. When investigating the mechanism of action, this includes altered G-protein signalling and calcium current activity.

7.2 Integrated mechanism of action of TTS-associated miRs

The overarching effect of AAV-miR *in vivo* in TTS was to increase the hypercontractility in the base and hypokinesia in the apex following adrenaline administration (Figure 5.15). This was reflected in the adrenaline concentration-response curves *in vitro*, with miR-16 and miR-26a in combination reducing the sensitivity to adrenaline in the apex, and increasing the maximal response in the base (Figure 3.6). The *in vitro* data clarifies the mechanism by which TTS-associated miRs are exerting their effects to suggest how AAV-miR is working *in vivo*.

The amplified response to adrenaline in the base is a feasible consequence of accentuated $G_{\alpha s}$ signalling and reduced adrenergic desensitisation, which can both occur secondary to a reduction in G_{β} , as observed here (Figure 6.5). Together, this would result in a greater maximal contractility to high concentration adrenaline, since $G_{\alpha s}$ activity would be generally increased and undergo reduced signal termination.

The reduction in sensitivity in the apex required synergism of both miRs to occur, suggesting multiple points of action within the same pathway, as we note that G_{β} is targeted by miR-16 and RGS4 by miR-26a (Figure 6.1 and Figure 6.2 respectively). This would combine to profoundly increase $G_{\alpha i}$ activity that we have directly demonstrated independently with each miR by restoration of baseline contractility following PTX pre-treatment (Figure 6.7). This would result in the observed hypokinetic changes with greater stimulus trafficking to $G_{\alpha i}$, and a greater proportion free to act. The resultant effect would be to counteract the positive inotropic effect of adrenaline, as seen in our pre-clinical rodent model of TTS (Paur *et al.*, 2012). This would reduce sensitivity to adrenaline and predispose to the negative inotropic changes observed. As described above, Figure 7.1 illustrates how alterations in G_{β} and RGS can influence the adrenergic signalling pathway and depending on the point of action in the signalling pathway, could confer increased or reduced contractility following β AR stimulation depending on numerous other factors to be discussed. Taken together, these reproduce key differences in apicobasal response to adrenaline as seen in TTS and provide an important insight into the mechanism of action of TTS-associated miRs.

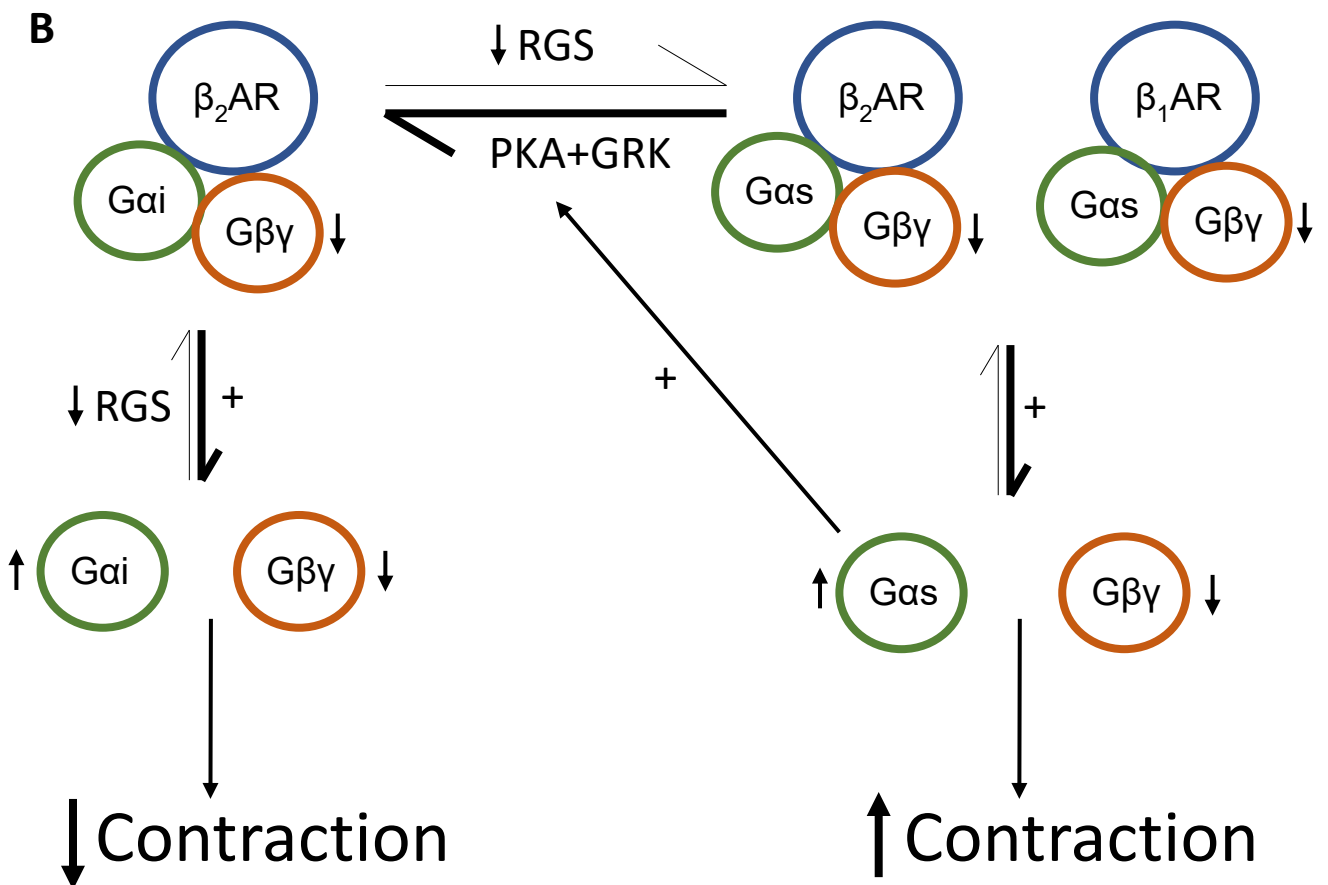
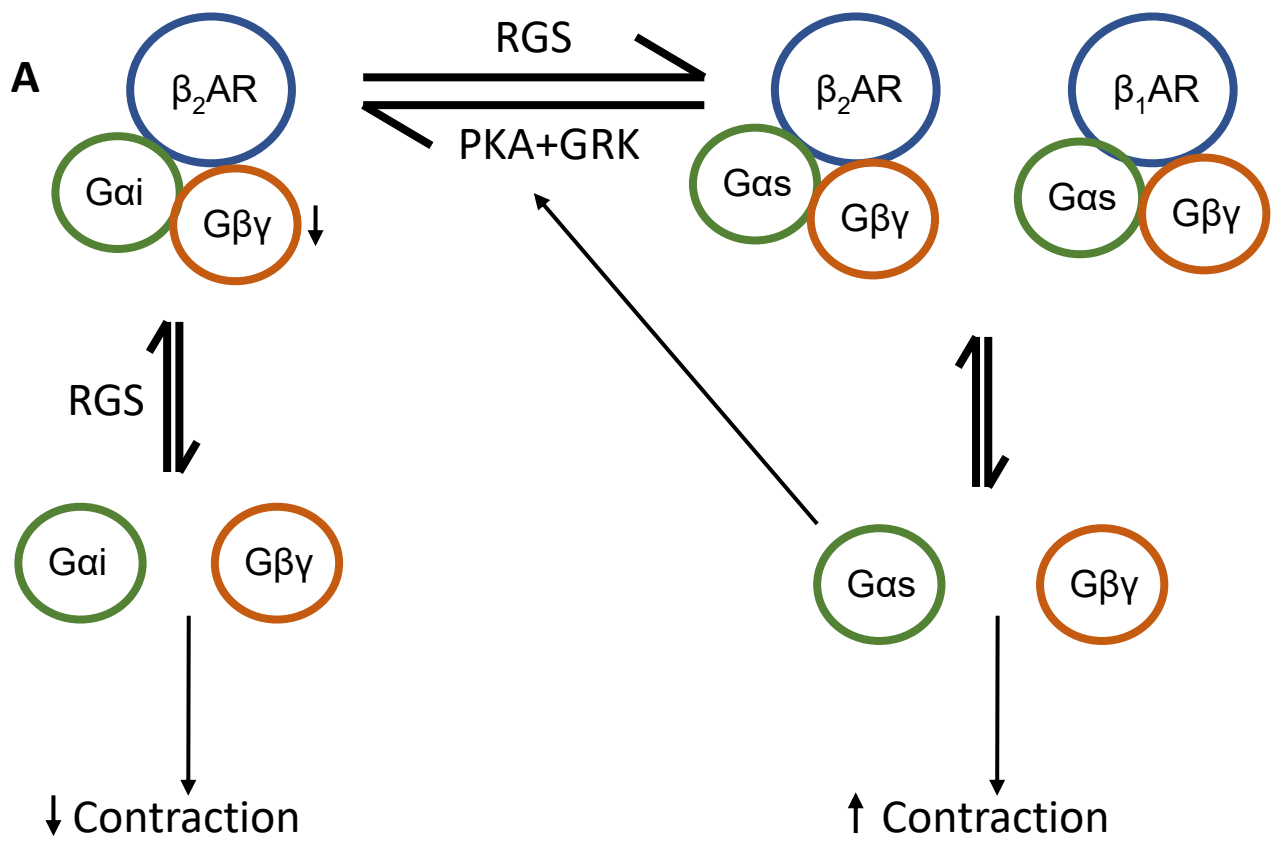


Figure 7.1: Schematic representation of the alterations in the β AR signalling pathway

A The β AR signalling pathway adapted from Paur et al. (Paur *et al.*, 2012). Upon activation by catecholamines, β_1 AR and β_2 AR can both signal via canonical G α s signalling pathways to result in an increase in contraction. As signal transduction occurs through this pathway, stimulus trafficking of the β_2 AR to β_2 AR-G α i can result, which is favoured by PKA and GRK activity and opposed by RGS. This pathway results in a reduction in contractility. Both G α s and G α i act by dissociating from G $\beta\gamma$ in order to subsequently exert their functional effect. G α i GTPase activity is accelerated by RGS to encourage resequentering by G $\beta\gamma$. G $\beta\gamma$ can independently act to desensitise adrenergic response by recruiting GRK to result in receptor internalisation (not shown). **B** Upon treatment with miR-16 and miR-26a, the schematic in Figure 7.1A is altered such that protein levels of RGS and G β are reduced. Reduced G β would result in increased activity of both G α s and G α i signalling pathways, with greater subsequent stimulus trafficking to β_2 AR-G α i. The reduction in RGS would favour β_2 AR-G α i signalling further by enabling more stimulus trafficking and reducing termination of G α i activity. Reduced G β would additionally result in less desensitisation of β AR-G α s response (not shown), potentiating both signalling arms.

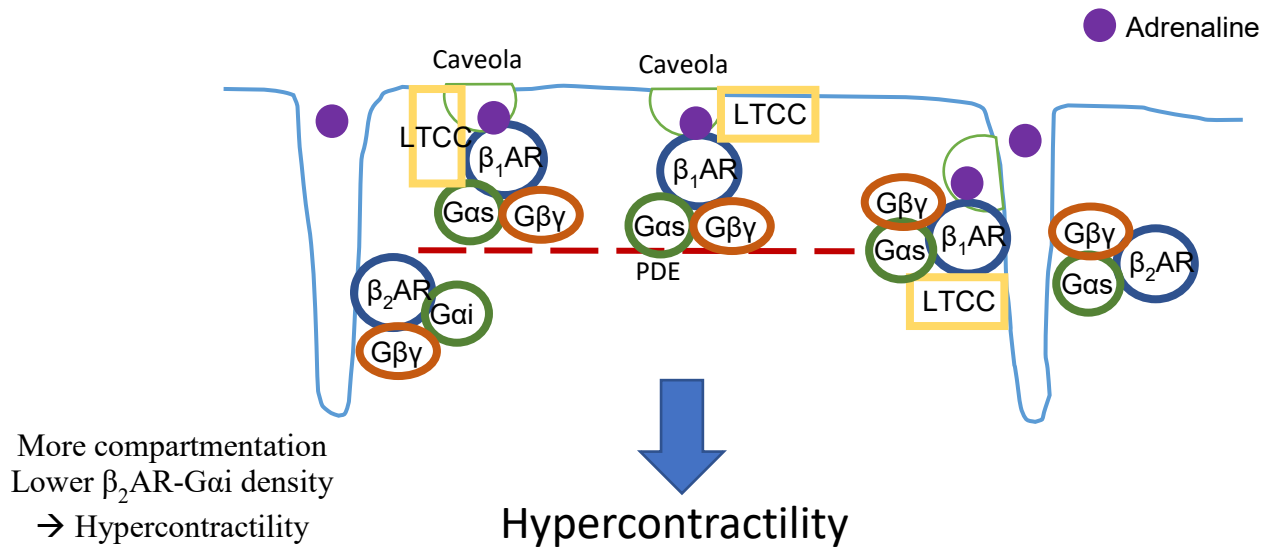
The divergent functional effect observed in apex and base in the *in vivo* and *in vitro* experiments conducted with miR-16 and miR-26a is also abundantly clear in the clinical TTS. This occurs despite a lack of difference between apex and base in protein expression following miR-16 and miR-26a. Although, this was expected, as the biological effect of miRs would be predicted to be similar (Ha and Kim, 2014) regardless of cellular location within the heart. It is extremely likely that the difference in functional outcome observed in TTS results from the inherent differences in adrenergic system and compartmentation recently demonstrated between apical and basal cardiomyocytes (Wright *et al.*, 2018), alongside the greater density of β_2 AR and greater β_2 AR response in the apex (Paur *et al.*, 2012; Wright *et al.*, 2018). This favours the negative inotropic adrenergic signalling axis at the apex (Paur *et al.*, 2012), although seemingly more so in post-menopausal women.

Indeed, Wright et al. recently provided further evidence to explain how the physiological differences present between apical and basal cardiomyocytes arise. Basal cardiomyocytes

exhibit a higher degree of microdomain organisation with greater T-tubular and caveolar densities (Wright *et al.*, 2018). This results in β_2 AR and cAMP signals that are smaller and shorter-lived in the base, consequent from regulation of phosphodiesterase 4 and caveolar organisation, constraining β AR-G α s-cAMP in the caveolin-containing compartment and localising cAMP produced (Wright *et al.*, 2018). They would therefore be less likely to produce β_2 AR phosphorylation and the resulting G α s to G α i switching. Thus, the highly regulated basal cardiomyocytes are prevented from undergoing the negative inotropic changes that occur in the apex.

Given that LTCC is known to colocalise with β_2 AR in caveolae (Balijepalli *et al.*, 2006), in highly compartmentalised basal cardiomyocytes, we suggest that the LTCC is localised within the same compartment as the G α s-coupled receptors and therefore preferentially phosphorylated by the cAMP. In the base, this results in increased LTCC activity due to enhanced channel phosphorylation. In the apex, the delocalisation of the LTCC from caveolae reduces this and permits direct interaction with the β_2 AR-G α i components, to decrease channel activity. Compartmentation may also sequester G α i away from the LTCC at the membrane, preventing the reduction in baseline contractility that occurs in apical cardiomyocytes following treatment with miR-16 or miR-26a *in vitro*. The effect of the inherent differences between apical and basal cardiomyocytes has on the alterations following miR-16 and miR-26a is summarised in Figure 7.2, where the feasibility of divergences in the signalling pathway is represented.

Basal cardiomyocyte with TTS-associated miRs



Apical cardiomyocyte with TTS-associated miRs

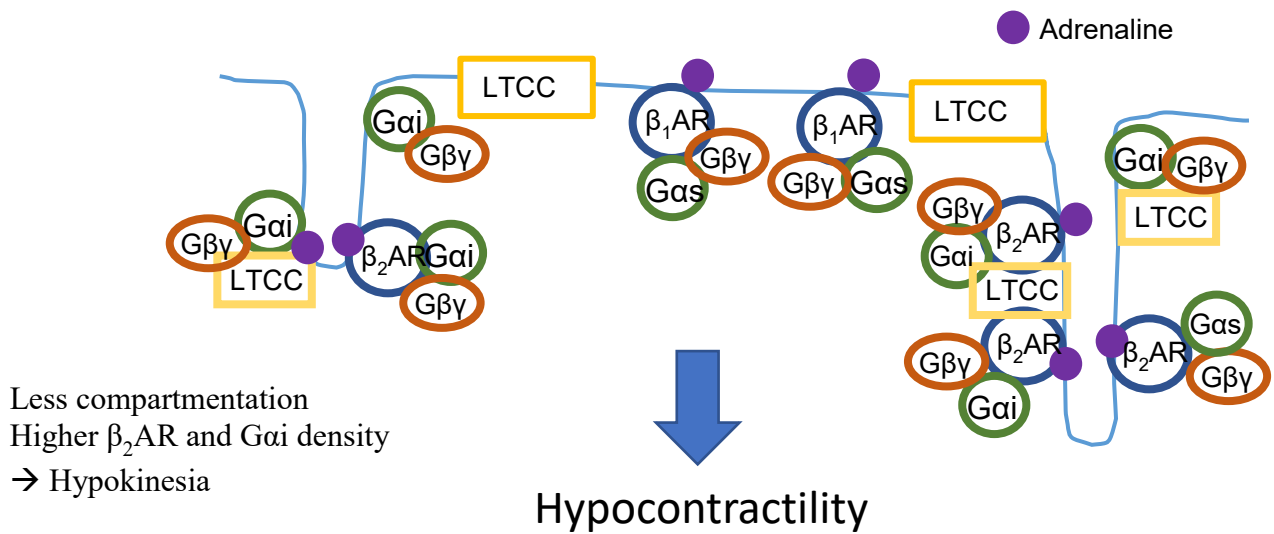


Figure 7.2: A schematic representation of how apicobasal differences in contractility arise in TTS following miR-16 and miR-26a

After exposure to miR-16 and miR-26a, the apex and base exhibit divergent changes in contractility following high concentration catecholamines, as seen in TTS. We have shown (Paur *et al.*, 2012) that the β_2 AR and/or $G_{\alpha i}$ are more concentrated in the apex, so the hypocontractile effects of that axis will outweigh the hypercontractile effects of β_1 AR or β_2 AR coupled to $G_{\alpha s}$. Additionally, we have recently shown (Wright *et al.*, 2018) that β AR- $G_{\alpha s}$ -cAMP is constrained in the caveolin-containing compartment and the cAMP produced is localised by phosphodiesterase activity. This happens to a greater extent in basal than apical cardiomyocytes, as apical cells are less compartmentalised. Here we suggest that the LTCC is localised within the same compartment as the $G_{\alpha s}$ -coupled receptors and therefore preferentially phosphorylated by the cAMP. In the base, this results in the LTCC being highly phosphorylated and therefore active. In the apex, the delocalisation of the LTCC reduces this activation as well as allowing direct interaction with the β_2 AR- $G_{\alpha i}$ components, which lead to a further decrease in activity.

The impairment of LTCC function specifically in the apex by TTS-associated miRs (Figure 6.6), and in combination with increased $G_{\alpha i}$ activity (Figure 6.7), provide explanation for the reduction in baseline contractility observed in the apex *in vitro* (Figure 3.3). This aligns with the reduction in calcium transient amplitude noted (Figure 3.9) and suggests the reduction in SR calcium load is secondary to reduced calcium entry into the cell through LTCC.

Interestingly, since LTCC can also be organised into caveolae with β_2 AR (Balijepalli *et al.*, 2006), this may further explain the differential effect observed between apex and base at rest and during stress as evidenced here and discussed above.

It is interesting that these changes occurred in the baseline contractility of miR treated cardiomyocytes, whereas baseline fractional shortening was unchanged in AAV-miR treated animals. This could be due to physiological differences *in vivo* that are able compensate for these resting differences in cardiomyocyte function. However, as heart rate was significantly reduced by AAV-miR, compensation from heart rate is unlikely to explain this difference as

rats exhibit a positive force-frequency relationship at this range (Janssen and Periasamy, 2007). Instead, the reduced heart rate (Figure 5.12) reflects the increased Gai activity demonstrated elsewhere. There are considerable temporal differences over which the miR upregulation occurs, with 48 hours *in vitro* versus 6 weeks *in vivo*. Furthermore, it is likely that the concentration of miRs administered *in vitro* is higher, since the increase in miR-16 and miR-26a is 5000-fold and 500-fold respectively, which can profoundly affect baseline contractility. *In vivo* it appears that a minor non-significant reduction in the apex and mid-LV does occur, and there is other evidence of increased Gai activity such as reduced heart rate and hypokinesis in the apex following adrenaline administration.

7.3 Translational application in human

miR-16 and miR-26a represent novel factors increased in TTS patients, and were first identified in the context of TTS in humans (Jaguszewski *et al.*, 2014). We have shown that when transfected into rat cardiomyocytes *in vitro*, they recapitulate pathognomonic changes present within the clinical TTS syndrome. Indeed, in a preclinical rat model of TTS, we have identified a predisposition of miR treated rats for the generation of TTS, exacerbating the classic contractility changes.

However, when considering the translational capacity of this data, the lack of effect of miR-16 and miR-26a in hiPSC-CMs which was discussed in Chapter 4 must be considered. Given that miR-16 and miR-26a were only shown to exert changes in baseline contractility and calcium current in apical but not basal rat cardiomyocytes *in vitro*, this suggests the effect of TTS-associated miRs is extremely specific. Indeed, the organisation of the adrenergic system appears to be particularly important, and this is not fully developed in hiPSC-CMs. It is perhaps unsurprising that hiPSC-CMs exhibiting a maturity comparable to mid-gestational

cardiomyocytes (Kane, Couch and Terracciano, 2015; Louch, Koivumäki and Tavi, 2015) do not undergo the same changes as a highly specialised adult cardiomyocyte.

Furthermore, whilst Borchert et al. observed numerous alterations both in general physiology and the adrenergic system of hiPSC-CMs generated from TTS patients (Borchert *et al.*, 2017) that align well with our findings in basal cardiomyocytes, miR-16 and miR-26a were not altered in this scenario. Therefore, their hiPSC-CM data is not directly comparable to the hiPSC-CM data we obtained in Chapter 4, and they did not identify any genetic differences in these cells via whole-exome sequencing.

It would not be possible to conduct a mechanistic study of this nature in humans, so *in vitro* and *in vivo* models must be used. When contextualising the relevance of the data we have obtained, it is important to consider that the origination of the association of miR-16 and miR-26a with TTS was in human and then applied to rats, wherein changes were observed that represented the clinical human syndrome. Indeed, we also replicated the baseline contractility changes seen within rat cardiomyocytes in healthy adult human cardiomyocytes, demonstrating the translational capacity of preclinical models. Therefore, it appears that the data presented in this thesis can be applied to the clinical TTS within humans.

Of note, the confirmation of the pathogenic role of miR-16 and miR-26a within TTS provides rationale for monitoring their levels in patients to guide therapy. In particular, they may be able to be used to assess possible risk of recurrence of TTS in the future, the chance of which is currently unknown. The use of anti-miRs in the prevention of TTS could be investigated in the capacity of modulating recurrence, since patients at risk could easily be followed. However, the application of anti-miRs for the primary prevention of TTS within the wider population would be extremely challenging, since it cannot be pre-emptively predicted which patients will develop TTS without population wide screening of miR-16 and miR-26a.

7.4 Role of miR-16 and miR-26a in the pathogenesis of TTS

The convincing role of β_2 AR-G α_i coupling in the pathogenesis of TTS was discussed at length in Section 1.2.7, but how this results in a specific subset of patients generally developing TTS is not clear. Here, we have clearly shown that miR-16 and miR-26a predispose to TTS in a preclinical rodent model.

TTS can be induced in the absence of miR-16 and miR-26a (Figure 5.2), although when are present, they predispose to TTS *in vivo* at lower adrenaline concentrations, and exacerbate the phenotypic changes in contractility (Figure 5.15). This demonstrates that when miR-16 and miR-26a are increased, they lower the threshold for TTS induction, making it more likely for TTS to develop at more modest catecholamine rises. This is in the context of post-menopausal women who are likely predisposed to TTS for other reasons, such as oestrogen deprivation (Lyon *et al.*, 2008). Therefore, when increased, it is clear that TTS-associated miRs could explain why certain patients develop TTS through the changes in the adrenergic system we have described. Furthermore, TTS-associated miRs could play an important role in assessing risk of recurrence of TTS in patients if they persist as described above.

Indeed, we have shown that they act by altering G-protein signalling to polarise the adrenergic system to generate these changes, with increased G α_i and G α_s activity, more stimulus trafficking to β_2 AR-G α_i and less β AR desensitisation. This represents a novel explanation to explain why a subset of patients may be predisposed to generate TTS over others.

Together the miRs produce hypercontractile changes in the base, and hypokinetic changes in the apex, depending on the inherent differences in the adrenergic system present within the apex and base. This may occur alongside differences in excitation-contraction coupling, with

reduced LTCC function specifically in the apex. This could also result in the negative inotropic changes in the apex after stress, owing to the β_2 AR-LTCC colocalization which has been observed (Balijepalli *et al.*, 2006).

miR-16 and miR-26a also provide the important link to neuropsychiatric disorders (Bai *et al.*, 2012; Katsuura *et al.*, 2012; Radu Enatescu *et al.*, 2016) that are frequent in TTS (Templin *et al.*, 2015), and we have demonstrated their probable importance in this context. Indeed, we observed altered animal behaviour, suggesting TTS-associated miRs may be interacting simultaneously with the brain and the heart. This could result in differences in background catecholamine levels, or differences in emotional processing following stress. Robust behavioural analysis is required to comment further about the specific effect TTS-associated miRs on activity and animal growth. Despite this, these observations provide strong preliminary evidence that miR-16 and miR-26a link TTS and psychiatric disorders.

7.5 Limitations

Throughout the course of this project, various limitations existed which required optimisation and careful consideration about the best way to approach experiments.

7.5.1 Culture of adult cardiomyocytes

The first major limitation encountered occurred with the 48 hour transfection of cardiomyocytes. Adult cardiomyocytes dedifferentiate in culture, losing their characteristic electrophysiological properties and striations (Zhang *et al.*, 2010). Indeed, we also noted alterations in the β_2 AR response. As previously discussed in Chapter 3, Chakir *et al.* identified regulator of G-protein signalling 2 (RGS2) as a key terminator of β_2 AR-G α_i signalling, which increased in the absence of agonist stimulation in culture (Chakir *et al.*, 2011). This amplifies β_2 AR response as β_2 AR-G α_i is inhibited. PTX usually has the effect of

increasing the β_2 AR response approximately five fold (Paur *et al.*, 2012), whereas it did not after culture (Appendix 2). As a result, given the usage of cultured cardiomyocytes in this study, whilst it is possible to note β_2 AR-G α s is unchanged, no conclusions pertaining to β_2 AR-G α i can be drawn from the direct assessment of β_2 AR response.

7.5.2 Paucity of healthy human cardiomyocytes

Another limitation concerned the lack of healthy human tissue available for experimentation. We were able to conduct one preliminary experiment with healthy human cardiomyocytes (Figure 4.2), but owing to the precious nature of this tissue, even this was viewed as a success. We considered using failing human hearts as these are more readily available, although as discussed in Section 4.4.1, they exist in a pathological state wherein many physiological parameters are altered, including response to catecholamines (Harding *et al.*, 1992). miR profile is also perturbed in heart failure, with reduced levels of miR-1 in heart failure (Kumarswamy *et al.*, 2012). As a result, cardiomyocytes from failing hearts were not used, as the findings would not be translatable to a normal physiological state and may confound further miR modulation. Additionally, it is not possible to get human tissue from TTS patients, as there is no indication to take a myocardial biopsy. Whilst the utilisation of human tissue was undertaken wherever possible, the increased reliance on non-human models was required.

7.5.3 Pre-existing role of TTS-associated miRs

Whilst we have evidence to suggest that miR-16 and miR-26a predispose to TTS, we do not know if these factors pre-exist in TTS patients. It would require a large longitudinal epidemiological study to investigate this which is currently not feasible. As such, we cannot currently address this question, or predetermine which patients will generate TTS, although this may be possible to screen in the future.

7.5.4 Low-throughput methods for functional analysis

Several techniques used for the assessment of the functional mechanism of action of miR-16 and miR-26a *in vitro* were low throughput. Specifically, pharmacological assessment of cardiomyocytes using Ionoptix (Section 2.4.4) was only able to measure approximately one cardiomyocyte in 90 minutes. There are higher throughput contractility systems that have recently become available, although at considerable expense.

7.5.5 Presence of non-myocytes in *in vivo* myocardial samples

Whilst the conclusions obtained from PCR and western blot in Chapter 6 were applied to the contractility changes within cardiomyocytes, it must be noted that non-myocytes are present that may also contain targets of miR-16 and miR-26a. Therefore, the role of TTS-associated miRs should be validated in these cell types as described in Section 7.6.4.

7.6 Future work

7.6.1 Effect of miR-16 and miR-26a on behaviour

We conducted a preliminary investigation and found that rat behaviour was altered with AAV-miR treatment. Whilst we saw an increase in activity, we do not know the cause of this, and whether it is linked to the concomitant association of miR-16 and miR-26a with stress, anxiety and depression (Bai *et al.*, 2012; Katsuura *et al.*, 2012; Radu Enatescu *et al.*, 2016). Indeed, we also noted a reduction of rat weight with AAV-miR, which we equally cannot attribute to anything specific. Therefore, comprehensive assessment of rat behaviour is required to understand the role miR-16 and miR-26a are playing in the psychosocial status of AAV-miR treated rats. This would provide an important explanation for increased frequency of neuropsychiatric disorders in TTS.

7.6.2 Differences between apical and basal cardiomyocytes

Further work is still required to explain how the apicobasal differences present within TTS occur. Wright et al. have recently produced data to highlight the differences present innately between apical and basal cardiomyocytes, although how this is altered to generate TTS is not clear. As such, this is a key area that requires further work to fully understand the disease mechanisms in TTS.

7.6.3 Downstream negative inotropic pathways

We have shown that the mechanism of the negative inotropic changes with TTS-associated miRs is G α i dependent, although we have not investigated the signalling pathways downstream of this. It is not clear if the direct action of G α i is solely responsible for the changes we have observed, or other downstream pathways such as p38 MAPK could also be exerting a direct negative inotropic effect as previously shown (Liao *et al.*, 2002; Paur *et al.*, 2012).

7.6.4 Role of TTS-associated miRs in other cell types

Given the systemic rise of catecholamines in TTS, and the circulating presence of TTS-associated miRs, it is likely that the non-cardiomyocyte fraction of the heart could similarly be affected. As such, the effect of miR-16 and miR-26a on endothelial cells, fibroblasts and immune cells should be investigated, especially with the endothelial dysfunction and long-term inflammatory and fibrotic changes that occur in TTS and have been discussed (Section 1.2.7).

7.7 Conclusion

Within this thesis, we have shown that TTS-associated miRs represent novel molecules that clarify the pathogenesis of this curious condition. They predispose to TTS *in vivo* by promoting apical hypokinesia and basal hypercontractility. This occurs by reducing sensitivity to adrenaline in the apex and increasing maximal response to adrenaline in the base through alterations in G-protein signalling and calcium handling.

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391–400. doi: 10.1161/CIRCEP.109.894329.

Appendices

1 Luciferase primer design

1.1 Rat CACNB1-3'-UTR and miR-16-5p

Cutting sites for restriction enzymes:

5' - SpeI (BcuI) ACT AGT

3' - HindIII AAG CTT

Sites underlined

Primer:

Forward:

AAA ACT AGT CAGGCTCTATGTGTTGGTGC

Reverse:

AAA AAG CTT CAGTGGGGTTGGGGATTG

CACNB1-3'-UTR (extraction):

miR-16-5p BS marked (position: 960-966, 963-969, 1602-1609, 1617-1623)

CTTGTCTCCCCCGGGAATGAGCTTTGTCTACAGCATACCCTGCCTGCTGCCATCA
GAAGAGAGGGGCCCTCTGCATCTGAGCCCCACATCTCCGTGCCGCCTGGGTGTGG
AGACCATGAGACCATGAATACGTTGGGTCCACCTCATTCTAAACCAAAAAACCTG
CTCTTTCACCTTCACCCCGAGGCCCCAGGGAACAGGCTCTATGTGTTGGTGCCCA

TGAGGTGACCGCCATTCAGGGGCTTCCTGAGGACCTTGGGTCTCCCCTAGGGGTC
TTGGTTGCTGCTGCTTCTCTGTATTTGCCTCTTGTGATCCTGTCCTTTCCCGTGTC
CACTTTCCCCAAGAGAGGCCTCGGTAGCCCCCTACCCCTGGGGTTCCCTTAGCCT
CGGCATCCCTGTAGTCCAGCAGCCCTGCCCCCTCCCCAGCATCACAGCTGGTCCAG
AGAGAGCCGATTGTGCCAACGAGGACTGGGGCCCTGCCCAGCTGCCCACCTCAGG
GATGGGCACCTCATGCCTGTCTCGCCACCTCCTGTGCCAATGTTGCCCCACCCCTT
CCGGGGGTGGGGCACAGCTTCCACTAACAGGTTAGAAGATAGCACTGCCCAACC
TTTCGCTCCCCGTCCCCATCTACTGTCTCGTGCCCATCTGTCTGTCTGTCTGTCTGT
ACTCCTCTAGGAGAGATATTTTGCCACATATAAAACCGCAGTTCTGTCCTTTGCG
GCCACCTCCTGAGCCTGTTTTCTTTGTTGCCACTACACTTTGTCAGCCCGGAAGCC
TCACTGTTAGGGAGCAAGGGGGCGTGCGAGCAAACGCTCTGCCCCAGGGCGGGCG
TCGAGGCTAAGGAGAGATGGTACTTTTATTCCACGGCTTCTGGATTGTCTGTAGG
TGTTGCTCATTGAGCCTCAAGCTCACATCTTCCCTGCCAAGGAGGGTGGGAGTGC
TGCTAACTGAGGTGCTGCTCACTGAGTGGGCTCAAGCCTCACTGGTTCACATCT
TTTTTTTTTTTTTTTTTTTTTTCCTTTTCTTTTTTTTTTCGGAGCTGGGGACCGAACCCA
GGGCCTTGCGCTCGCTAGGCAAATCCCCAACCCCACTGGTTCACATCTTAAAGGC
ACTTTTTATTTTTGCCCCCTTTCTGCTTTGACCTCCCTGTGCCCTAGCAGCTCACCTC
CCTAGGGAAGAGTGAGCTTCAACTCCGCTGGGACTCAGAGGTTTGCCTGCATCTG
CCTCCTGACTGCTAGGATGAAAGACACACGTATGCCACACCCTCAGCAACCAGT
TTGTTGCTGGGTTTTG

Using 1-based sequence positions

```
PRODUCT SIZE: 890, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00
TARGETS (start len)
```

301

1.2 Rat CACNA1C-3'-UTR and miR-26-5p

Cutting sites for restriction enzymes:

5' - SpeI (BcuI) ACT AGT

3' - HindIII AAG CTT

Sites underlined

Primer:

Forward:

AAA ACT AGT GGGAGTTCAAAATCTGGGGC

Reverse:

AAA AAG CTT AACAGCAGAAGGGAGCAGAA

CACNA1C-3'-UTR (extraction):

miR-26-5p BS marked (position: 1389-1396)

CAATTTTCCTTAAGTCAAAGCAACTTTTTTTCCCCCTTTTCGGTTTGGTTGAGC
ATCACGACCTGCAGGTAAACAGTCAGTTTAGATAAGGCGGTGTGTGCCTGGG
CGACTGAGCTGGGCTCCTTTCTGGGCATATGGATTGGTTCAGGAGAGAAGAA
ATATGGGCATCTTTGCGTCACACGTGTGTCCATAGTGGGTCGTCTTGTGTGTCT
GTGCATCCACACGGTACGTGAGCACCCACCCACCCCGCACAAAGTCTGT
AGATCCCCGTTTCGGGTTTGACTGCAGGGAGTTCAAAATCTGGGGCTATTCGAA
AGCAAGAACAAACCACTGTCTCTGCTTCTGCTTCTGAAACGAGAATCTGGTAA

CTGCATTTTCTGTCCCACGAGATATGCAAAAGCAATGCAATAATATCCATTTT
AAAATACGATTGTGAGTTGTGTCAGCATTAAAATTCTATTTTAAAAAACATG
AAATTTAAGGGGAAAACTCAAGAAGACATTTTGCTTCGATATATTCTGTGTA
ATGTTTTATTGCATTGATAATGTTTCTGTTGAAGAACTGTTATACTTGAATT
CAGGTCAGTTTCAGTATTTTTCAAATATTTTTTTAAACTGAATTGCAATTGTG
CCAAGCAAATATAATGACTTAAGTTTGTTTTAAAATTCACTTCTTGTATATTTT
GCTGCATGTAAAGTAAATCATTTTGTATTTGGAGTGTGACAAGCTTTACCTTT
GAGCTCTTAAGTGCTTTTTCTATATGTGGTGGGGGGAAGATGGTTTTCTTTCA
TTTGCAAAGTGAACAAAGGTGTCGCCCCGTGTAAGTCCTCATGCTGCTCTCTG
TTGGCAGGTCTGGGAGGTGCCATTTTGCATATCTTGTGTTTTGTTTCCATATCC
CTCAGTATTTCCAAAACCTGGGGTTTAGCTTGCCCATGCCGCATCTTCCTTTGC
GAGATCAGGATGTGTTCCACCCAGGTCTTCTGCTCCCTTCTGCTGTTCTCTGTG
GAGAATTGTCAAAG

Primer3 Output

No mispriming library specified
Using 1-based sequence positions

OLIGO	<u>start</u>	<u>len</u>	<u>tm</u>	<u>gc%</u>	<u>any th</u>	<u>3' th</u>	<u>hairpin</u>	<u>seq</u>
LEFT PRIMER	290	20	58.81	55.00	0.00	0.00	0.00	
GGGAGTTCAAATCTGGGGC								
RIGHT PRIMER	1005	20	59.23	50.00	0.00	0.00	0.00	
aacagcagaagggagcagaa								
SEQUENCE SIZE: 1026								
INCLUDED REGION SIZE: 1026								

PRODUCT SIZE: 716, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00

```
1 CAATTTTCCTTAAGTCAAAGCAACTTTTTTCCCCCTTTTCGGTTTGGTTGAGCATCACG
61 ACCTGCAGGTTAACAGTCAGTTTAGATAAGGCGGTGTGTGCCTGGGCGACTGAGCTGGGC
121 TCCTTTCTGGGCATATGGATTGGTTCAGGAGAGAAGAAATATGGGCATCTTTGCGTCACA
181 CGTGTGTCCATAGTGGGTCGTCTTGTGTGTCTGTGCATCCACACGGTACGTGAGCACCCC
241 ACCCCACCCCCGCACAAAGTCTGTAGATCCCCGTTTCGGGTTTGACTGCAGGGAGTTCAAA
    >>>>>>>>>>
301 ATCTGGGGCTATTTCGAAAGCAAGAACAAACCACTGTCTCTGCTTCTGCTTCTGAAACGAG
    >>>>>>>>>
361 AATCTGGTAACTGCATTTTCTGTCCCACGAGATATGCAAAAGCAATGCAATAATATCCAT
421 TTTAAAATACGATTGTGAGTTGTGTCAGCATTAATAATTCTATTTTAAAAAACATGAAAT
481 TTAAGGGGAAAACTCAAGAAGACATTTTGCTTCGATATATTCTGTGTAATGTTTTATTG
541 CATTGATAATGTTTCTGTTGAAGAACTGTTATACTTGAATTCAGGTCAGTTTCAGTATT
601 TTTCAAATATTTTTTTTAAACTGAATTGCAATTGTGCCAAGCAAATATAATGACTTAAGT
661 TTGTTTTTAAATTCACCTTCTGTATATTTTGCTGCATGTAAAGTAAATCATTTTGTATTT
721 GGAGTGTGACAAGCTTTACCTTTGAGCTCTTAAGTGCTTTTTTCTATATGTGGTGGGGGGA
781 AGATGGTTTTTCTTTCATTTGCAAAGTGAACAAAGGTGTCGCCCCGTGTAAGTCCTCATG
```


(primer3_results.cgi release 4.0.0)

1.3 Rat RGS4-3'-UTR and miR-26-5p

Cutting sites for restriction enzymes:

5' - SpeI (BcuI) ACT AGT

3' - HindIII AAG CTT

Sites underlined

Primer:

Forward:

AAA ACT AGT CCTTGTTGCCTAGCCCTACA

Reverse:

AAA AAG CTT GTCTTCACTTTGCAGCCACA

RGS4-3'-UTR (extraction):

miR-26-5p BS marked (position: 1328-1335)

GGTTGGTCTGGAGATGGATGTTTGAGAGGAATGAAGGAATGCGGTCCAGGCCTT
GTTGCCTAGCCCTACAAATGACTACTGCTTAGGTGAGGGCAGAAGACCCCGAGG
TTATCTTAATTCTTTCCTCTCTTGATAAGGTGCTCTCCTATTCCTACCCCCAAAGA
ACGTCACCTGTCAGATGAGCCTCTTTACTTCCCCGTGGTTGGTTGCTCACGTTAGG
AACCATGGCCACCTGCAAGTCATTCTAAGCTACCCGCTTACATTTTTCAAAAGCA
TCACCAGGGGGAAAGGTTATGCTGTCCCAGGAGGAAGTAAGCTCTTATGAGCTG
TAAACCCCTTTTTGGTTTTCTGCTTTGAGGTAAGAGCTTGTTTCATCCCATCCCCAA
CACCAAAGTTAGCAGAGACCAACGGAAGGAGAGAGGAAAGAAAAAAAAAAGA

AAGAGAAGAGAAAAGAGGAGGACGGGATATCAGTTGGTAGGACTACTGTTTGG
AGCTTTTTTAAGGCACGTGAAATTACTTGAAAATTTCTCATTGTGTTATTTATGTTG
TTTTGTACAATGTTTTTATTACTGTATTGTTTTATAAATGGAGGTACAGGATATTA
CCCGAATTATTAATGAATGCCCAAGAAGTAATTTTCTTCTCATTCTCTAAAAGT
CCTGCCTTTGAGAGCGCACACAGACTCGTGCATACTGCAGGCACAGCTCCAGTTT
AAATTCCATGCATGAGCACCATTGTGGCTGCAAAGTGAAGACACTCATAGTGTG
AATCCAAGTCTCACCATTATTGAAGAAGTCAGTTTTCTAACAGTACTGTTGTCTCT
CTTGACAGTCTTGCGTATAATATCCCCAGAGCTCCCCCATCAGTGGTCCTAACTC
CTTTTTGTAATACACAAGTCCACAGTCGTGTCTGTATAACTCTCCGGTCTTGCCCT
AATATTATCATGACTTTGAGATTGTACCTGTACTCCCAGACTGCTGTCTCACAAA
TAGGTCTGGGAAAAGTCATTCTGAATGAGAAGTAAATTATTTTATGTAATACATC
TTGAGTGTGTCTTCAGTTGTATTTGCCATCGCTCTTTGGTCTGGCGAGCCTGCCTG
CTGCTGGCCCCTGCAAAGGACGCTCCTTCCAGATGCATGTCTGTTTCTGCCATGT
GCTTTATAGGCCTCAGCGCTGATGCTTCCAATGAAACACATAGATCTGAATGATA
ACAGGAAATAAATGCTTCCTAC

PRODUCT SIZE: 704, PAIR ANY TH COMPL: 0.00, PAIR 3' TH COMPL: 0.00

310

841 AATATCCCCAGAGCTCCCCCATCAGTGGTCCTAACTCCTTTTGTAAATACACAAGTCCAC

901 AGTCGTGTCTGTATAACTCTCCGGTCTTGCCCTAATATTATCATGACTTTGAGATTGTAC

961 CTGTACTCCCAGACTGCTGTCTCACAAATAGGTCTGGGAAAAGTCATTCTGAATGAGAAG

1021 TAAATTATTTTATGTAATACATCTTGAGTGTGTCTTCAGTTGTATTTGCCATCGCTCTTT

1081 GGTCTGGCGAGCCTGCCTGCTGCTGGCCCCCTGCAAAGGACGCTCCTTCCAGATGCATGTC

1141 TGTTTCTGCCATGTGCTTTATAGGCCTCAGCGCTGATGCTTCCAATGAAACACATAGATC

1201 TGAATGATAACAGGAAATAAATGCTTCCTAC

KEYS (in order of precedence):

>>>>> left primer

<<<<< right primer

ADDITIONAL OLIGOS

	<u>start</u>	<u>len</u>	<u>tm</u>	<u>gc%</u>	<u>any th</u>	<u>3' th</u>	<u>hairpin</u>	<u>seq</u>
1 LEFT PRIMER	364	20	59.00	50.00	0.00	0.00	0.00	
AGCTTGTTTCATCCCATCCCA								
RIGHT PRIMER	1088	19	58.91	57.89	0.00	0.00	0.00	
GCCAGACCAAAGAGCGATG								
PRODUCT SIZE: 725, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00								
2 LEFT PRIMER	81	20	59.09	55.00	0.00	0.00	0.00	
GCTTAGGTGAGGGCAGAAGA								
RIGHT PRIMER	859	20	57.66	55.00	14.64	0.00	0.00	
GGGGAGCTCTGGGGATATTA								
PRODUCT SIZE: 779, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00								
3 LEFT PRIMER	184	20	59.02	55.00	0.00	0.00	0.00	
CCTCTTTACTTCCCCGTGGT								
RIGHT PRIMER	924	22	59.06	50.00	0.00	0.00	0.00	
CCGGAGAGTTATACAGACACGA								
PRODUCT SIZE: 741, PAIR ANY_TH COMPL: 0.21, PAIR 3'_TH COMPL: 0.00								
4 LEFT PRIMER	181	20	59.10	55.00	0.00	0.00	0.00	
GAGCCTCTTTACTTCCCCGT								
RIGHT PRIMER	931	21	57.90	52.38	9.46	0.00	0.00	
GGCAAGACCGGAGAGTTATAC								
PRODUCT SIZE: 751, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 6.42								

Statistics

con	too	in	in	not		no	tm	tm	high	high
high	high									
hair-	sid	many	tar	excl	ok	bad	GC	too	too	any_th 3'_th
pin	poly	end								
	ered	Ns	get	reg	reg	GC%	clamp	low	high	compl compl
	X	stab	ok							

Left	2794	0	0	0	0	337	0	1210	307	0	0
5	102	0	833								
Right	3050	0	0	0	0	406	0	1544	341	0	2
162	37	0	558								

Pair Stats:
considered 2434, unacceptable product size 2427, primer in pair overlaps a
primer in a better pair 319, ok 7
libprimer3 release 2.4.0

(primer3_results.cgi release 4.0.0)

1.4 Rat ADRBK1-3'-UTR and miR-16-5p

Cutting sites for restriction enzymes:

5' - SpeI (BcuI) ACT AGT

3' - HindIII AAG CTT

Sites underlined

Primer:

Forward:

AAA ACT AGT GCCCACC CGCCTTTTATAAA

Reverse:

AAA AAG CTT CTGGGTTCTTATGCTGTGCC

ADRBK1-3'-UTR (extraction):

miR-16-5p BS marked (position: 601-607)

TTCACCCATCTGCTGCCCCGCCTGCCACCCGCCTTTTATAAACCTCTAATTTATTT
TGTTGAATTTTATTATTTGTTTTCCCGCAAAGCGGAAAAGGTTTTATTTTGTAAT
TATTGTGATTTCTGTGGCCCCAGCCTGGCCCCAGCCCCAGGGAGGGGCCTGCTT
GCCTCAGCTCCTGCTGCCACCAACCCAGCCACTGTCTAGCACCCCTGCCCCGCCC
CCACCGCATCCCCCTAGGGTCCTTGCTCCCAGTATCTTCCTACTGGGAAGAGGAG
CAACCCTCAGCAGCCCTCTTGCCCCTCCATGGGGTGAAGCCACAGTCGGTGGGTG
TGTCACTTCAGGCTCTGTGGGCTACACTCAGCCTGAGGCCAGCTGGGTGGCCCA
GCACCCCTACCCTAGGAAGGTGCAACACAGGGATACTACTTGAACCTTCCTATCA

TAGCTCCCATGCCACAGAGGGACAGACCTCTCTGTCCTGCCCTCTTGCCACTGGT
GAGAGAAGGCCCTTGTCTCCCCAGACTTGGCGGTCTCCACAGTGACGGGCTCCT
GGGTCCTTGTTTAGGAAAAGCCTGTGTGTGACACCTGCCTCATCAGGAGGCTGCC
ATTGGCAGTGGCTTGCTGCTGGCCTCCCTGCAAGTCCCCTCTTCCCATTGCGTGCC
TGGACTCAGGAGCATAGAAAAACGACTGTAGATTGTTGGGCCACACTGGCCTAG
CCTGAACCTAGATCCCTAACTCTGGGCTGGGTGAGGCTCATCTGCCCAGGACCAC
AGTGGGACAGCCTGGGCAGGTGGGTGGCACAGCATAAGAACCCAGCTGGGGCC
GGCCCTCCCTGTCCTGGCTGGTCAGTGTGCCCCCACCCACACTGTCCAGC

```
No mispriming library specified
Using 1-based sequence positions
WARNING: Unrecognized base in input sequence
```

PRODUCT SIZE: 813, PAIR ANY TH COMPL: 0.00, PAIR 3' TH COMPL: 0.00

315

1.5 Rat GNB1-3'-UTR and miR-16-5p

Cutting sites for restriction enzymes:

5' - SpeI (BcuI) ACT AGT

3' - HindIII AAG CTT

Sites underlined

Primer:

Forward:

AAA ACT AGT TTCAAGTTGTAACCGTGGCG

Reverse:

AAA AAG CTT CAGAGATTCATTGGCCCTGC

GNB1-3'-UTR (extraction):

miR-16-5p BS marked (position: 1269-1275)

CATATTCCTTTATGTTTCAGTAGTTAAGAGAACCAGAGCCCATGGGGGTGTCTGAC
TGTCAGTGCATTTTTGTCCCTGGCTTGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
GTCTCATGTCACAGATGACTGTTTCGCACAAACACAGCATAGTGGAAGAAAAAA
TTCAGAAGCAAAGTAGCCAAGCACATGTTATGATGGCCACGAATGCTTGGTCAG
AATCCTTCCCTTTGCTCAGCAGTGAAGTTATCCCAGCACCCCTTCCCTCCTCACTG
TCCAGTTTCTGTTCTTTCCTTGGGTTTCCTGTGAAGTTGGAGGGAAGTTTGTAGTC
ATGTGACACTAGCCGTCCTGCCCCTGCTGTCAGATTTTTTTTTCTCCAGAGAGGTGC
CTGTCCTGTGCTTGGATAGTCAGTTTGTGTATGAAACAATGTACAAATCAATGTT

TTGAAAATAATGATCTCAGACTTTCTAAGTTAAATTTTAAACAATTTGATTGTTTG
CCATATTGGGTGGGTTTACTCTTAGAATCGCATGCTGTAGAAATGCTCTAAAGTG
CATATAGGACTCAGTCCTTAGATGTTCTTTTTATTTAAGAAATAACCTCTTCAAG
TTGTAACCGTGGCGGCTTTGTCCATTACTGTTGCTATTCTGCACACATATCGGAA
GCAGCAAGCGCTTTACACACTCGAGTAGTGGGATAAGTCACTGTTTTCTTTACTT
TACAAGATGATAGTCCTGTTGGGAATGACTGCTGCTGGCTTCAGGAAGGGAGG
GAAGCTGCTCCGCTGCCTCTTTGCCACTTCCTGGAAGCAGCCTCCATGGAACAGC
CTTTGCATCTGAGGCAACCATTTTTACACCGTGCAGATCTTCGTGGTTATCATCTC
AAAGCCATGTTTCAGCAGCAGCGTTTGGACAGCAGTGTTCTGTGGGCCCTCCCATC
CCCCTATATCCTGCACCCCTCATAGCAGTAGTGGCTTCGCCATTCTGTTTTCTGCA
GCGTCCTGTACAAAACCTGTGCTGTGACTGTGCGGTAGGCCTGGACATGGCAAAG
AGAATACAGCGAAGCCCCTTCTCTTCCTACCAAACGACTCTGTAGAGCTCTCTGC
ACCCTGTACCCTTTCCACCTTTTGTATTTAATTTTAAAGTCAGTGTACTGCAAGGA
GGCTGGATGCAAGATAGATACTATATTAACCTGTACTGTTATTTAAGATGTAATA
AAGCAGTTTGACATGAGGGACTCTGGTGTGATGGTTATTTGAGGGTCAGCTTCAG
TGTGTGAAGGTAGCCACATGCCCCAACTGCTGACTCAACAGTTATATAATGTCAT
GAGAGATCCTTATATTATAAAACTGTCAGCTCAGGCTGAGTGCGGTAGTGCACAC
TTTTAATCACAACACTCAGGAGGCAGGGCCAATGAATCTCTGAGGTTACGACCA
GCCTGATCTACTTTGTAAA

PRODUCT SIZE: 818, PAIR ANY TH COMPL: 0.00, PAIR 3' TH COMPL: 0.00

319

```
KEYS (in order of precedence):
>>>>> left primer
<<<<< right primer
```

	<u>start</u>	<u>len</u>	<u>tm</u>	<u>gc%</u>	<u>any th</u>	<u>3' th</u>	<u>hairpin</u>	<u>seq</u>
1 LEFT PRIMER cttccctcctcactgtccag	264	20	59.10	60.00	0.00	0.00	0.00	
RIGHT PRIMER gttttgtacaggacgctgca	1013	20	59.06	50.00	0.00	0.00	0.00	
PRODUCT SIZE: 750,	PAIR	ANY_TH	COMPL: 0.00,	PAIR	3'_TH	COMPL: 0.00		
2 LEFT PRIMER caagcgctttacacactcga	672	20	58.86	50.00	22.51	0.00	0.00	
RIGHT PRIMER tgcttcctgagtggttgat	1407	20	58.94	50.00	0.00	0.00	0.00	
PRODUCT SIZE: 736,	PAIR	ANY_TH	COMPL: 0.37,	PAIR	3'_TH	COMPL: 0.00		
3 LEFT PRIMER atgatggccacgaatgcttg	198	20	59.26	50.00	8.16	0.00	0.00	
RIGHT PRIMER ctactgctatgaggggtgca	972	20	58.88	55.00	0.00	0.00	0.00	
PRODUCT SIZE: 775,	PAIR	ANY_TH	COMPL: 0.00,	PAIR	3'_TH	COMPL: 0.00		
4 LEFT PRIMER ttattcccagcacccttccc	250	20	59.37	55.00	0.00	0.00	0.00	

RIGHT PRIMER 1127 20 58.93 55.00 0.00 0.00 0.00
aaggtggaaaggggtacaggg
PRODUCT SIZE: 878, PAIR ANY_TH COMPL: 12.56, PAIR 3'_TH COMPL: 12.56

Statistics

	con	too	in	in	not		no	tm	tm	high	high
high		high									
	sid	many	tar	excl	ok	bad	GC	too	too	any_th	3'_th
hair-	poly	end									
	ered	Ns	get	reg	reg	GC%	clamp	low	high	compl	compl
pin	X	stab	ok								
Left	4179	0	0	0	0	913	0	1694	420	0	0
35	96	0	1021								
Right	4389	0	0	0	0	655	0	1279	935	0	0
14	38	0	1468								

Pair Stats:

considered 394, unacceptable product size 389, primer in pair overlaps a
primer in a better pair 147, ok 5
libprimer3 release 2.4.0

(primer3_results.cgi release 4.0.0)

1.6 Rat GNG12-3'-UTR and miR-16-5p

Cutting sites for restriction enzymes:

5' - SpeI (BcuI) ACT AGT

3' - HindIII AAG CTT

Sites underlined

Primer:

Forward:

AAA ACT AGT CCTGAAACTTCCTGCTGCAG

Reverse:

AAA AAG CTT CGTGAGGGTATGTGGGCTAT

GNG12-3'-UTR (extraction):

miR-16-5p BS marked (position: 976-983)

ACCCTGAAACTTCCTGCTGCAGCATCAGCCCTTCAGAGTTACAGCGTTATCCTGA
CTCTGTGTAGGTGAATAACAGAGTCTTTCTGTGTATTTTTGTACAAAAGAAGGT
AATATAATCTGCATTATAACGAGCTGATGCTGTTCTCTCTCCTCTCTTGGAATTGT
GCTGGCCCCTTAGTCTACAATAAACTGTGCCAGTTTAAAAACCTAAGGCTAAAAC
TGAAAGCCCTTTGATGGGGTCTTCATTCATATCAGTCATTTGGGCTTCTGTGATCC
TGAGGCTAAGAAAAGGGAAGATACTCTCGGGAGGACCGTGGGCAGCTACACTCC
AGGCTCTTGATCTCTGCTGGATTGGGGGTGGCCACCTCAGAACTTCCACTCTCA
TGACTGGCATGGAAGAATGAACTGAGAGCCTCATGTGGAGAGGGGAGCAGAAA

TCGTGAAAACAGCTGCTCTCCTGGGCCCAGCTTTATAGGCAACCCTTTCTCCTCA
CCAGCATGATAGATAGAGCATCTCTCCTTTGCTGCTAAAGGCAGCTGTTTTCAGG
GGGATAGTCCCTCCTTCCCAGTTAACTGGGTGAACCCCAGTGGGGGAAAAACAC
TCAAGCAATATACAGACGCTTGAATTCTTTGCAGACGTCTGCAGTTCCAACACTA
CCGTGTTCTCAAACCACCCTAGGGGAAAGGCCCTTCCAAACGGGGGCTTGCATTC
TGTATAACAGTTCAAACATCTAGCAGTTGGGTCTTGATGTCCTCAATTTTACTCTG
ATCATGAAAATAGCCACATACCCTCACGGTCTCTAGTAGGGATCCCAGAGGCA
CATAACCTGCCACGACCAAGGAGGTATTCTGACTTTGTCACAGTGAATATTTAAA
ATAATCAGTTAGAAGCCCACACCAAACCCAAAGCCTGTGATATTTTAGTTCATTA
AGGTAAC TACTAATGAAGGATTTTAAAAAGTG TTC

PRODUCT SIZE: 795, PAIR ANY TH COMPL: 0.00, PAIR 3' TH COMPL: 0.00

841 AGGAGGTATTCTGACTTTGTACAGTGAATATTTAAAAATAATCAGTTAGAAGCCCACACC

901 AAACCCAAAGCCTGTGATATTTTAGTTCATTAAGGTAAGTACTAATGAAGGATTTTAAAA

961 AGTGTTTC

KEYS (in order of precedence):

>>>>> left primer

<<<<< right primer

ADDITIONAL OLIGOS

	<u>start</u>	<u>len</u>	<u>tm</u>	<u>gc%</u>	<u>any_th</u>	<u>3'_th</u>	<u>hairpin</u>	<u>seq</u>
1 LEFT PRIMER	167	20	59.09	55.00	0.00	0.00	0.00	
gctggcccccttagtctacaa								
RIGHT PRIMER	918	20	58.66	50.00	0.00	0.00	0.00	
atcacaggcctttgggtttgg								
PRODUCT SIZE: 752, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00								
2 LEFT PRIMER	160	20	58.89	55.00	0.00	0.00	0.00	
gaattgtgctggcccccttag								
RIGHT PRIMER	901	20	58.57	50.00	0.00	0.00	0.00	
tggtgtgggcttctaactga								
PRODUCT SIZE: 742, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00								
3 LEFT PRIMER	26	20	58.27	55.00	0.00	0.00	0.00	
cagcccttcagagttacagc								
RIGHT PRIMER	842	20	58.91	55.00	7.12	0.00	0.00	
cttggtcgtggcagggttatg								
PRODUCT SIZE: 817, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00								
4 LEFT PRIMER	43	21	58.83	47.62	0.00	0.00	0.00	
agcgttatcctgactctgtgt								
RIGHT PRIMER	754	20	58.83	55.00	0.00	0.00	0.00	
ggacatcaagacccaactgc								
PRODUCT SIZE: 712, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00								

Statistics

	con	too	in	in	not		no	tm	tm	high	high
high		high									
hair-	sid	many	tar	excl	ok	bad	GC	too	too	any_th	3'_th
pin	ered	end									
	ok	Ns	get	reg	reg	GC%	clamp	low	high	compl	compl
Left	X	stab	ok								
9	1535	0	0	0	0	246	0	749	113	0	0
23	0	395									
Right	1531	0	0	0	0	377	0	665	136	0	0
2	19	0	332								

Pair Stats:

considered 231, unacceptable product size 226, primer in pair overlaps a primer in a better pair 134, ok 5

libprimer3 release 2.4.0

(primer3_results.cgi release 4.0.0)

1.7 Rat ADRBK1-3'-UTR and miR-26-5p

[same constructs as ADRBK1 : miR-16-5p]

Cutting sites for restriction enzymes:

5' - SpeI (BcuI) ACT AGT

3' - HindIII AAG CTT

Sites underlined

Primer:

Forward:

AAA ACT AGT GCCCACCCGCCTTTTATAAA

Reverse:

AAA AAG CTT CTGGGTTCTTATGCTGTGCC

ADRBK1-3'-UTR (extraction):

miR-26-5p BS marked (position: 405-412)

TTCACCCATCTGCTGCCCCGCCTGCCACCCGCCTTTTATAAACCTCTAATTTATTT
TGTTGAATTTTATTATTTGTTTTCCCGCAAAGCGGAAAAGGTTTTATTTTGTAAT
TATTGTGATTTCTGTGGCCCCAGCCTGGCCCCAGCCCCAGGGAGGGGCCTGCTT
GCCTCAGCTCCTGCTGCCACCAACCCAGCCACTGTCTAGCACCCCTGCCCCGCCC
CCACCGCATCCCCCTAGGGTCCTTGCTCCCAGTATCTTCCTACTGGGAAGAGGAG
CAACCCTCAGCAGCCCTCTTGCCCCCTCCATGGGGTGAAGCCACAGTCGGTGGGTG

TGTCAC TTCAGGCTCTGTGGGCTACACTCAGCCTGAGGCC CAGCTGGGTGGCCCA
GCACCCCTACCCTAGGAAGGTGCAACACAGGGATTACTACTTGAACTTTCCTATC
ATAGCTCCCATGCCACAGAGGGACAGACCTCTCTGTCCTGCCCTCTTGCCACTGG
TGAGAGAAGGCCCTTGTCTCCCCAGACTTGGCGGTCTCCCACAGTGACGGGCTCC
TGGGTCCTTGTTTAGGAAAAGCCTGTGTGTGACACCTGCCTCATCAGGAGGCTGC
CATTGGCAGTGGCTGCTGCTGGCCTCCCTGCAAGTCCCCTCTTCCCATTGCGTGCC
TGGACTCAGGAGCATAGAAAAACGACTGTAGATTGTTGGGCCACACTGGCCTAG
CCTGAACCTAGATCCCTAACTCTGGGCTGGGTGAGGCTCATCTGCCCAGGACCAC
AGTGGGACAGCCTGGGCAGGTGGGTGGCACAGCATAAGAACCCAGCTGGGGCC
GGCCCTCCCTGTCCTGGCTGGTCAGTGTGCCCCCACCCACACTGTCCAGC

```
No mispriming library specified
Using 1-based sequence positions
WARNING: Unrecognized base in input sequence
```

PRODUCT SIZE: 813, PAIR ANY TH COMPL: 0.00, PAIR 3' TH COMPL: 0.00

328

1.8 Rat CACNB2-3'-UTR and miR-26-5p

Cutting sites for restriction enzymes:

5' - SpeI (BcuI) ACT AGT

3' - HindIII AAG CTT

Sites underlined

Primer:

Fwd 1:

AAA ACT AGT TGTTGCTTTCGAACCCACTG

Rev 1:

AAA AAG CTT GTGGAGTGCTGTTCTGAGGA

CACNB2-3'-UTR (extraction):

miR-26-5p BS marked (position: 1437-1443)

AGCAGCTAGTGTCTGTGGTAGTCACTGCACTGGAGTATCCAAGCGGAGCCCACC
ATGTGCACATGCGTGTGTGAGTGTGAGAGTGAGTGGCTGAGACAAAGTGGAATG
CAGAAGACTAATGCGGAAACCAGAGGCTGATGGACCTGGCTTTGCATTTAACTG
TTGTATGTTGCTTTTCGAACCCACTGAGCAGCCAGGGAATGTGAGGAAGCTTGCTG
CCAAAGGAACTAGGGAAGCACTTAGTGGCTGACTCCCTGCTTTTGCCCCTGAAGA
GAGAAAACATGGGCGTCTTGTACTGTGAGTGTTCCACTGGAAATGGACAATCTTT
GTGTGTCAGAGTATTTTGTTTTAGTAAGAAATGTTTACACAGCTTGTGCAATTATT
TCGTAGGGAAATAAATTTTATAACTTGTCCCACTTCACTTTCTAAACGTGCCTAT

TGCTCCTCTTGTTTCATCCTCAGCCACCGCTCTTCTTCCCCTGCACCAAGCTGTGCA
GCTGCACTGTCGGAATCTAGAAGTGCTTCTTAGAACCAGTTCTGCTAAATCT
TTAGAAGTTATCAGGGCAAAAATGGAATGACTTGAAGCAAAAAAGTTGAAAC
ATTTTCTTTTACGAGACATCTCTAAGGGAGAACGTAGAAGACACTTTTTCACACC
TCAATTTGCTTTCGTTAAGAAGCTTAACTGCTCTTAGCCGAGGCTATCCAATCTC
TGTCTCTCAAGAGCCCAGTGCTGATGGAGAGGTAAGACTGTATGAGCTCCACAC
GCCCCTAGCATTCGAAGCCTTCGCAGGAAAATGTCTATCTGTGATTACCCAACCA
GATCAGTATATACTAAAACAAGTAAGTTACAATAAATATATTTTTGTTTTGTTTCC
GGGTTACTTCACATTCTTCTGAAATATTCTTAGGCATTTCGTATGAAAAGCAGTTTG
ACATTTACTACTTTATACTATACCCAAGAAAACCTCAAGCATTTAAAACGTGAGTT
GCATATGTATTTGACCAGTGGGTACCTGCTTAGAGAATGAAATTTTCCTCAGAA
CAGCACTCCACGCTTACAGAAATACAGACGATACCCAGCAATC

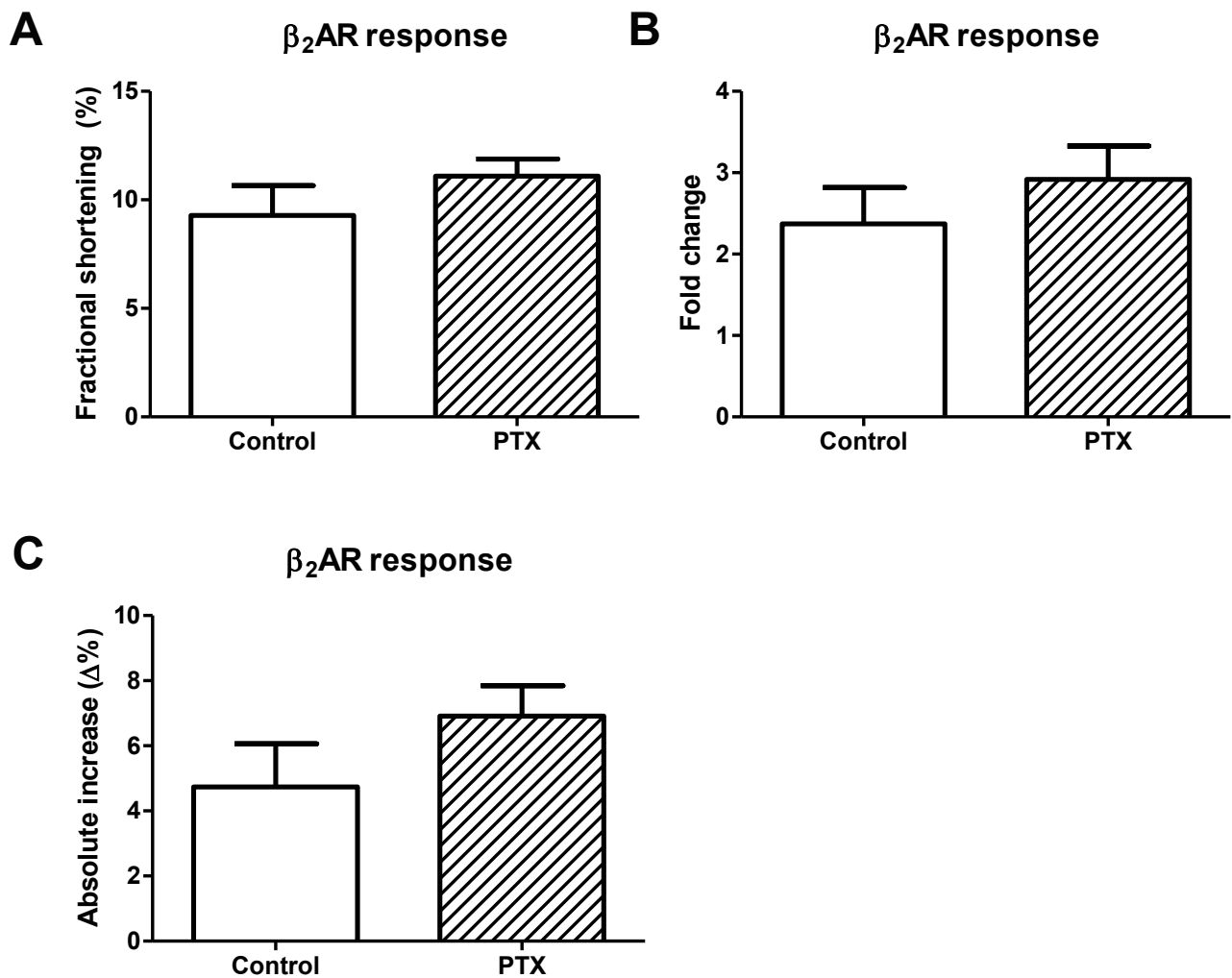
PRODUCT SIZE: 890, PAIR ANY TH COMPL: 7.45, PAIR 3' TH COMPL: 1.14

332

libprimer3 release 2.4.0

(primer3_results.cgi release 4.0.0)

2 Pertussis toxin pre-treatment in cultured cardiomyocytes undergoing β_2 AR response



Appendix 2. 1: β_2 AR response after 48-hour culture with PTX pre-treatment

(A) Peak β_2 AR response. (B) Fold change from baseline in β_2 AR response. (C) Absolute change from baseline in β_2 AR response. N=6. Statistical comparison made using Student's t-test.

3 Comparison of statistical methods with hierarchical clustering

	Student's t-test	Hierarchical clustering
Pre-miR-26a <i>versus</i> pre-miR control	p=0.0021	p=0.0018
Anti-miR-26a <i>versus</i> anti-miR control	p=0.4761	p=1.0000

Appendix 3. 1: Statistical comparison between Student's t-test and hierarchical clustering